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13. Abstract (Maximum 200 Words)

The AP-1 transcription factor is a central component of many signal transduction pathways. We have shown that blocking AP-1 by over-expressing a dominant negative form of cJun (cJun-DN, Tam67) inhibits breast cancer cell growth. We hypothesize that inhibition of AP-1 blocks the cell cycle, reverses TAM-resistance of breast cancer cells, and suppression of AP-1 in vivo causes regression of existing breast tumors. In the present study, we demonstrated that TAM67 inhibits breast cancer growth both *in vitro* and *in vivo*. We determined the mechanism by which AP-1 blockade inhibits breast cancer growth. Our studies suggested that TAM67 inhibits breast cancer growth predominantly by inducing CDK inhibitors (such as P27), suppressing G1 cyclins expression and reducing CDKs activity, thus inducing a cell cycle block. We also showed that TAM67 induces apoptosis in serum-free condition in breast cancer cells. We are currently investigating the molecular mechanism by which TAM67 causes a cell cycle block. Over the next year we will investigate whether inhibition of AP-1 activity reverses tamoxifen-resistance of breast cancer cells.

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INTRODUCTION

The AP-1 transcription factor is a central component of signal transduction pathways in many cells. The AP-1 family consists of multiple Jun (cJun, JunB, and JunD), Fos (cFos, FosB, Fra-1, and Fra-2), ATF/CREB (ATF1, ATF2, ATF3, CREB1, CREB2) family members and Jun dimerization partners (JDP1 and JDP2). It was shown that AP-1 is involved in controlling cellular proliferation, differentiation, apoptosis, and oncogene-induced transformation. However, most of this work has been done in fibroblasts. Relatively few studies of the function of AP-1 have been performed in epithelial cells. Thus the exact role of this transcription factor family in controlling the proliferation and transformation of epithelial cells is not known. We and others have demonstrated that Jun and Fos family members are variably expressed in human breast tumors, and AP-1 is activated by a variety of important growth factors such as EGF, IGFs, and estrogen. Recent studies found correlations between high phospho-cJun expression and decreased overall survival in breast cancer (1); and the expression of FosB correlated with ER-positivity while expression of Fra1 showed a strong negative correlation with ER positivity (2). AP-1 complexes may be involved in regulating transcription of the ER gene as well (3). These results indicated that AP-1 proteins might play a role in the pathogenesis and growth of breast tumors. In addition, AP-1 activity has been shown to increase when human breast cancers become resistant to tamoxifen (4,5). The cJun overexpression in MCF 7 breast cancer cells produces a tumorigenic invasive and hormone resistant phenotype (6,7). All of these studies suggest that AP-1 transcription factors may also be critical for the growth of tamoxifen-resistant or drug-resistant breast cancer cells. To investigate the role of AP-1 in regulating breast cell growth, we have developed MCF-7 breast cancer cell lines that express an inducible cjun dominant-negative mutant (cJun-DN) under the control of the Tet-off system. This cJun dominant-negative mutant lacks the transactivation domain of cJun, yet retains its DNA-binding and dimerization domains (the "TransActivation domain Mutant", TAM-67). We have shown that the TAM67 inhibits AP-1 activity and suppresses breast cancer growth [8,9]. In the present study, we first showed that TAM67 inhibited breast cancer cell growth both in vitro and in vivo. We then investigated the cellular biologic mechanism by which TAM67 inhibits cell growth. We have demonstrated that TAM67 in breast cancer cells caused growth inhibition by suppressing G1 cyclins expression and reducing CDKs activity, thus inducing a cell cycle block. We also showed that the expression of Tam67 induced apoptosis in the absence of serum. Our data suggested that inhibition of AP-1 blocked cell cycle, and induced apoptosis in absent of serum condition in breast cancer cells. We are currently investigating the molecular mechanism by which TAM67 causes a cell cycle block. Over the next year we will investigate whether inhibition of AP-1 activity reverses tamoxifen-resistance of breast cancer cells.

BODY

Statement of Work

Specific Aim 1: Determine the mechanism(s) by which AP-1 blockade inhibits breast cancer cell growth

Time Line	Task
Months 1-12	1). Determine whether AP-1 blockade affects the cell cycle and cell cycle regulators in breast cancer cells; 2). Determine whether AP-1 blockade induces apoptotic cell death and alters the expression of critical apoptosis regulators.

Specific Aim 2: Determine whether inhibition of AP-1 activity reverses tamoxifen-resistance of breast cancer cells *in vitro*

Time Line	Task
Months 1-6	Introduce the Tet-Off system into the LCC-2 cell line and screen clones expressing cJun-DN.
Months 7-12	Investigate effect of expression of cJun-DN on AP-1 activity and expression of AP-1 dependent genes in LCC2 cells
Months 13-24	Determine whether AP-1 blockade reverses TAM-resistance of LCC2 breast cancer cells.

Specific Aim 3: Determine whether inhibition of AP-1 suppresses the growth of established human breast cancer cells or reverses TAM resistance in nude mice

Time Line	Task
Months 13-24	Inject MCF-7 Tet-Off cJun-DN into nude mice to determine the effect of AP-1 blockade on the growth of human breast cancer cells <i>in vivo</i>
Months 25-36	Inject LCC2 Tet-Off cJun-DN into nude mice to determine whether AP-1 blockade reverses TAM resistance <i>in vivo</i>

Specific Aim 1. Determine the mechanism by which AP-1 blockade inhibits breast cancer cell growth.

Task 1: Determine whether AP-1 blockade affects the cell cycle and cell cycle regulators in breast cancer cells. We first determined the effect of AP-1 blockade, TAM67, on cell growth by performing cell proliferation assay, we demonstrated TAM67 inhibit breast cancer cell growth (see Fig.1). We next investigated the effect of AP-1 blockade on DNA synthesis and the cell cycle using a ³H-thymidine incorporation assay and flow cytometry. The results of the ³H-thymidine uptake assay showed that TAM67 dramatically inhibited ³H-thymidine uptake in MCF-7 cells (see Fig.2). Flow cytometry also showed that expression of cells of TAM67 reduced the proportion of cells in S phase, and increased the proportion in the G₀/G₁ phase (see Fig.3). Thus, in the presence of serum, the expression of TAM67 blocked the cell cycle by causing a G₁ arrest.

Task 2: Determine whether AP-1 blockade induces apoptotic cell death. We first performed TUNEL assay to measure apoptosis. We found the expression of TAM67 dramatically increased the cell apoptotic rate in serum free condition, but there is no significant difference in full serum condition (see Fig. 4A). To further demonstrate the involvement of apoptosis in the inhibition of MCF-7 cell growth, we then performed western blotting to measure the cleavage of PARP, a hallmark of apoptosis. When the

MCF-7 Tet-Off TAM 67 cells were cultured in serum-containing medium, we also observed no PARP cleavage neither in TAM67 induced or uninduced conditions. Under serum-free condition, we did not see obvious PARP cleavage when TAM67 was not expressed, however, when TAM67 was induced the PARP cleaved band was clearly observed (see Fig. 4B). Our study showed that TAM67 induced apoptosis in serum-free condition.

Task 3: We are now extending these studies to determine whether AP-1 Blockade alters the expression and/or activity of cell cycle regulators. We first performed western-blotting to determine the effect of TAM67 on Rb phosphorylation, we found that TAM67 caused Rb hypophosphorylation (see Fig. 5). We next performed luciferase assays to determine whether AP-1 blockade inhibits E2F activity (see Fig. 6). The results from these experiments demonstrated that TAM67 decreased E2F activity, and thus blocked the cell cycle. Next, we performed western-blotting to determine the effects of Tam67 on the expression of cell cycle regulatory proteins. We discovered that TAM67 decreased cyclin Ds (D1, D2, D3), cyclin E, CDK4, and CDK6 expression, while increase CDK inhibitor p27 expression (see Fig. 7). Next, we used CDK2 and CDK4 kinase assays to determine whether TAM67 reduced CDK activity. We demonstrated that both CDK2 and CDK4 activity were reduced by TAM67 expression (see Fig. 8). Our study suggests that TAM67 inhibits breast cancer growth predominantly by inducing inhibitors of cyclin dependent kinases (such as p27), suppressing G1 cyclins expression and reducing CDKs activity, thus inducing a cell cycle block.

Specific aim 2. Determine whether inhibition of AP-1 activity reverses tamoxifen-resistance of breast cancer cells in vitro.

Task 1: Introduce the Tet-Off system into the LCC-2 cell line and screen clones expressing cJun-DN.

Task 2: Investigate effect of expression of cJun-DN on AP-1 activity and expression of AP-1 dependent genes in LCC2 cells

Task 3: Determine whether AP-1 blockade reverses TAM-resistance of LCC2 breast cancer cells.

These studies have not yet begun, I will perform these studies in year 2 and 3 because we have not yet obtained the Tamoxifen-resistant cell line LCC-2.

Specific aim 3. Determine whether AP-1 blockade suppresses breast cancer growth and reverses tamoxifen resistance in vivo.

Task 1: Inject MCF-7 Tet-Off cJun-DN into nude mice to determine the effect of AP-1 blockade on the growth of human breast cancer cells *in vivo*. To investigate the effect of AP-1 blockade on *in vivo* tumor growth, we injected the MCF-7 Tet-Off TAM67 cells into nude mice receiving doxycycline to suppress the expression of the AP-1 inhibitor. After the mice developed tumors, they were randomized to either continue to receive Dox or not. In mice not receiving Dox, the expression of TAM67 was induced, and tumor growth was inhibited, while the tumors in mice receiving Dox continued to grow. We have completed this task. These results are included in the attached manuscript.

Task 2: Inject LCC2 Tet-Off cJun-DN into nude mice to determine whether AP-1 blockade reverses TAM resistance *in vivo*. This task has not yet begun.

KEY RESEARCH ACCOMPLISHMENTS

We studied the mechanism by which AP-1 blockade inhibits breast cancer cell growth. Our data demonstrated that the AP-1 blockade, TAM67, inhibited breast cancer growth both *in vitro* and *in vivo*. TAM67 suppressed cyclin D and E expression, increased p27 expression, decreased CDk2 and CDk4 kinase activity, caused Rb hypophosphorylation and reduced E2F activity, thus results a G1 cell cycle block leading to cell growth inhibition. We also observed that TAM67 induced MCF7 breast cancer cell apoptosis in serum free condition.

REPORTABLE OUTCOMES

1. The paper "Inhibition of AP-1 Transcription Factor Activity Causes Global Signal Transduction Blockade and Inhibits Breast Cancer Growth" was accepted by Oncogene.
2. The paper "AP-1 Blockade in Breast Cancer Cells Causes Cell Cycle Arrest by Suppressing G1 Cyclin Expression and Inducing p27" is being submitted to Oncogene.
3. Poster presentation " AP-1 blockade inhibits breast cancer growth by inducing a G1 cell cycle block" at Era of Hope DOD Breast Cancer Research Program Meeting, 2002.

CONCLUSIONS

AP-1 blockade, TAM67, inhibits breast cancer growth both *in vitro* and *in vivo*. TAM67 inhibits breast cancer growth predominantly by inducing the expression of inhibitors of cyclin dependent kinases (such as p27), suppressing G1 cyclins expression and reducing CDKs activity, thus causing a cell cycle block. This effectively blocks breast cell proliferation. TAM67 also induces apoptosis in cells grown in serum free condition.

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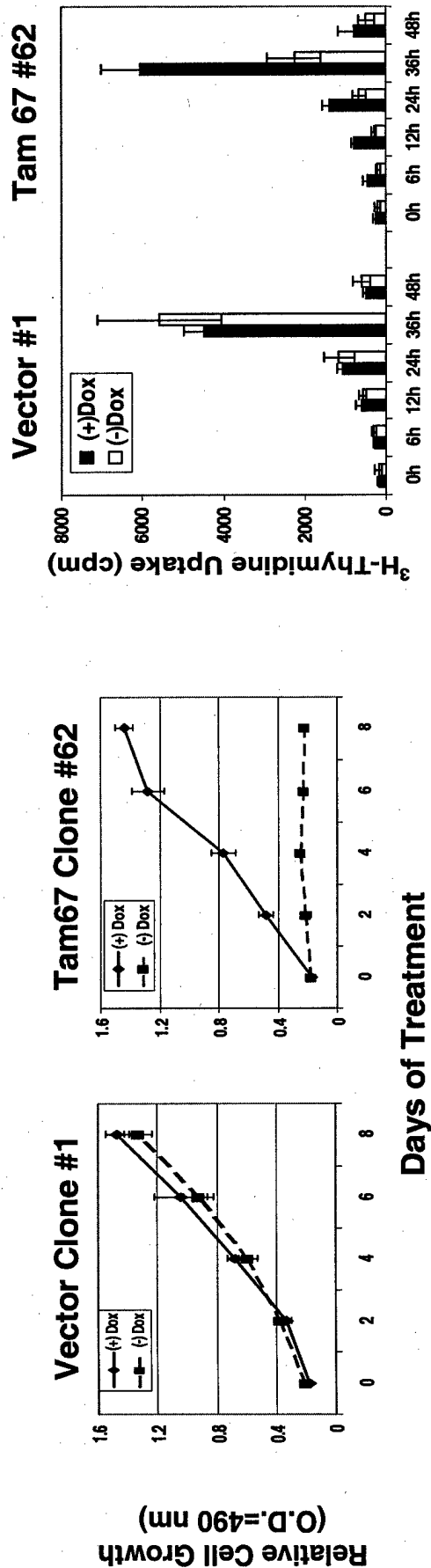


Fig. 1. TAM67 inhibition of MCF 7 cell growth both in (a) serum present and (b) serum absent conditions. MCF 7 Tet off TAM67 cells were cultured in the presence or absence of DOX for 5 days, starved of fetal bovine serum for 2 more days. The cells were then stimulated with serum or without serum, then the cell proliferation was determined by MTS assay.

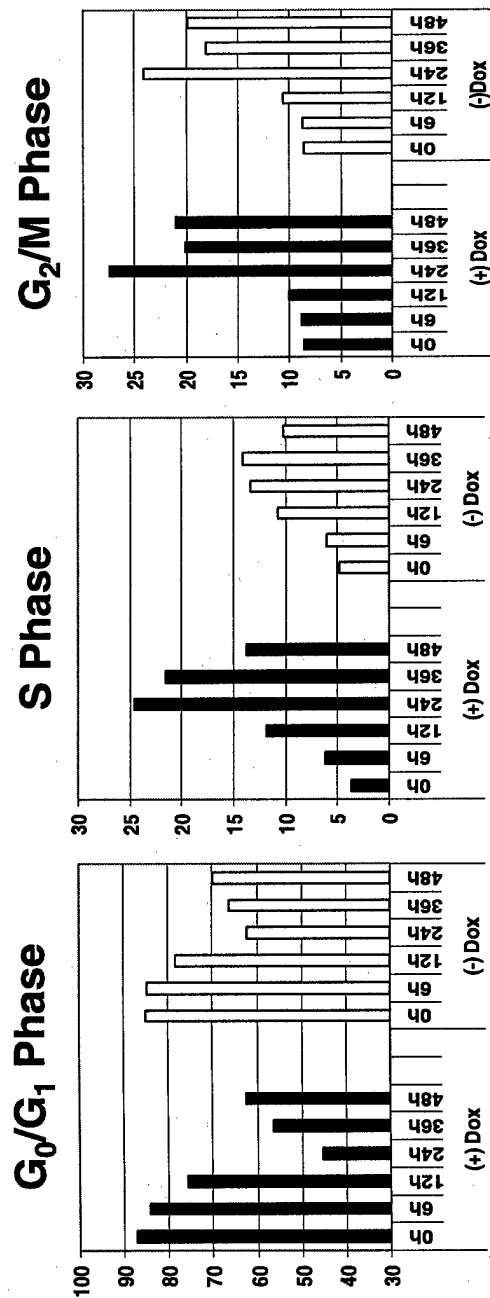


Fig. 2. TAM67 inhibition of ^3H -thymidine uptake in MCF 7 cells. MCF 7 Tet off TAM67 cells were cultured in the presence or absence of DOX for 5 days, starved of fetal bovine serum for 2 more days. The cells were then stimulated with serum or without serum, and ^3H -Thymidine incorporation assay was performed at time points.

Fig. 3. TAM67 inhibits normal cell cycle by causing G₁ arrest. MCF 7 Tet off TAM67 cells were cultured in the presence or absence of DOX for 7 days, 48 hours before harvest the medium is changed to serum-free to synchronize cells. Then Flow Cytometry Assay was performed. a: TAM67 increased cell numbers in G₀/G₁ phase. b: TAM67 caused reduced cell numbers in S phase. c: TAM67 did not dramatically affect the cell distribution in G₂/M phase.

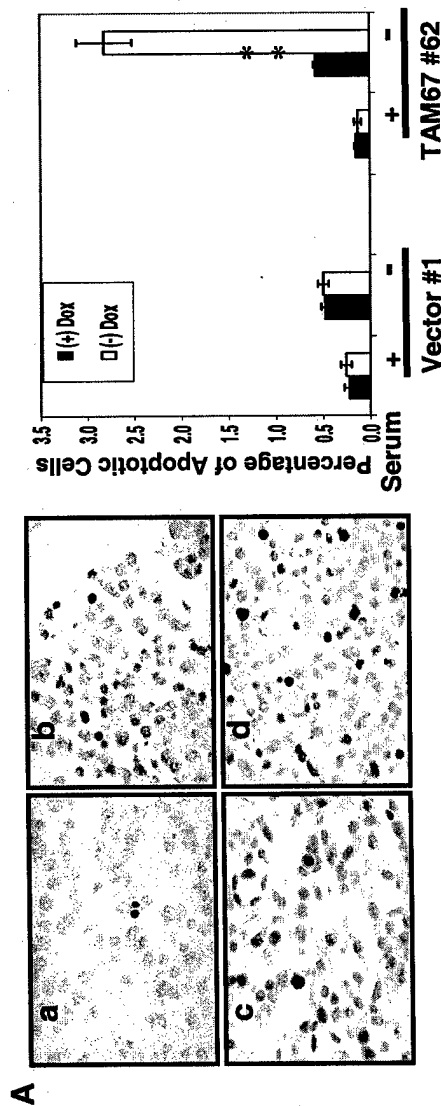


Fig. 4. TAM67 cause apoptosis in serum-free conditions. MCF 7 Tet off TAM67 cells were cultured in the presence or absence of DOX for 7 days, in the last 2 days cells were cultured in medium with serum or without serum. **A.** TUNEL assay was used to measure the apoptotic cells in different conditions. **a,** DOX (+), serum (+); **b,** DOX (+), serum (-); **c,** DOX (-), serum (+); **d,** DOX (-), serum (-). **B.** Western-blotting assay was performed to measure the cleavage of PARP, a hallmark of apoptosis.

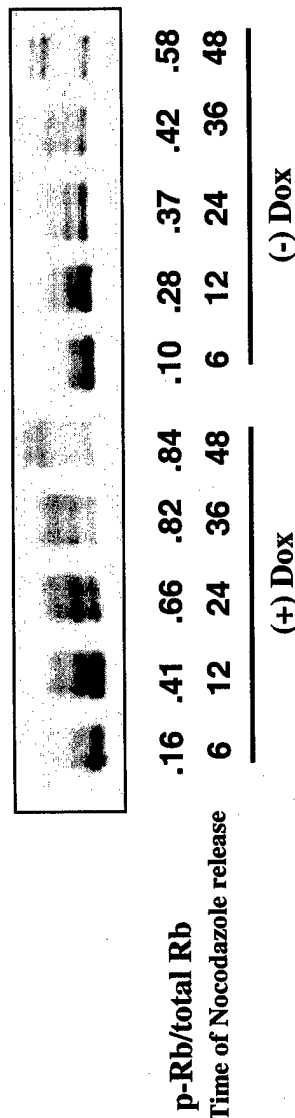


Fig. 5. TAM67 cause Rb hypophosphorylation. MCF 7 Tet off TAM67 cells were cultured in the presence or absence of DOX for 7 days, and synchronized using nocodazole, then Rb phosphorylation status was determined by western-blotting assay. Rb hypophosphorylation was observed in the absence of DOX condition.

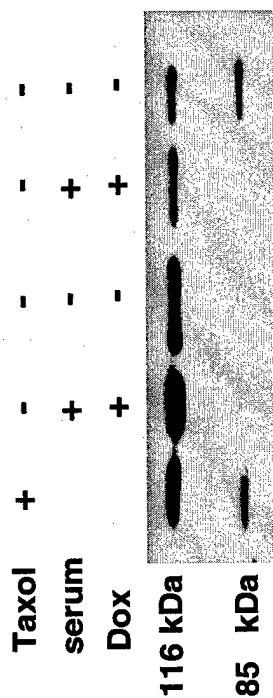


Fig. 6. TAM67 decreased E2F1 activity. MCF 7 Tet off TAM67 cells and MCF 7 Tet off vector cells were cultured in the presence or absence of DOX for 7 days, then the cells were cotransfected with the E2F1-luc reporter gene and pRL-TK, luciferase activity was measured and normalized with the Renilla activity. E2F1 activity was decreased in DOX absence condition in Tam67 cells, while there is no difference in vector cells between DOX present and absent conditions.

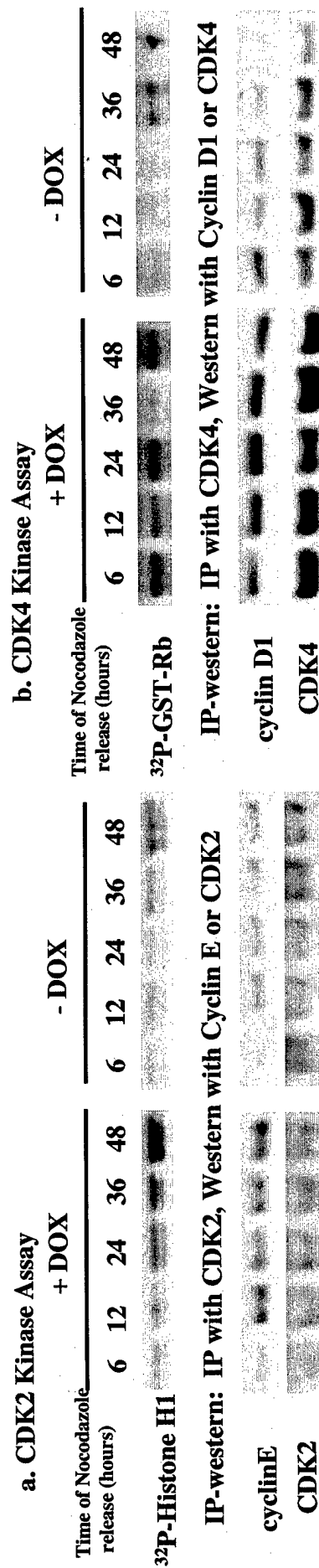
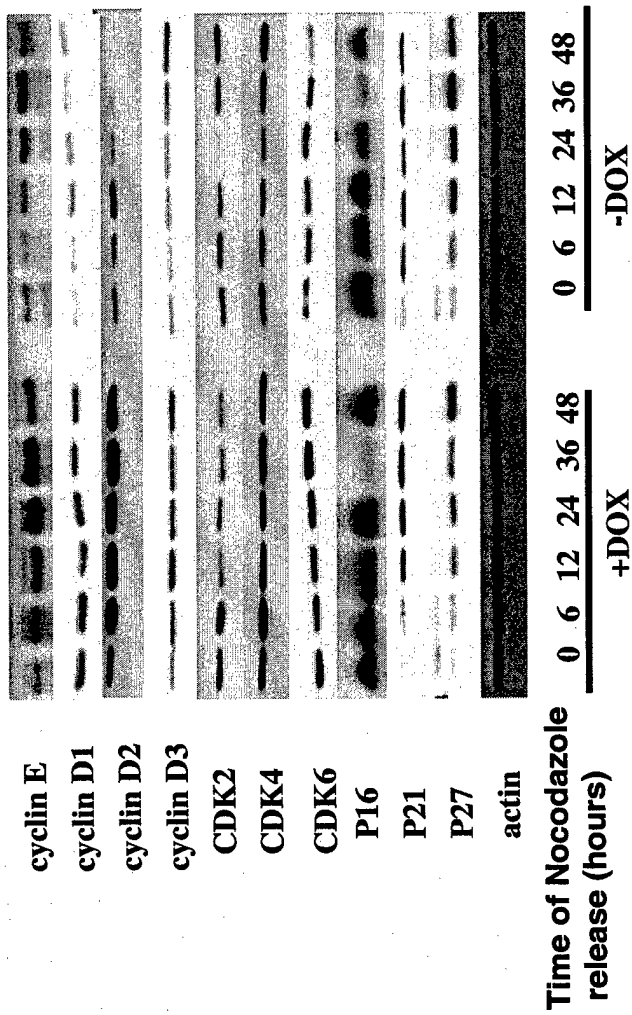


Fig. 8. Effect of TAM67 on CDK 2 and CDK 4 kinase activity. MCF 7 Tet off TAM67 cells were cultured in the presence or absence of DOX for 7 days, synchronized using nocodazole for 18 hours, then cells in M phase were replated in full medium and harvested at several time points. CDK 2 and CDK4 kinase assay were performed as described in materials and methods. CDK2 and Cyclin E protein expression in CDK2/Cyclin E complex, CDK4 and Cyclin D1 proteins expression in CDK4/Cyclin D1 complex were determined by immunoprecipitation-western blotting. a: TAM67 suppressed Cyclin E expression and CDK 2 kinase activity, while did affect CDK 2 protein expression. b: TAM67 inhibited Cyclin D1 and CDK4 expression, and suppress CDK4 activity in some time points.

Inhibition of AP-1 Transcription Factor Causes Global Signal Transduction Blockade and Inhibits Breast Cancer Growth

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Running Title: AP-1 inhibition causes global signal transduction blockade

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Abbreviations: AP-1, activating protein-1; TAM-67, cJun Dominant-negative mutant; Dox, doxycycline; ER, estrogen receptor; ERE, estrogen response element; EGF, epidermal growth factor; IGF, insulin-like growth factor; TGF- α , transforming growth factor- α ; TPA, 12-O-tetradecanoylphorbol-13-acetate; bFGF, Basic fibroblast growth factor; HRG- β , Heregulin-beta;

Abstract

AP-1 transcription factors play a critical role in signal transduction pathways in many cells. We have investigated the role of AP-1 in controlling proliferative signals in breast cells, and have previously shown that AP-1 complexes are activated by peptide and steroid growth factors in both normal and malignant breast cells. In this study, we investigated the role of AP-1 in transducing proliferative signals induced by peptide and steroid growth factors. We used MCF-7 clones that express a specific inhibitor of AP-1, a dominant-negative cJun mutant (TAM67), under the control of an inducible promoter to investigate the role of AP-1 in regulating breast cancer growth. In the presence of doxycycline (Dox), the AP-1 inhibitor was not expressed, and the MCF-7 clones proliferated normally in response to serum stimulation. However, when Dox was withdrawn, TAM67 was expressed, AP-1 activity was inhibited, and serum-induced proliferation was blocked. We next investigated whether the mitogenic response to specific growth factors also requires AP-1. MCF-7 Tet-Off-TAM67 cells were grown in the presence of increasing concentrations of IGF-1, EGF, heregulin- β , bFGF, or estrogen under un-induced and induced conditions. These studies showed that the AP-1 inhibitor completely blocked proliferation in response to the peptide growth factors (IGF-1, EGF, heregulin- β , and bFGF), and partially blocked the response to estrogen. To investigate the effect of AP-1 blockade on *in vivo* tumor growth, we injected the MCF-7 Tet-Off TAM67 cells into nude mice receiving doxycycline to suppress the expression of the AP-1 inhibitor. After the mice developed tumors, they were randomized to either continue to receive Dox or not. In mice not receiving Dox, the expression of TAM67 was induced, and tumor growth was inhibited, while the tumors in mice receiving Dox continued to grow. Analysis of the tumors from these mice showed that the

expression of TAM67 caused reduced proliferation of the breast cancer cells without inducing apoptosis. These results demonstrate that AP-1 blockade suppresses mitogenic signals from multiple different peptide growth factors as well as estrogen, and inhibits the growth of MCF-7 breast cancer cells both *in vitro* and *in vivo*. These results suggest that novel agents specifically targeting AP-1 or its activating kinases could be promising agents for the treatment of breast cancer.

Introduction:

Breast cancer is one of the most common malignancies in women, and is the leading cause of death for women between the ages of 40 and 55 in the United States (1,2). During the last two decades, breast cancer has been intensively studied, and recently new treatments for this disease have emerged. Drugs that inhibit the ability of estrogen to activate the estrogen receptor (e.g., tamoxifen) are used to prevent and treat breast cancer. Drugs that block growth factor receptors, such as antibodies specific for the epidermal growth factor receptor, or for ErbB2 (Her2/neu), have previously been shown to inhibit breast cancer cell proliferation (2-4) and are now being used to treat breast cancer patients. These drugs target specific molecules, which are expressed by only a sub-set of breast cancers. Many of these drugs inhibit individual signal transduction pathways, and may ultimately be ineffective, since several different signal transduction pathways can stimulate breast cell proliferation. It may be more effective to inhibit signal transduction at a more distal point in the cascade, where many mitogenic signals converge. Since transcription factors, the nuclear proteins that control DNA transcription and gene expression, are the most distal components of these converging mitogenic signal transduction pathways, they could be good targets for new therapeutic agents.

In this study we investigated whether inhibition of the AP-1 transcription factors suppresses breast cancer growth. The AP-1 family is a key family of transcription factors transducing multiple signals, including mitogenic and stress induced signals. These transcription factors are complexes of DNA-binding proteins made up of homodimers of Jun family members or heterodimers of Jun and Fos family members. AP-1 functions by regulating AP-1-dependent downstream genes, or by interacting with transcriptional co-activators or integrators, such as Jab-1,

CBP or p300 (5,6). AP-1 transcription factors are expressed in most cell types, and are activated by specific kinases, which are themselves activated by diverse signals such as growth factor stimulation, exposure to UV light, oxidative stress, tumor promoters, or oncogene overexpression or activation (7).

In fibroblasts, AP-1 plays a critical role in cell proliferation. The expression of both *c-jun* and *c-fos* is rapidly increased in many cell types in response to mitogens such as serum or EGF (7-9). Microinjection and knockout experiments have shown that both Jun and Fos protein are necessary for fibroblast growth. Bravo and coworkers showed that microinjection of Fos- or Jun family member- specific antibodies blocks DNA synthesis and S phase entry in fibroblasts (10,11). Other studies using *c-jun* null mutation mouse embryonic fibroblasts demonstrated that these cells have reduced growth and lose the response to growth factor stimulation (12). These results suggest AP-1 complex is necessary for the proliferation of these cells.

In breast cells, previous studies have suggested that growth factors and hormones, such as IGF, EGF, estrogens and retinoids, can modulate AP-1 transcriptional activity (13-16). Other studies demonstrate that ER and AP-1 interact to regulate the expression of certain estrogen- and/or tamoxifen-regulated genes (17). Activation of AP-1 may also contribute to tumor cell invasive capacity (18) and tamoxifen resistance (18-21). These previous studies provide indirect evidence to suggest that the AP-1 transcription factor is an important regulator of breast cancer cell growth, invasion, and resistance to anti-estrogens.

To directly investigate whether AP-1 controls breast cell growth, we have used a specific inhibitor of AP-1, the dominant negative *c-Jun* mutant, TAM67, to block AP-1 activity in breast cancer cells. We have previously investigated the effect of AP-1 blockade on the growth of different breast cells using TAM67 (22). Results from these studies demonstrated that TAM67 blocks AP-1

activation in normal, immortal and malignant breast cells. In the present study we have explored the role of AP-1 in controlling the *in vitro* and *in vivo* growth of MCF-7 breast cancer cells. For these experiments, we used MCF-7 clones that express TAM67 under the control of an inducible promoter. Using these clones, we demonstrated that the expression of TAM67 inhibited AP-1 activity and inhibited MCF-7 cell growth *in vitro*. AP-1 blockade also completely suppressed MCF-7 cell growth induced by these peptide growth factors, and partially inhibited growth in response to estrogen. Studies of MCF7 xenografts in nude mice demonstrated that AP-1 blockade also inhibited the MCF-7 tumor growth *in vivo*. Investigation of the tumors from these mice showed that TAM67 caused decreased proliferation of the breast cancer cells without inducing apoptosis. Thus, AP-1 blockade inhibits the growth of MCF-7 breast cancer cells both *in vitro* and *in vivo*. These studies also suggest that agents that block AP-1 activation could be promising agents for the prevention or treatment of breast cancer.

Materials and Methods

Cell Culture and Transfection

The generation of the MCF-7 Tet-Off TAM67 Clones #62, #67 and vector clones #1, #3 has been previously described (22). The cells were maintained in Improved MEM (high zinc option, Life Technologies, Grand Island, New York) with 100 $\mu\text{g/ml}$ of genitacin and 100 $\mu\text{g/ml}$ of hygromycin. The cells were transfected using Fugene 6 reagent (Roche, Indianapolis, Indiana) according to manufacturer's recommendations.

Western Blot Analysis

Equal amounts of total cellular protein extract were electrophoresed on a 12% acrylamide denaturing gel and transferred by electroblotting onto a nitrocellulose membrane (Bio-Rad, Hercules, California). The primary antibody used was rabbit anti-cJun Ab-1 (Oncogene Science, Cambridge, MA). Donkey anti-rabbit antibody (1:4,000, Amersham, Piscataway, New Jersey) was used as secondary antibody. Blots were developed using the enhanced chemiluminescence (ECL) procedure (Amersham, Piscataway, New Jersey).

Luciferase Assay to Measure AP-1 and ER activity

AP-1 transcriptional activity in cells was measured using the Dual-LuciferaseTM Reporter Assay (Promega, Madison, Wisconsin) according to manufacturer's protocol. The cells were co-transfected with the Col-Z-Luc reporter gene containing the luciferase gene linked to 1100 bp of the human collagenase gene promoter which contains a single AP-1 binding site (TGAG/CTCA) and pRL-TK, a Renilla construct for normalizing of transfection efficiency. To determine the AP-1 activity stimulated by growth factors, the cells were treated with EGF (100 ng/ml, Life

Technologies, Grand Island, New York), IGF-1 (100 ng/ml, GroPep, Australia), heregulin- β 1 (10 ng/ml, R&D System, Minneapolis, Minnesota), bFGF (10 ng/ml, Life Technologies, Grand Island, New York), 17- β -estradiol (10^{-9} M, Sigma, St. Louis, Missouri), or DMSO, respectively for 6 hours before harvest. Transfected cells were lysed 36 hours after transfection and luciferase activity was measured with equal amounts of cell extract using a microplate luminometer (Labsystems, Helsinki, Finland) and normalized with the Renilla activity.

To measure estrogen receptor activity, the Vit-ERE-TK-Luc construct was employed instead of Col-Z-Luc to perform the luciferase assay. The cells were starved of estrogen for at least 24 hours in phenol red-free medium with 5% charcoal-stripped serum, and then treated with 17- β -estradiol (10^{-9} M) for 12 hours to stimulate the ERE activity before harvest.

Cell Growth Assays

Cell proliferation assay of stably transfected and Tet-Off cell lines

The CellTiter 96TM AQueous Non-Radioactive Cell Proliferation Assay (MTS assay; Promega, Madison, WI), performed according to manufacturer's protocol, was used to measure breast cancer cell growth. Approximately 12,000 cells were seeded in wells a 24 well plate and doxycycline or vehicle was added to block or induce the expression of TAM67 by MCF-7 Tet-Off TAM67 cells. A solution containing a 20:1 ratio of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazo-lium) and PMS (phenazine methosulfate) was added to the cells for 2 hours at 37^o C and absorption at 495 nm was determined. Each data point was performed in quadruplet, and the results were reported as mean absorption +/- standard error.

Cell proliferation assay of breast cells treated with serum and specific growth factors

The MTS assay described above was used to measure MCF-7 breast cancer cell growth after stimulation with specific growth factors, including EGF (0 to 100 ng/ml), IGF-1 (0 to 100ng/ml), heregulin- β 1 (0 to 10 ng/ml), bFGF (0 to 10ng/ml), or estradiol (0 to 10^{-9} M), respectively. The cells were cultured in medium with or without doxycycline for 4 days, and then seeded in 24 well plates in full medium. The cells were allowed to attach overnight, and then starved for 24 hours in 5 % charcoal-stripped or serum-free (with 10 mM Hepes; 1 μ g/ml transferrin; 1 μ g/ml fibronectin; 200 μ M glutamine; 1x trace element Biofluids Division, BSI, Rockville Maryland), phenol red-free medium. The cells were then cultured at 37 $^{\circ}$ C for 0 to 8 days with different concentrations growth factors or estrogen. Cells were harvested every other day and the MTS assay was done as described above to measure proliferation. Each data point was performed in quadruplet, and the results were reported as mean absorption +/- standard error.

Nude Mouse Xenograft Experiments

96 athymic Balb/C nude mice (Harlan Teklad, Madison, Wisconsin) were randomized to four groups. Estrogen pellets (Innovative Research of America, Sarasota, Florida) were injected into all animals to stimulate the development and growth of tumors. The next day, mice from each group were injected into the fat pad with approximately 5×10^6 cells/mouse of four different MCF-7 clones (MCF-7 Tet-Off TAM67 clones #62, #67 and vector clones #1, #3), and fed with Doxycycline-containing water (200 μ g/ml). After tumors developed and reached the size of 100 mm ^{3} , the mice were randomized to receive doxycycline-free or doxycycline-containing water to induce or suppress the expression of TAM67. The tumor sizes were measured twice a week and tumor volumes were estimated according to the formula: (long dimension) x (short dimension) ^{2} /2. Tumor growth rates of different groups were calculated and statistically analyzed as described below in Statistical Analysis.

Immunohistochemical Analysis

Tumor tissues were collected from sacrificed nude mice. The samples were fixed in 10% neutral buffered formaldehyde overnight and then embedded in paraffin. Tissue sections were then mounted on slides and processed for either hematoxylin & eosin staining or immunohistochemical staining. For immunohistochemical studies, tissue sections were cut at 4 μ m and mounted onto slides. Slides were deparaffinized, and then endogenous peroxidase was blocked with 0.1% sodium azide in 3% hydrogen peroxide in 1 x auto buffer. Slides were then rinsed in PBS, and nonspecific binding was blocked with 10% albumin. Because the TAM67 gene was FLAG-tagged, an anti-FLAG antibody was employed as first antibody (1:10,000, M2, Sigma, St. Louis, Missouri), followed by a biotinylated rabbit anti-mouse antibody (1:100), and peroxidase activity was visualized using DAB chromagen intensified with 0.2% osmium tetroxide. For immunohistochemical staining of phospho-Histone H3, the anti-phospho-Histone H3 monoclonal antibody (1:400, Upstate, Lake Placid, New York) was employed, followed by biotinylated anti-rabbit antibody (1:200). The slides were counterstaining with 1 % methyl green.

TUNEL Assay

Paraffin-fixed tumor tissues were cut at 3-4 microns and mounted to slides. The slides were baked overnight at 58°C and deparaffinized, and were digested in proteinase K for 15 min at 37°C. 3% hydrogen peroxide was used to block endogenous peroxidase. The slides were incubated with avidin solution, then biotin solution for 15 min respectively, and then incubated with TdT reaction cocktail (TdT (Roche, Indianapolis, Indiana), 1:400; Manganese cation, 1:50; d-UTP-biotin 16, 1:100) for 2hrs at 37°C. Freshly prepared horseradish peroxidase labeled streptavidin (Dako, Carpinteria, California) at a 1:200 was added, and peroxidase activity was visualized using DAB chromagen. Counterstaining was done with 0.05 % methyl green.

Statistical Analysis

Tumor growth *in vivo* was approximately exponential, but varied slightly from animal to animal. To compare the growth rates of tumors in animals treated with Dox or not, we estimated individual growth rates by linear regression of logtransformed tumor volumes on time, and then compared the growth rates by student's t-test. Growth rates were summarized by means and 95% confidence intervals.

Results

Expression of cJun dominant-negative mutant inhibits basal and induced AP-1 activity

TAM67 (Fig. 1a) is a mutated form of c-Jun that can specifically inhibit AP-1 activity in different cell types(23). Using this mutant, we previously isolated MCF-7 clones that express TAM67 under the control of an inducible promoter, the tet-off promoter(22). In the present study, we used these MCF-7-Tet-Off-TAM67 clones to determine the effect of AP-1 blockade on breast cancer growth *in vitro* and *in vivo*. First, the expression of TAM67 protein in independent clones was determined using Western Blotting (Fig. 1b). As can be seen, no TAM67 protein was expressed in the presence of doxycycline, while high levels of TAM67 protein were seen when the cells were cultured in the absence of doxycycline.

To demonstrate the effects of TAM67 on basal AP-1 activity and AP-1 activity induced by peptide growth factors and estrogen, we performed luciferase reporter assays using an AP-1-dependent reporter construct in the presence and absence of doxycycline in our MCF-7 TAM67 Tet-Off cell clones #62 and #67. For these experiments, the cells were starved of all growth factors and stimulated with the individual growth factors (EGF, IGF-1, HRG- β , bFGF or estradiol). These studies demonstrated that all tested growth factors stimulated AP-1 activity in MCF-7 cells (Fig. 1c). TAM67 repressed the basal level of AP-1 activity, and also inhibited AP-1 activity induced by each of these growth factors and estrogen (Fig. 1c).

TAM67 inhibits MCF-7 cell growth induced by serum and by polypeptide growth factors

We next examined whether TAM67 expression inhibited MCF-7 breast cancer cell growth induced by serum or by individual growth factors. First, we tested the effect of TAM67 on

serum-induced MCF-7 cell growth. Different concentrations (0.5, and 5.0 %) of fetal bovine serum were used to stimulate the growth of serum-starved MCF-7 Tet-Off TAM67 clone cells (#62), and vector-transfected cells (clone #1), in the presence or absence of doxycycline. We found the expression of TAM67 after the removal of Dox from the culture medium totally inhibited MCF-7 cell growth stimulated by fetal bovine serum. These cells proliferated normally after serum stimulation in the presence of Dox (#62 +Dox). Vector transfected cells grew well after serum stimulation in the presence or absence of Dox (Fig. 1d).

To determine whether AP-1 blockade inhibited proliferation induced by specific peptide growth factors, we treated the MCF-7 cells with different concentrations of peptide growth factors to stimulate cell growth in the presence or absence of Dox (EGF, 0-100 ng/ml; IGF-1, 0-100 ng/ml; heregulin- β , 0-10 ng/ml; bFGF, 0-10 ng/ml). The results from these experiments showed that all these peptide growth factors stimulated the proliferation of both vector- and TAM67-transfected MCF-7 cells (Fig. 2). When the MCF-7 Tet-Off TAM67 cells were cultured in the absence of Dox, TAM67 was induced and the cell growth stimulated by growth factors EGF, IGF-1, heregulin- β , bFGF was totally inhibited (Fig. 2a, 2b, 2c and data not shown for bFGF). The vector-transfected cells responded equally well to these growth factors in the presence or absence of Dox (Fig. 2).

TAM67 partially inhibited estrogen-induced MCF-7 cell growth

MCF-7 breast cancer cells express the estrogen receptor and proliferate in response to estrogen stimulation. This proliferative response is caused by estrogen activating the estrogen receptor, which may or may not involve the AP-1 transcription factor. AP-1 and the estrogen receptor have been previously shown to affect each other through transcription factor cross talk (13, 17, 24, 25). To investigate the effect of AP-1 blockade on estrogen-induced gene expression, we

used the Vit-ERE-TK-Luc reporter construct which has a classical ERE upstream of the TK promoter and luciferase gene to measure estrogen-induced gene expression. The TAM67 clones #62, #67 and Vector clones #1, #3 were transfected with this estrogen-inducible construct, and were treated or not treated with estrogen in the presence and absence of Dox. All these clones responded well to estradiol, both in the presence and absence of Dox (Fig. 3a). The results demonstrated that AP-1 blockade induced by expression of TAM67 did not block estrogen receptor transactivating activity. Thus, the inhibition of estrogen-induced growth by TAM67 is likely due to blocking signals independent or downstream of classical estrogen-induced transcription.

To investigate whether AP-1 blockade inhibits estrogen-induced growth, we measured the proliferative response to estrogen using our MCF-7 Tet-Off TAM67 cell line. MCF-7 Tet-Off TAM67 cells and vector-transfected cells were treated with different concentrations of estradiol ($0-10^{-9}$ M) to stimulate cell growth in the presence or absence of Dox. The results from these experiments showed that estrogen stimulated the proliferation of MCF-7 cells and that the growth was dose-dependent both in the vector clone and the TAM67-expressing clone (Fig. 3b). When the MCF-7 Tet-Off TAM67 cells were cultured in the absence of Dox, TAM67 was induced and the cell growth stimulated by estrogen was suppressed. However, while TAM67 totally inhibited peptide growth factor-induced growth, it did not totally block estrogen-induced growth. (Fig. 3b).

TAM67 inhibits MCF-7 xenograft tumor growth in nude mice

Since TAM67-induced AP-1 blockade inhibited MCF-7 cell growth, we next investigated whether TAM67 could also inhibit breast tumor growth *in vivo* in nude mice. For these experiments, we utilized two MCF-7 Tet-Off TAM67 cell clones (clones #62, #67) and two vector clones (#1, #3). These cells were injected into the mammary fat pad of nude mice that received estrogen pellets to stimulate the development and growth of tumors as described in Materials and Methods. After the

tumors developed in nude mice and the tumor sizes were greater than 100 mm³, we randomized the mice of each group to either receive or not receive doxycycline to suppress or induce the expression of TAM67. Tumor size was then measured. The size of the tumors as a function of time is shown in Figs. 4a and 4b. As can be seen, tumors from vector-transfected cells grew rapidly when the mice were fed with water containing or not containing Doxycycline (Fig. 4a). In the presence of Dox, the TAM67 tumors also grew well. However, when the mice were fed with water without Dox, the tumor growth was dramatically reduced (Fig. 4b).

We also calculated and compared the growth rates of tumors in each group. There was no significant difference in the average growth rate of the vector-transfected clones treated with or without Dox (Fig. 4c). However, in both TAM67-transfected clones, tumor growth rates were significantly lower in the absence of Dox. These results demonstrate that AP-1 blockade in established breast tumors suppresses their growth *in vivo*.

We next examined the histologic appearance of these tumors. No obvious necrosis was observed when TAM67 was induced (Fig. 4d). We can see strong expression of TAM67 (as seen by immunohistochemical staining for the FLAG tag) in tumor tissues collected from TAM67-mice that were fed with water without Dox, while in tumor tissues from TAM67 mice that were fed with doxycycline-containing water and from Vector-mice, there was no expression of TAM67.

TAM67 inhibits proliferation without inducing apoptosis

To better understand the mechanism by which AP-1 blockade affects the growth of breast cancer cells, we investigated whether TAM67 inhibited proliferation or induced apoptosis in the mouse tumor tissues. Phosphorylation of histone H3 correlates closely with mitosis (26, 27). Thus, we chose immunohistochemical staining using anti-phospho-Histone H3 to determine proliferation

in the tumor tissues. There were fewer cells expressing phospho-Histone-H3 in tumors expressing TAM67, compared to tissues not expressing TAM67, both in TAM67 clones #62 and #67 (Fig. 5a). These differences were statistically significant (Fig. 5b).

We next used the TUNEL assay to measure apoptosis in the tumor tissues. Tumors isolated from mice injected with vector clones and TAM67 clones, and grown in the presence or absence of doxycycline, were studied. We observed no differences of apoptotic rates in any of these tumor tissues (Fig. 5c).

Discussion

The above results demonstrate that expression of a cJun dominant negative protein inhibits peptide growth factor-induced activation of the AP-1 transcription factor, and inhibits breast cancer cell growth. In addition, the data show that this AP-1 inhibitor also suppresses estrogen-induced growth of breast cancer cells. AP-1 blockade suppressed the growth of breast cancer cells both *in vitro* and *in vivo* in nude mice. The present results show that this suppression of tumor growth was caused by inhibition of proliferation without inducing apoptosis. These results demonstrate that mitogenic signal transduction in breast cancer cells can be blocked at a distal point at which signals from multiple peptide growth factors and estrogen converge. By blocking signal transduction at the point where these multiple signals converge, one can potentially overcome the problems of receptor downregulation or utilization of alternative growth factor pathways that can occur with other agents that target individual growth factor pathways.

Previous studies have demonstrated that the AP-1 transcription factor is an important regulator of proliferation, transformation, and apoptosis, depending on the cell type. We and others have used the cJun dominant negative mutant, TAM67, to investigate the role of AP-1 in several different cell types. These studies have shown that in fibroblasts, AP-1 is an important regulator of proliferation and transformation (28, 29). Other studies, done in neuronal cells and in hematopoietic cells, show that AP-1 regulates apoptosis (30, 31). The current study demonstrates that in breast cancer cells, AP-1 is a critical regulator of proliferation.

We and others have investigated the function of AP-1 in normal and malignant breast cells. These previous studies have shown that AP-1 family members are expressed in normal and malignant breast cells, that peptide growth factors and estrogen induce AP-1-dependent

transcriptional activation, and that the anti-estrogen, tamoxifen, can also activate the AP-1 transcription factor. Increased AP-1 activity in breast cancer cells can also lead to tamoxifen resistance. Thus, overexpression of cJun induces tamoxifen resistance in MCF7 breast cancer cells (18). In addition, selection for tamoxifen resistance leads to upregulation of AP-1 activity in breast cancer cells. Dumont and coworkers isolated a hormone-resistant clone of MCF7 cells, that were found to be tamoxifen-resistant and have increased AP-1 activity (32). We have also shown that MCF7 xenografts that acquire tamoxifen resistance by being chronically treated with tamoxifen in vivo, develop increased AP-1 activity at the time they develop tamoxifen resistance by increasing the expression and activity of the cJun activating kinase, JNK (20). Studies of the expression and activity of AP-1 in human breast tumors also demonstrate that AP-1 activity is increased in tamoxifen-resistant breast cancer cells. Johnson and co-workers showed that AP-1 DNA binding activity and JNK activity were increased in tamoxifen-resistant human breast cancers as compared to untreated ER-positive breast cancers (19). All of these results show that the AP-1 transcription factor is an important transducer of mitogenic signals in breast cells.

The results reported here represent the first direct demonstration that the AP-1 transcription factor is essential for mitogenic signal transduction induced by many different growth factors (EGF, TGF α , heregulin, bFGF, IGF-1, and estrogen). A possible mechanism for this general block of proliferative signals is shown in Figure 6. As shown in this figure, peptide growth factors bind their respective membrane bound receptors, and activate cytoplasmic signal transduction cascades. These signals are transduced to the nucleus where AP-1 is activated by phosphorylation, and AP-1-dependent genes are induced. TAM-67 is able to block AP-1 activity, block the expression of these AP-1-dependent genes, and ultimately block proliferation induced by these peptide growth factors. In breast cancer cells treated with estrogen, estrogen is able to bind to the

estrogen receptor, and activate estrogen receptor-dependent genes, either through the "classical pathway" of ER-regulated genes, or through a "non-classical pathway" that activates genes that do not have classical EREs within their promoters. The expression of some of these genes, particularly those with an ERE within their promoter (the classical pathway), may not be directly affected by the expression of TAM-67. However, it is possible that these estrogen-induced genes include peptide growth factors or their receptors. In that case, TAM-67 could inhibit the estrogen-induced signal transduction indirectly by inhibiting the subsequent peptide growth factor signals.

Another way TAM-67 could inhibit estrogen-induced proliferation is by blocking the expression of estrogen-induced genes that use the "non-classical pathway" of estrogen regulated genes (see Figure 6). Some genes activated by estrogen do not have classical estrogen response elements, but instead have AP-1 sites, within their promoters (13, 25, 33, 34). Expression of these genes is induced by estrogen binding to the estrogen receptor, which then binds to and activates AP-1 transcription factors. These activated AP-1 complexes bind to the AP-1 sites and induce the expression of these "estrogen-induced" genes. We predict that TAM-67 would inhibit the expression of such estrogen-induced, AP-1-dependent genes. Thus, TAM-67 may inhibit estrogen-induced growth by inhibiting the expression of a subset of estrogen-induced, AP-1-dependent genes that are involved in regulating proliferation.

Given the potent ability of TAM-67 to block peptide hormone-induced breast cell growth, it may be possible to combine agents that block AP-1 with anti-estrogens to obtain total signal transduction blockade. In that case, peptide hormone mitogenic pathways, non-classical ER pathways, and classical ER pathways would all be blocked. Such total blockade may be the most effective way to suppress breast cancer growth and avoid the outgrowth of resistant breast cancer clones.

The AP-1 inhibitor described in these studies would be difficult to develop as a therapeutic agent for the treatment of breast cancer. It might have significant toxicity, and it would need to be delivered to breast cancer cells via gene therapy techniques. A more practical application of the present results would be to use small molecule inhibitors of the upstream activating kinases to block AP-1 activation. Such kinases would include either Jun-N-terminal kinases or MAP kinases. Small molecule inhibitors of these kinases are now being developed and are currently being testing in Phase I trials. Our results suggest that such agents either alone, or in combination with anti-estrogens, have significant promise for the treatment and prevention of breast cancer.

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Figure Legends:

Fig. 1a: Schematic diagram of the c-Jun and TAM67 proteins.

Fig. 1b: Expression of TAM67 in MCF-7 Tet-Off TAM67 clones #62 and #67 measured by Western blot as described in "Materials and Methods". The cells were cultured in the medium without Dox for 0-7 days, and lysates prepared and loaded onto the gel. A Western blot to actin was used to demonstrate equal loading.

Fig. 1c: TAM67 inhibition of basal, peptide growth factor-induced, and estrogen-induced AP-1 activity in MCF-7 Tet-Off TAM67 clone #62. The cells were cultured in the presence or absence of Dox for 5 days to suppress or induce the expression of TAM67. The cells were then starved of all growth factors for 2 more days (in the presence or absence of Dox). The cells were then transfected with and AP-1 dependent reporter, and then stimulated with different growth factors for 6 hours. Cell lysates were prepared, and luciferase assays were performed as described in "Materials and Methods".

Fig. 1d: TAM67 inhibition of serum-induced cell growth. MCF-7 Tet-Off TAM67 cells (clone #62) were cultured in the presence or absence of Dox for 5 days, after which time the cells were starved of fetal bovine serum for 2 more days. The cells were then stimulated with serum (at 0.5 % and 5 % concentrations) and cell growth was determined as described in "Materials and Methods". MCF-7 Tet-Off Vector clone #1 was used as control. *indicates statistical significant difference ($P < 0.05$).

Fig. 2. TAM67 inhibition of peptide growth factor-induced cell growth. MCF-7 Tet-Off TAM67 cells (clone #62) or MCF Tet-Off vector (clone #1) were cultured in the presence or absence of Dox

for 5 days, after which time the cells were starved of all growth factors for 2 more days (in the presence or absence of Dox). The cells were then stimulated with different concentrations of growth factors. Growth was determined as described in "Materials and Methods". **Fig. 2a:** MCF-7 Tet-Off vector cells stimulated with EGF; **Fig. 2b:** Cells stimulated with IGF-1; **Fig. 2c:** Cells Stimulated with HRG- β .

Fig. 3. TAM67 partially inhibits estrogen-induced cell growth but does not block ER-induced transcriptional activity. **Fig. 3a:** Effects on ERE-activity. MCF-7 Tet-Off TAM67 clones #62, #67 and Vector clones #1, #3 were cultured in the presence or absence of Dox for 5 days, and were starved in phenol red-free medium with charcoal stripped serum for 2 more days. The cells were then transfected with an ERE-luciferase reporter, then were stimulated with 17- β -estradiol overnight. The luciferase reporter assays were performed according to the "Materials and Methods".

Fig. 3b: Effect on estrogen-induced growth. MCF-7 Tet-Off TAM67 cells (clone #62) or MCF-7 Tet-Off vector cells (clone #1) were cultured in the presence or absence of Dox for 5 days, after which time the cells were starved of all growth factors for 2 more days (in the presence or absence of Dox). The cells were then stimulated with different concentrations of 17- β -estradiol, and cell growth was determined as described in "Materials and Methods". MCF-7 Tet-Off Vector clone #1 was used as control.

Fig. 4: TAM67 inhibits MCF-7 xenograft tumor growth in nude mice. The MCF-7 Tet-Off TAM67 clones #62, #67 and vector clones #1, #3 were injected into Balb/C nude mice and maintained as described in "Materials and Methods". When the tumors reached 100 mm³, the mice were

randomized to either continue to receive Dox or not. Tumor growth was measured and is plotted as a function of time after randomization. Each line indicates the growth of each individual tumor.

Fig. 4a: Growth of MCF-7 Tet-Off vector cells in the presence or absence of doxycycline.

Fig. 4b: Growth of MCF-7 Tet-Off Tam67 cells in the presence or absence of doxycycline.

Fig. 4c: Average growth rates of the MCF-7 tumors in the presence (+) or absence (-) of doxycycline. P values to determine statistical significance are shown.

Fig. 4d: H&E staining and immunohistochemical staining for the Flag tag are shown to demonstrate the expression of TAM67.

Fig. 5: Immunohistochemical staining of phospho-Histone H3 and TUNEL assay in tumor tissues.

Fig. 5a: Tumor tissues from vector clones or TAM67 clones in the presence or absence of doxycycline were stained with anti-phospho-Histone H3 antibody as described in "Materials and Methods". (1) Vector clone (+) Dox; (2) Vector clone (-) Dox; (3) TAM67 clone (+) Dox; (4) TAM67 clone (-) Dox. **Fig. 5b:** The average percentage of phospho-Histone H3 positive cells in each clone is shown. *indicates statistical significant difference ($P < 0.05$). **Fig. 5c:** The percentage of TUNEL positive (apoptotic) cells of 2 Vector Clones and 2 TAM67 Clones in the presence or absence of Dox is shown. There is no difference in % apoptotic cells between tumors grown in the presence or absence of doxycycline.

Fig. 6. Proposed mechanism by which TAM67 blocks signal transduction In breast cancer cells, peptide growth factor pathways are activated by peptide growth factors binding to their receptors, which activate signal transduction kinases, such as ERK, JNK, p38 and PI3-K. These kinases in turn phosphorylate Jun and Fos proteins, causing activation of AP-1 and induction of genes regulating

proliferation. This pathway is blocked by TAM67 (left). For estrogen-induced growth, there appear to be at least two pathways. In the classical estrogen-induced pathway, estrogen binds to estrogen receptor, which then binds to estrogen responsive elements (ERE) in the promoter of target genes to cause cell growth. This pathway may not be blocked by TAM67 (middle). In the non-classical estrogen-induced pathway (right), estrogen binds to ER, ER interacts with AP-1 and induces the expression of AP-1-dependent genes that have AP-1 sites within their promoters, which promote cell growth. This pathway is likely blocked by TAM67.

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Figure 1A

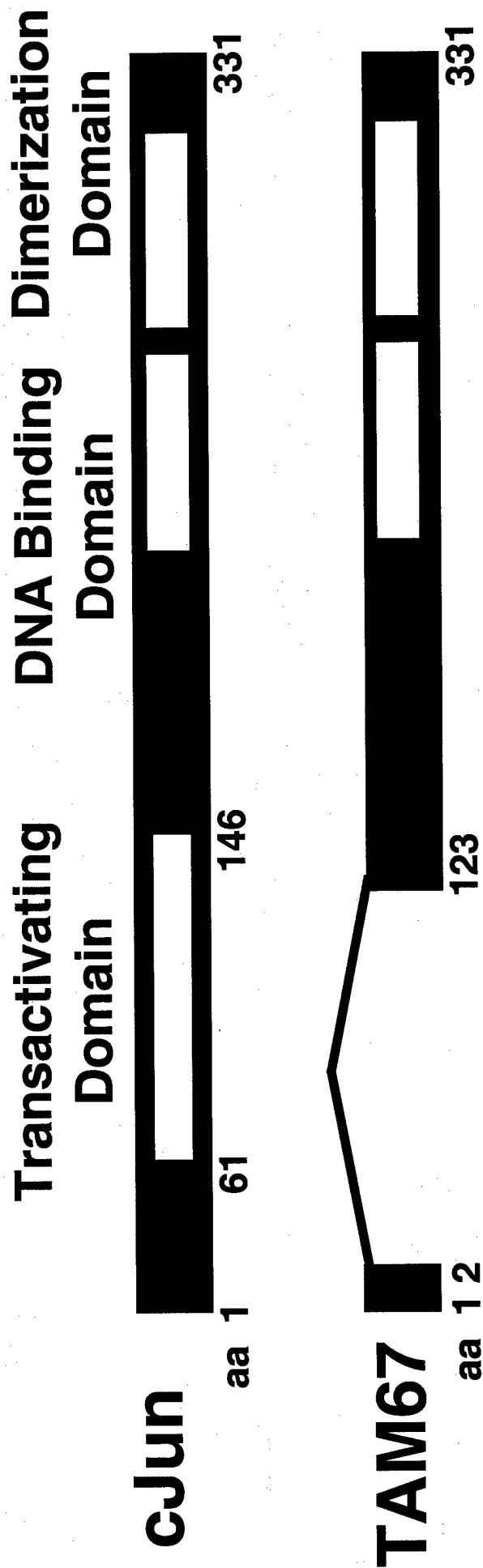


Figure 1B

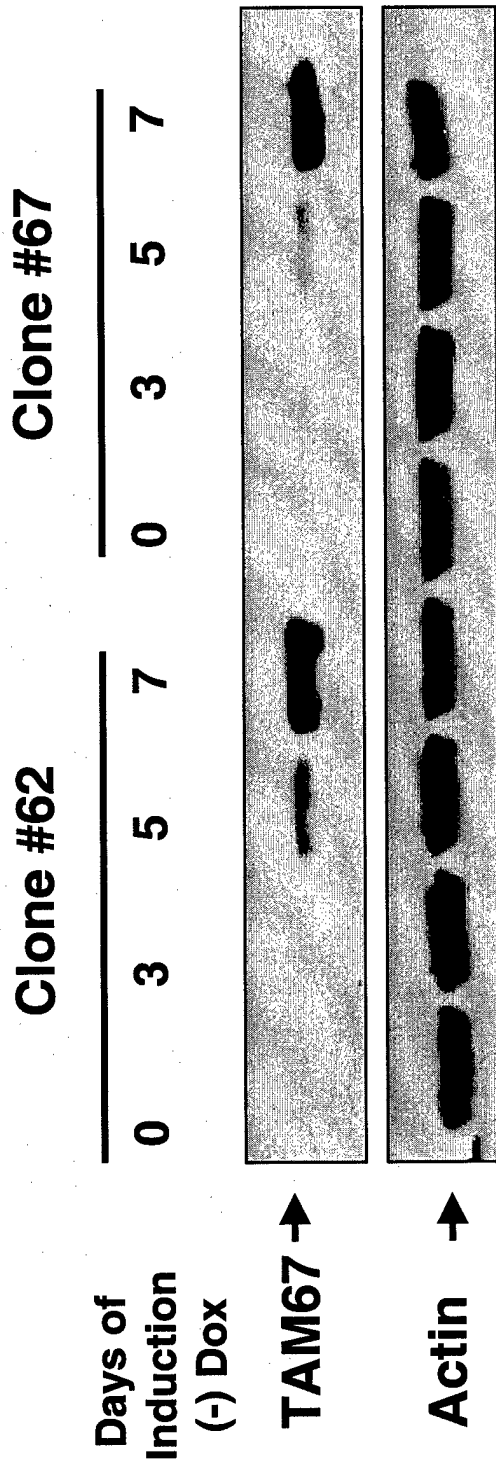


Figure 1C

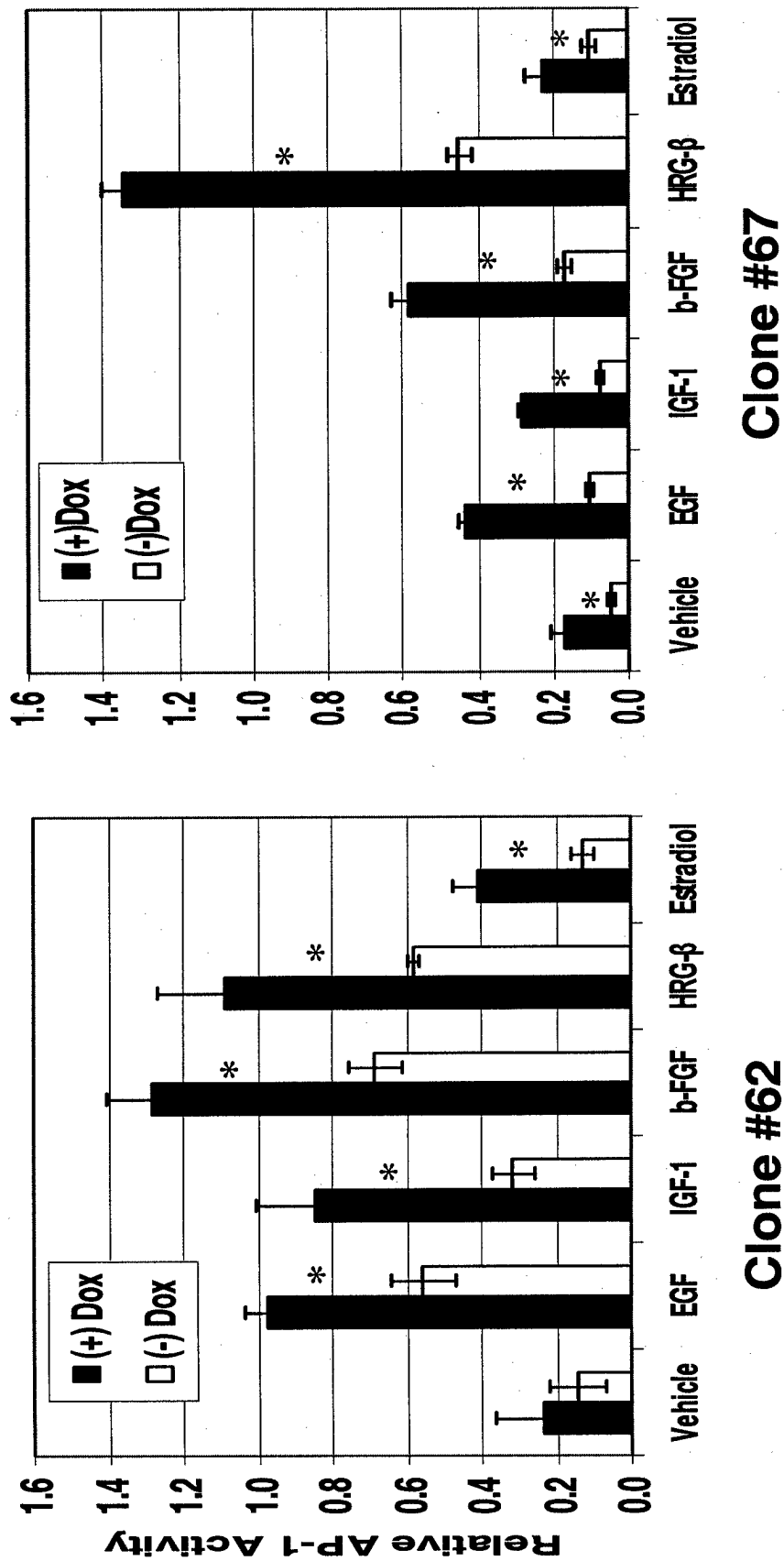
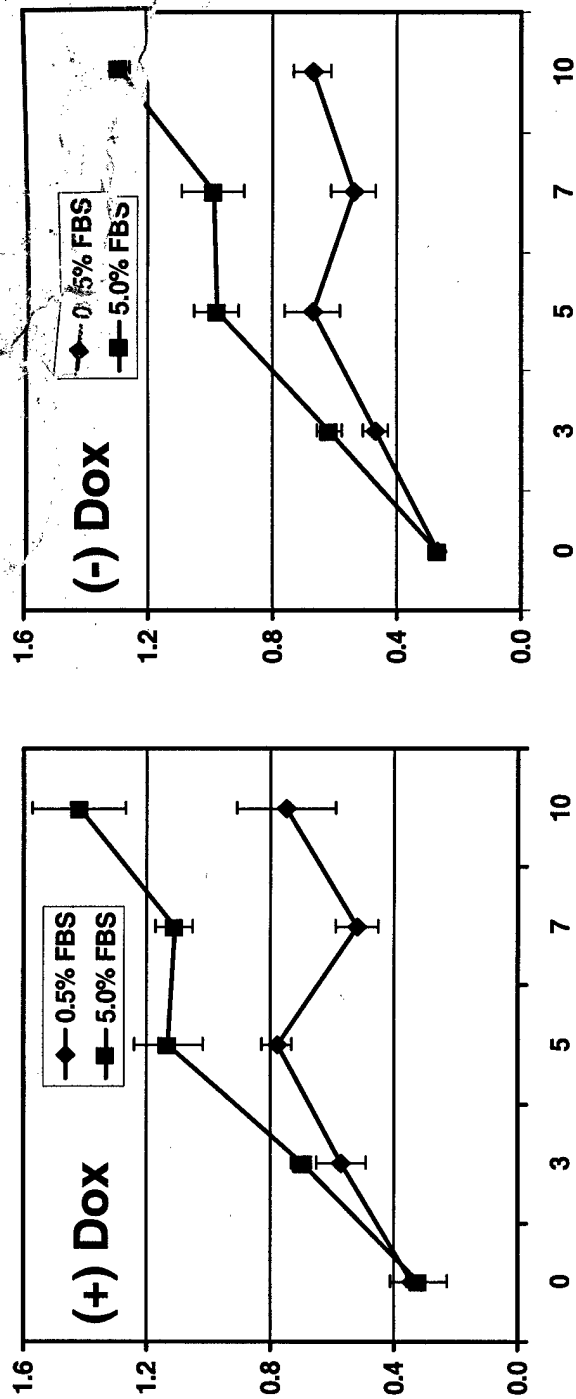


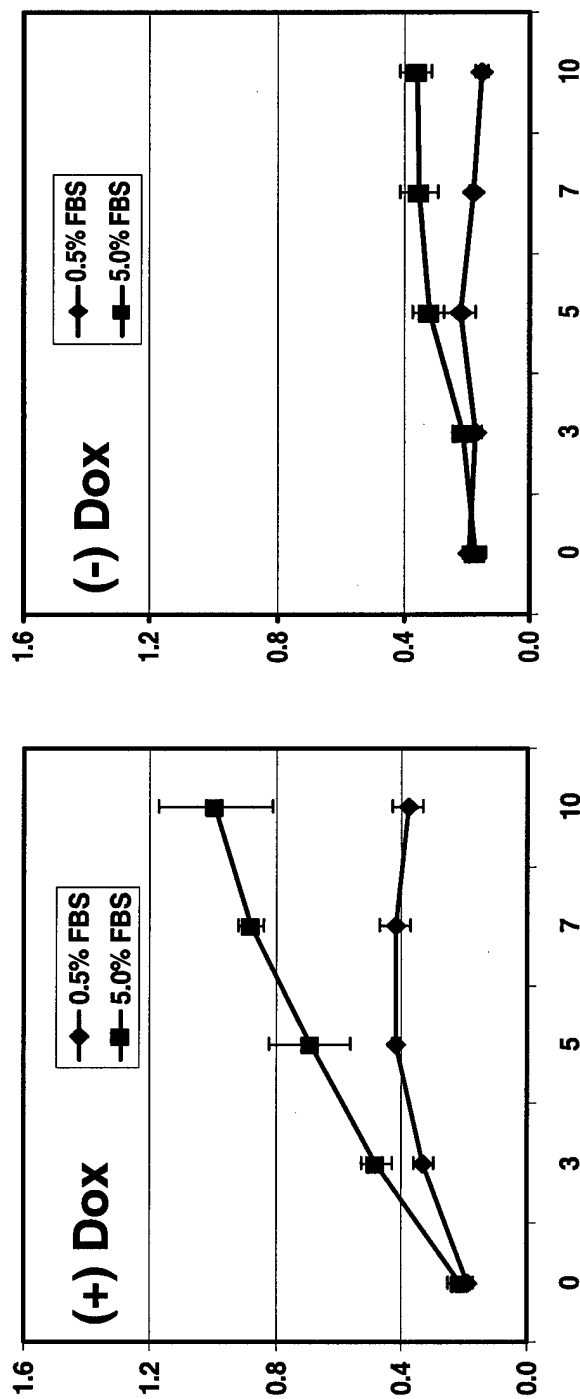
Figure 1D

Relative Cell Growth
(O.D. 490 nm)

MCF-7 Tet-Off Vector Clone #1



MCF-7 Tet-Off TAM67 Clone #62

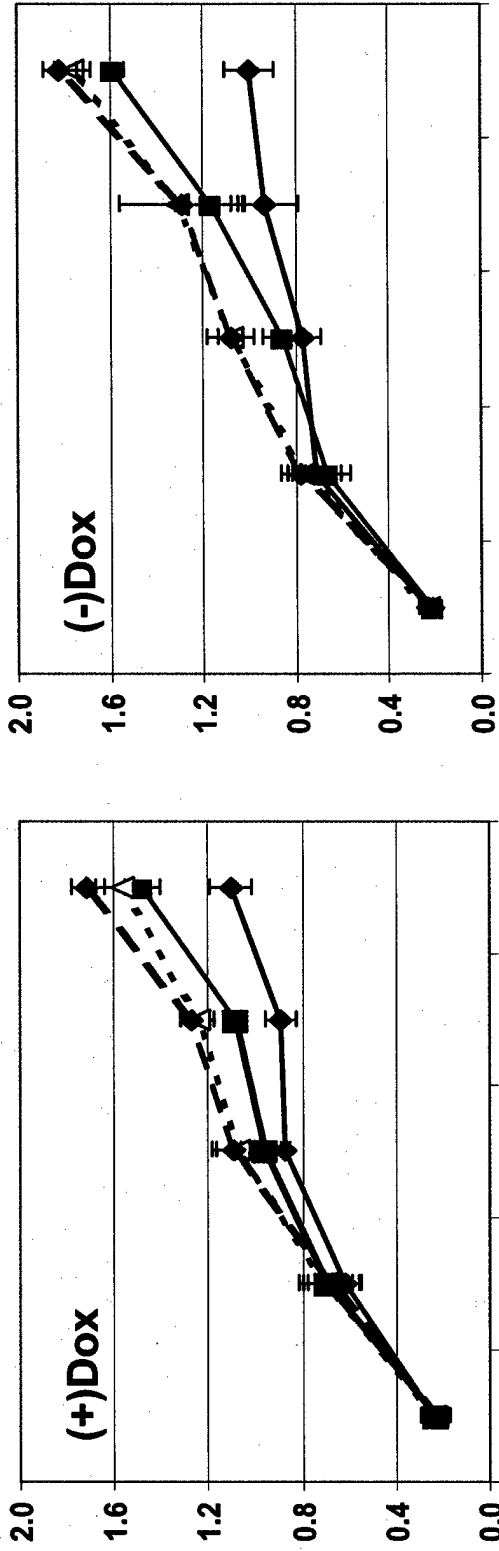


Days of Growth

Figure 2A

Relative Cell Growth
(O.D.=490 nm)

MCF-7 Tet-Off Vector #1



MCF-7 Tet-Off TAM67 #62

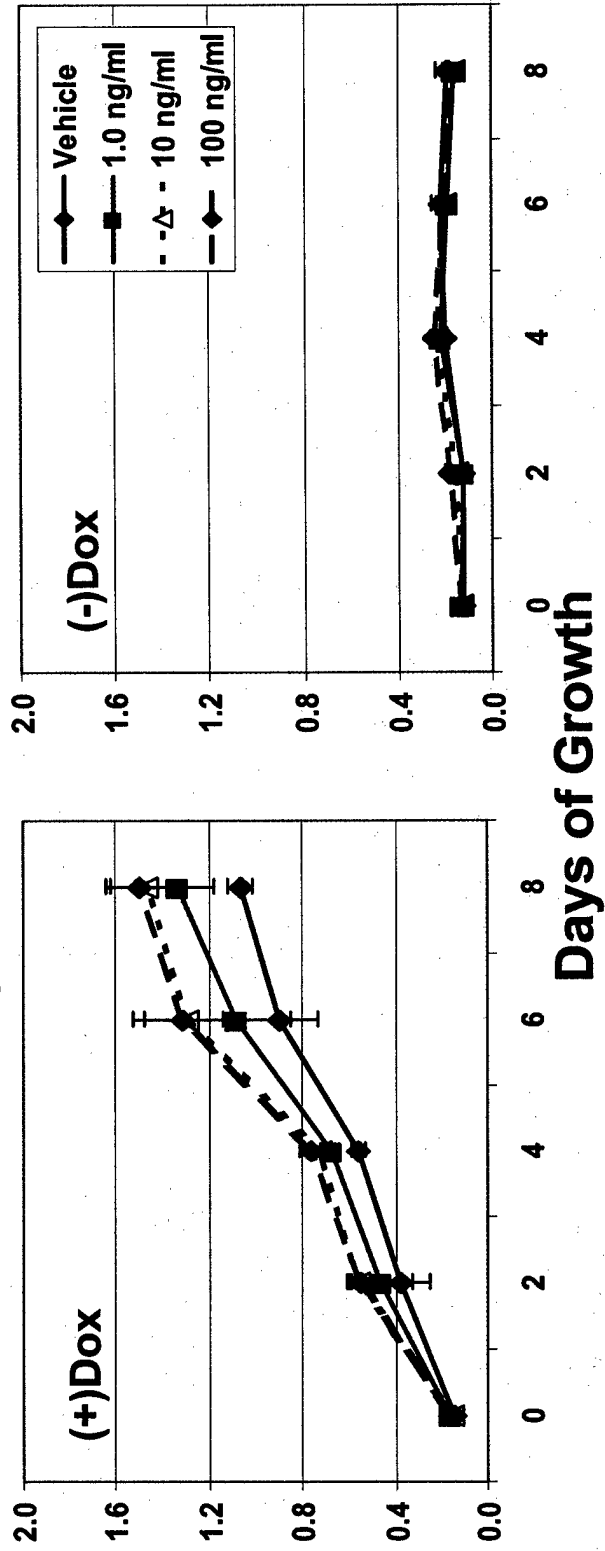
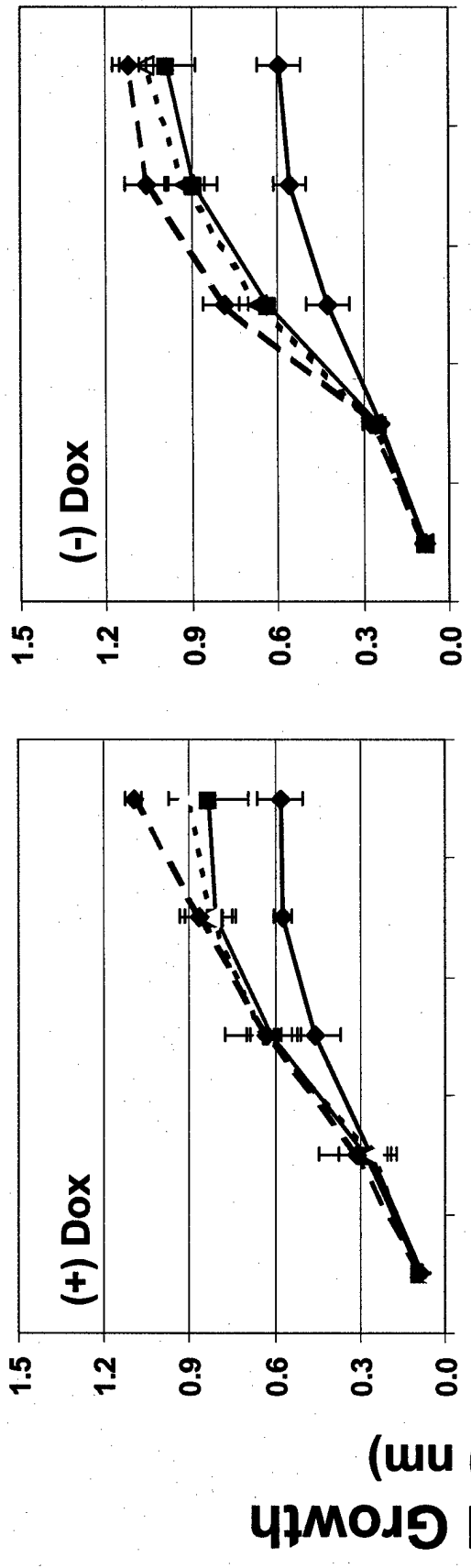
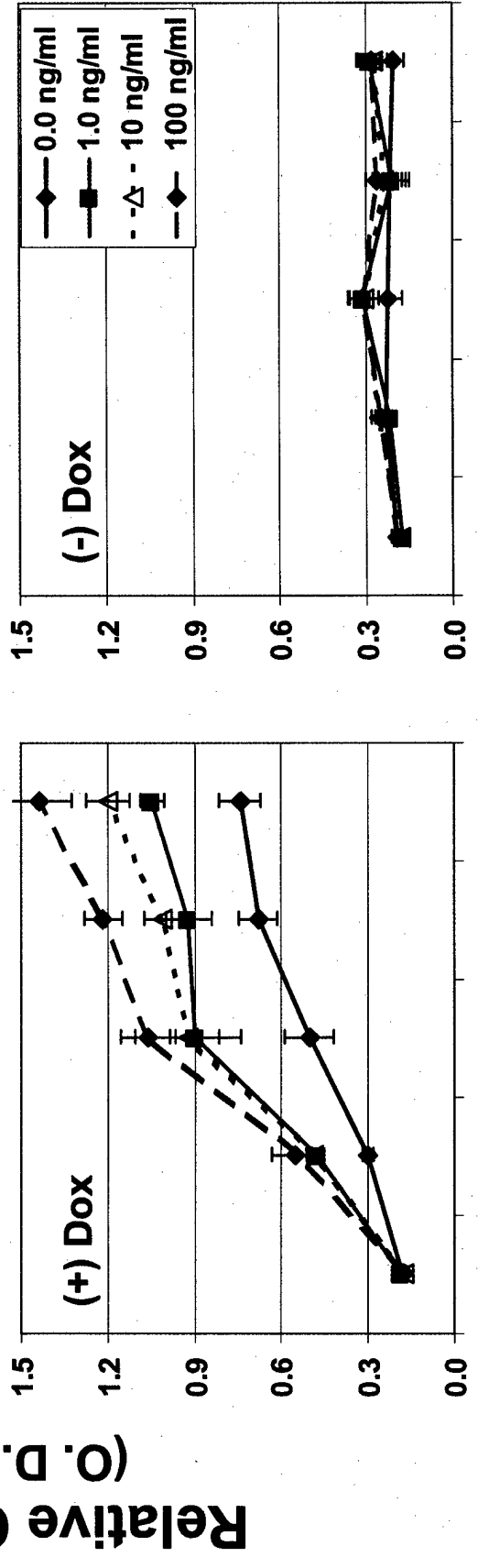


Figure 2B

MCF-7 Tet-Off Vector #1



MCF-7 Tet-Off TAM67 #62



Days of Growth

Figure 2C

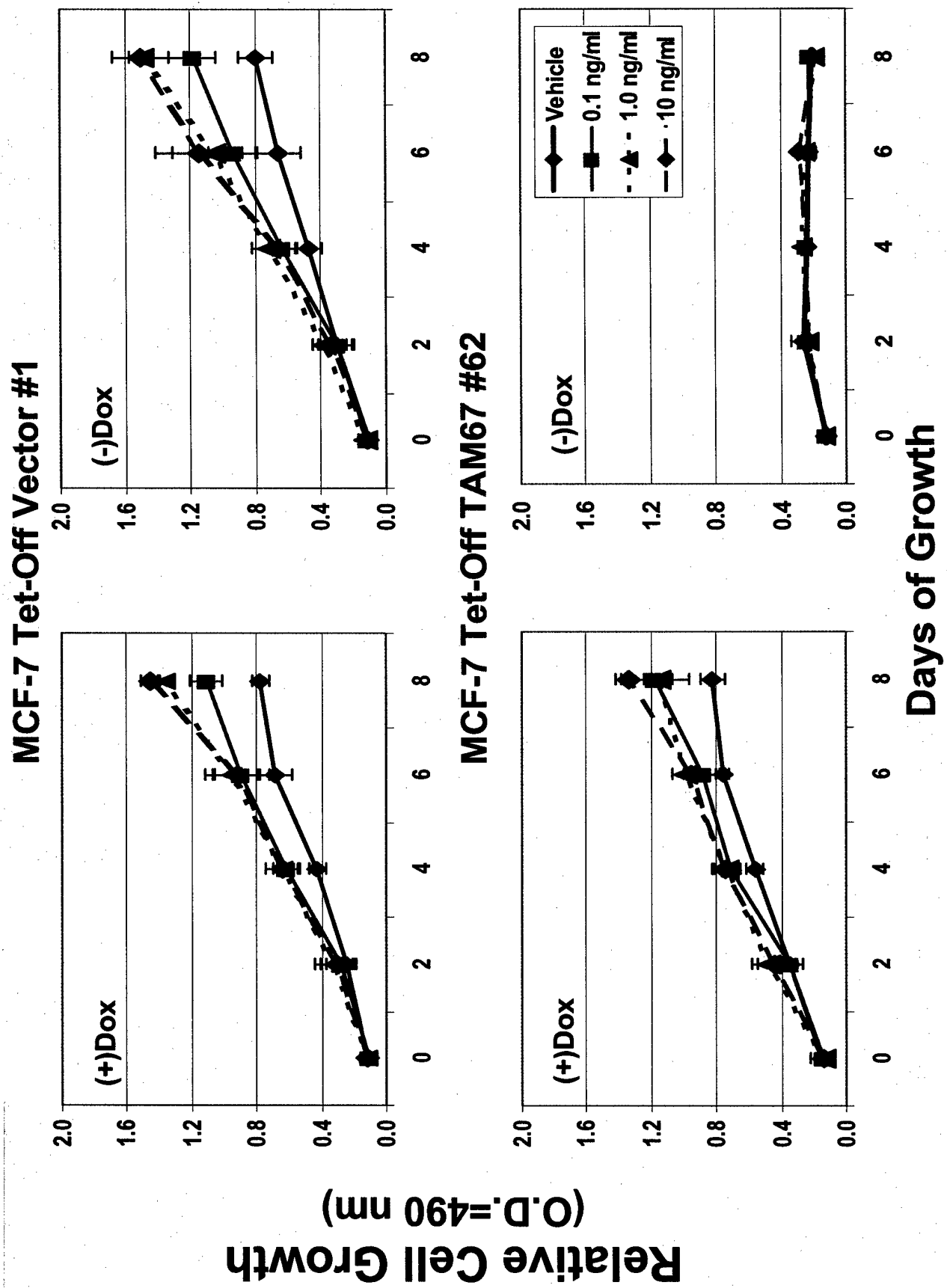


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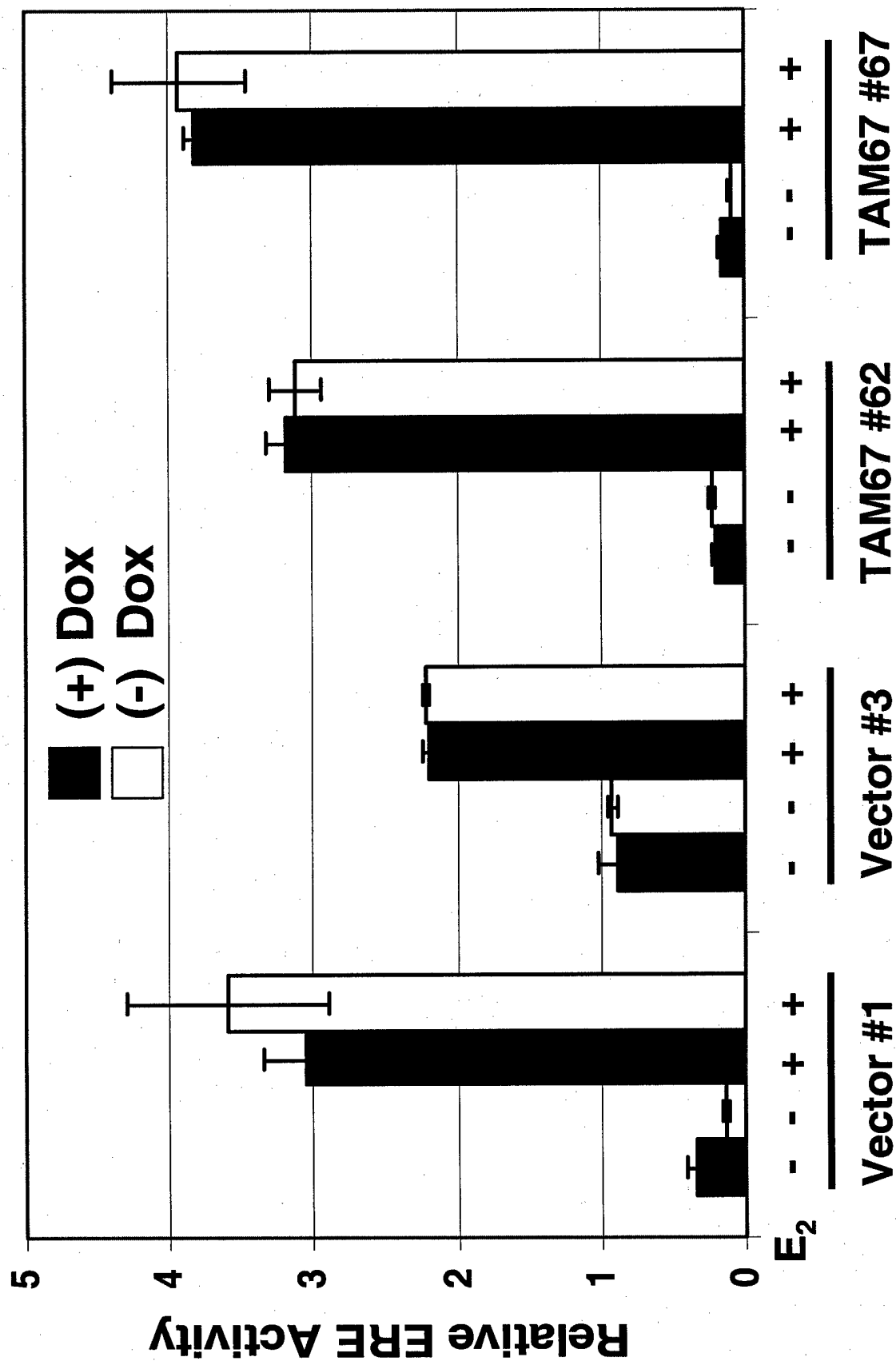


Figure 3B

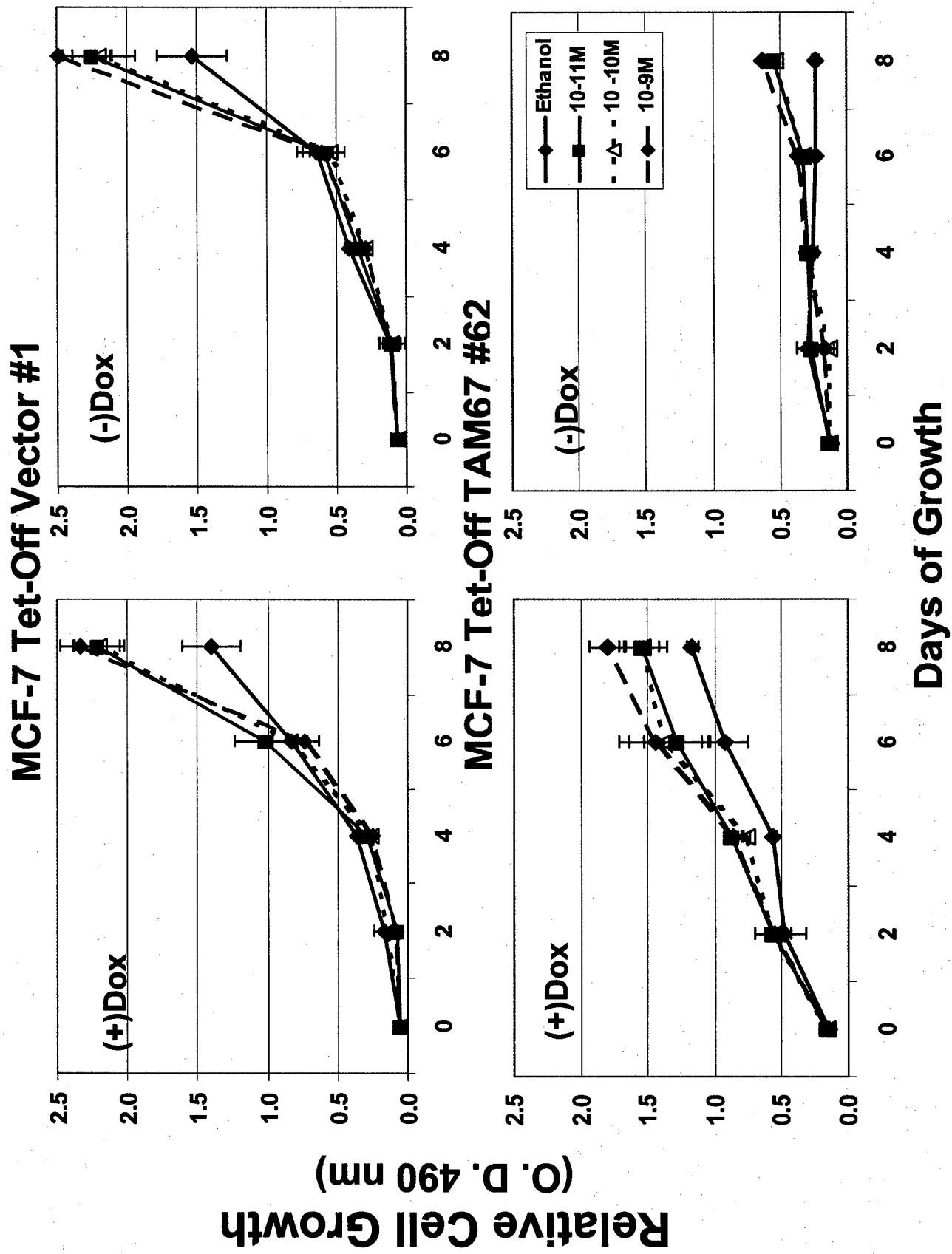


Figure 4A

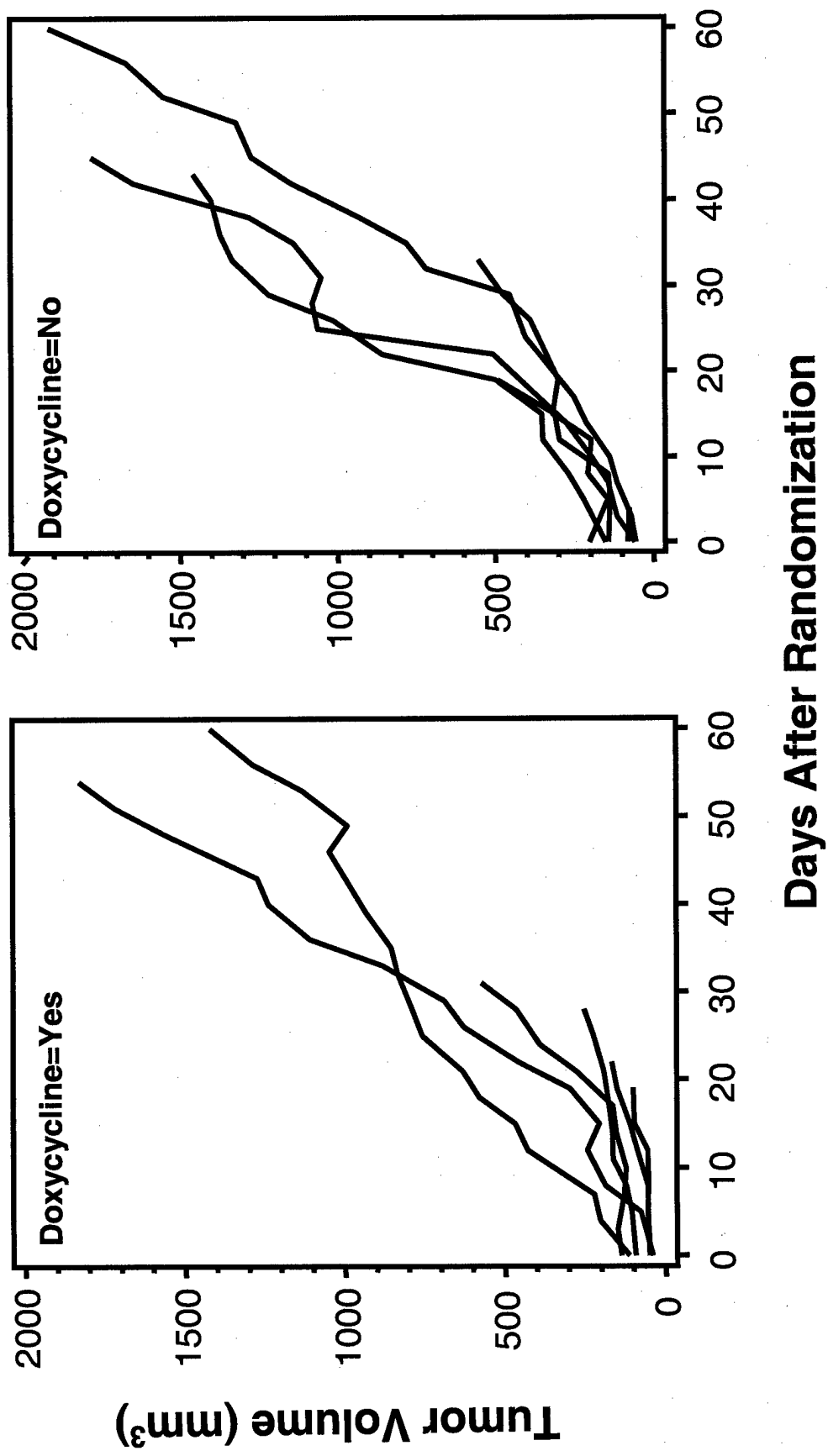


Figure 4B

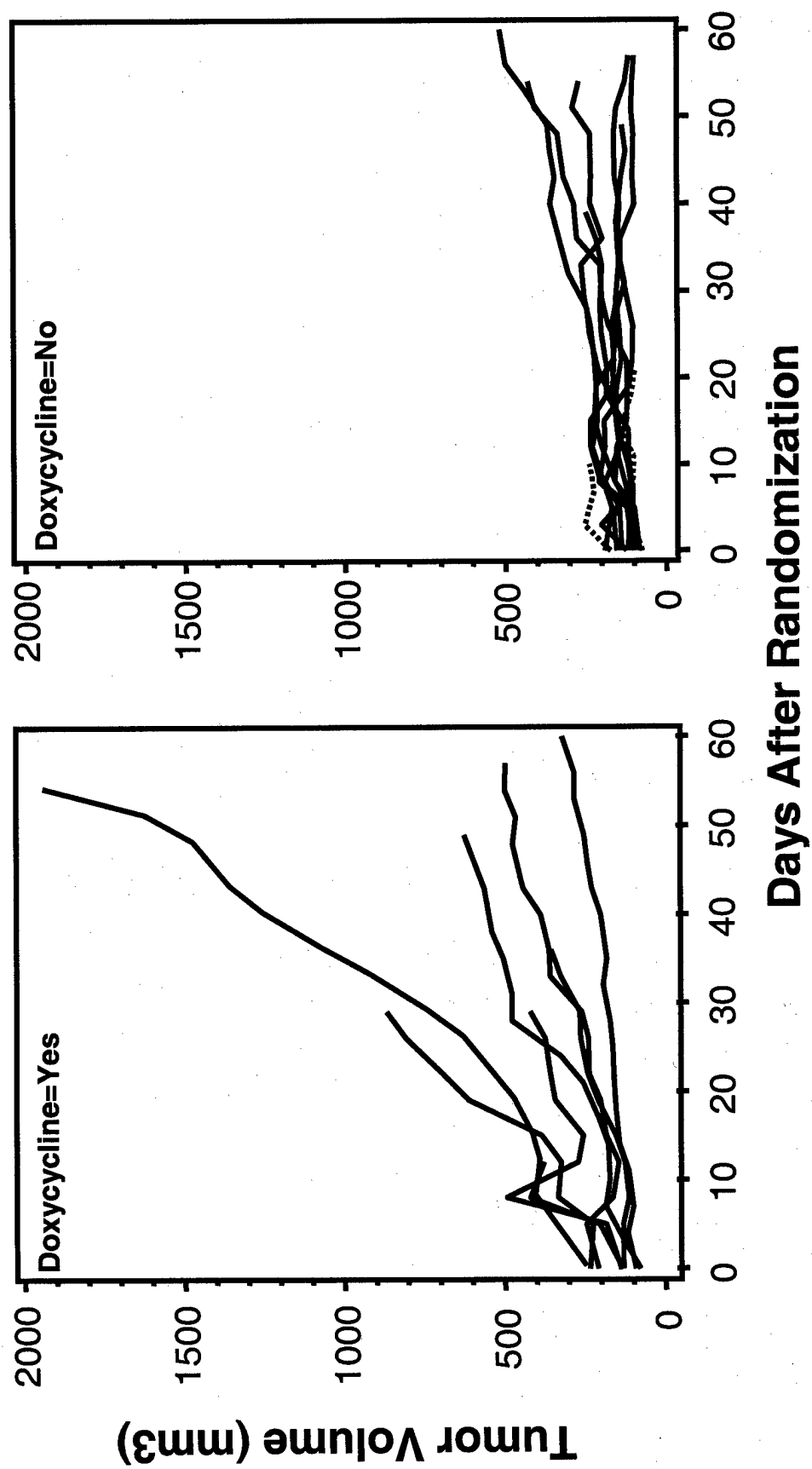


Figure 4C

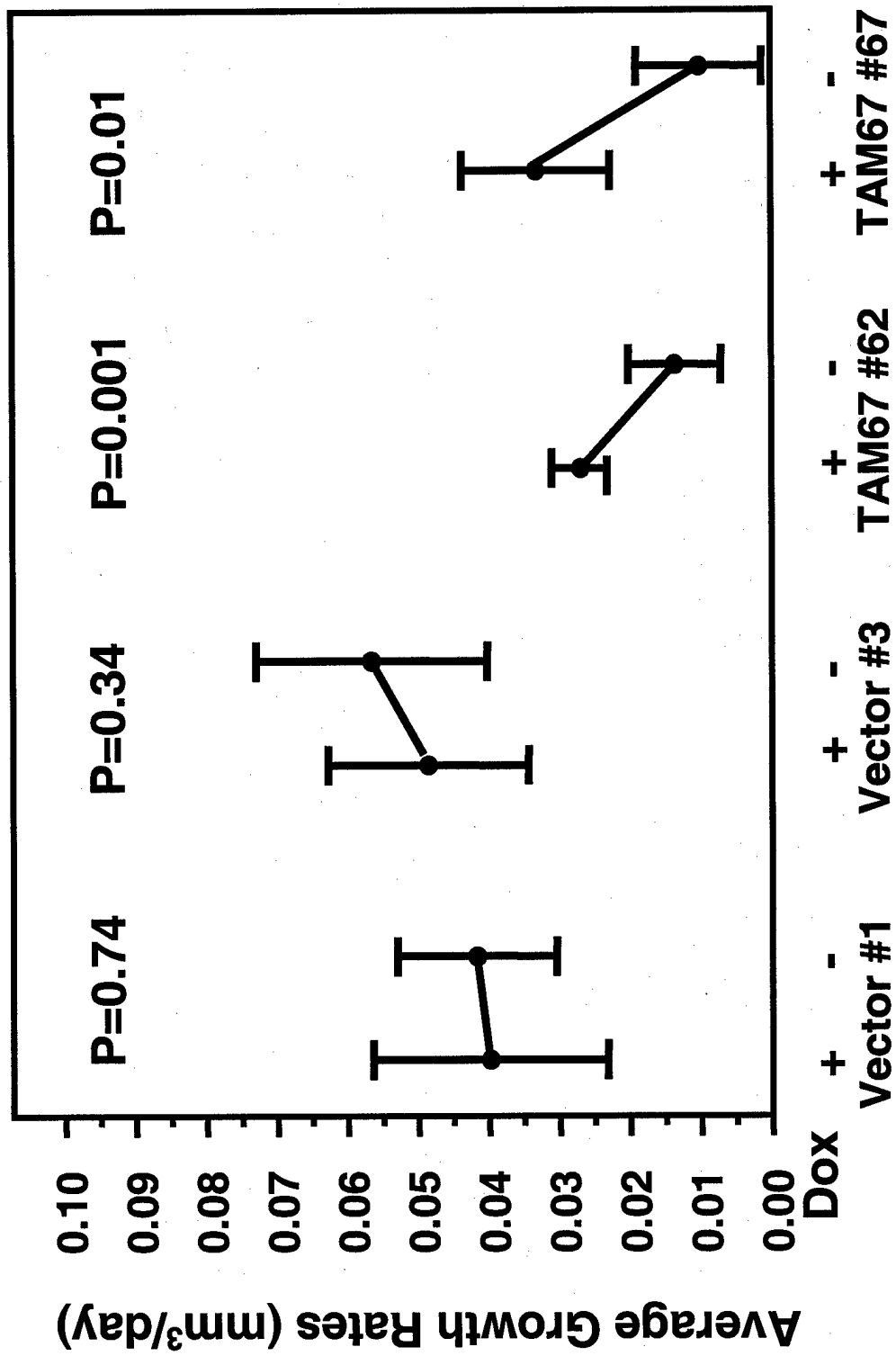
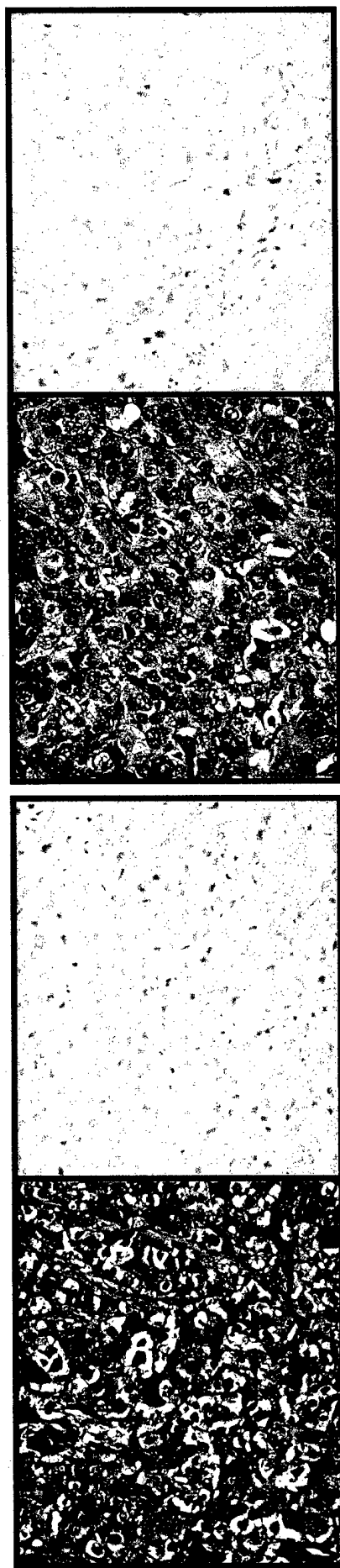
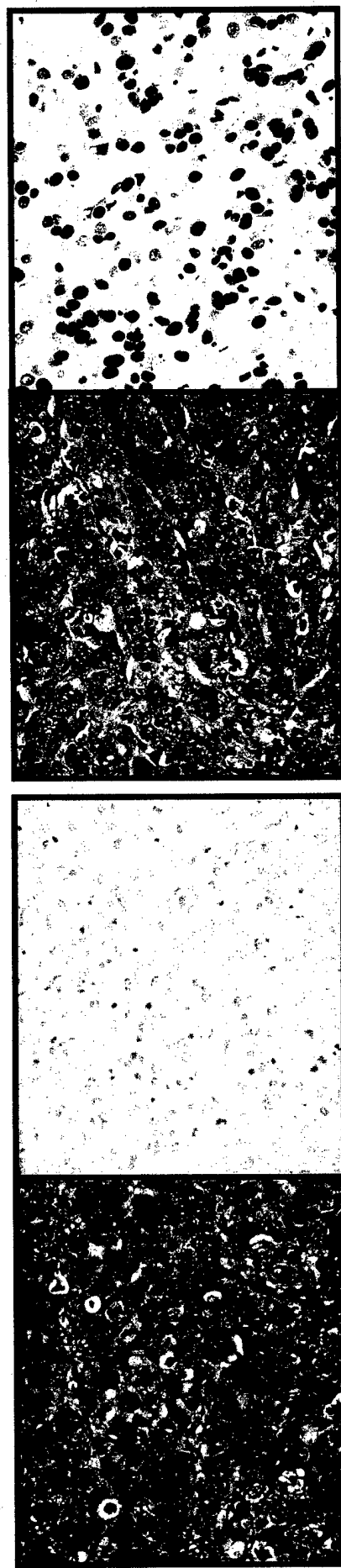


Figure 4D



Vector clone #1 (+) Dox **Vector clone #1 (-) Dox**



TAM67 #62 (+) Dox **TAM67 #62 (-) Dox**

Figure 5A

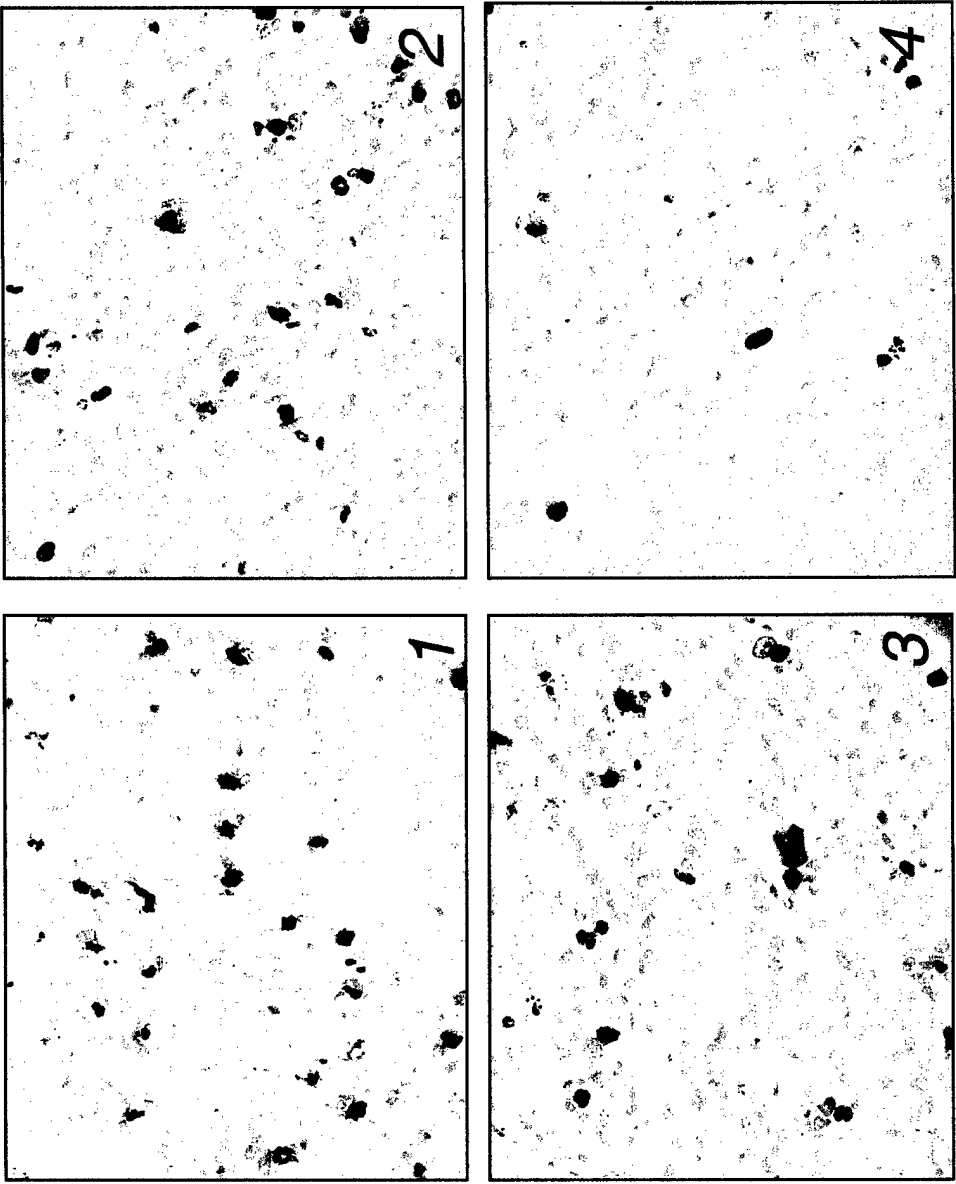


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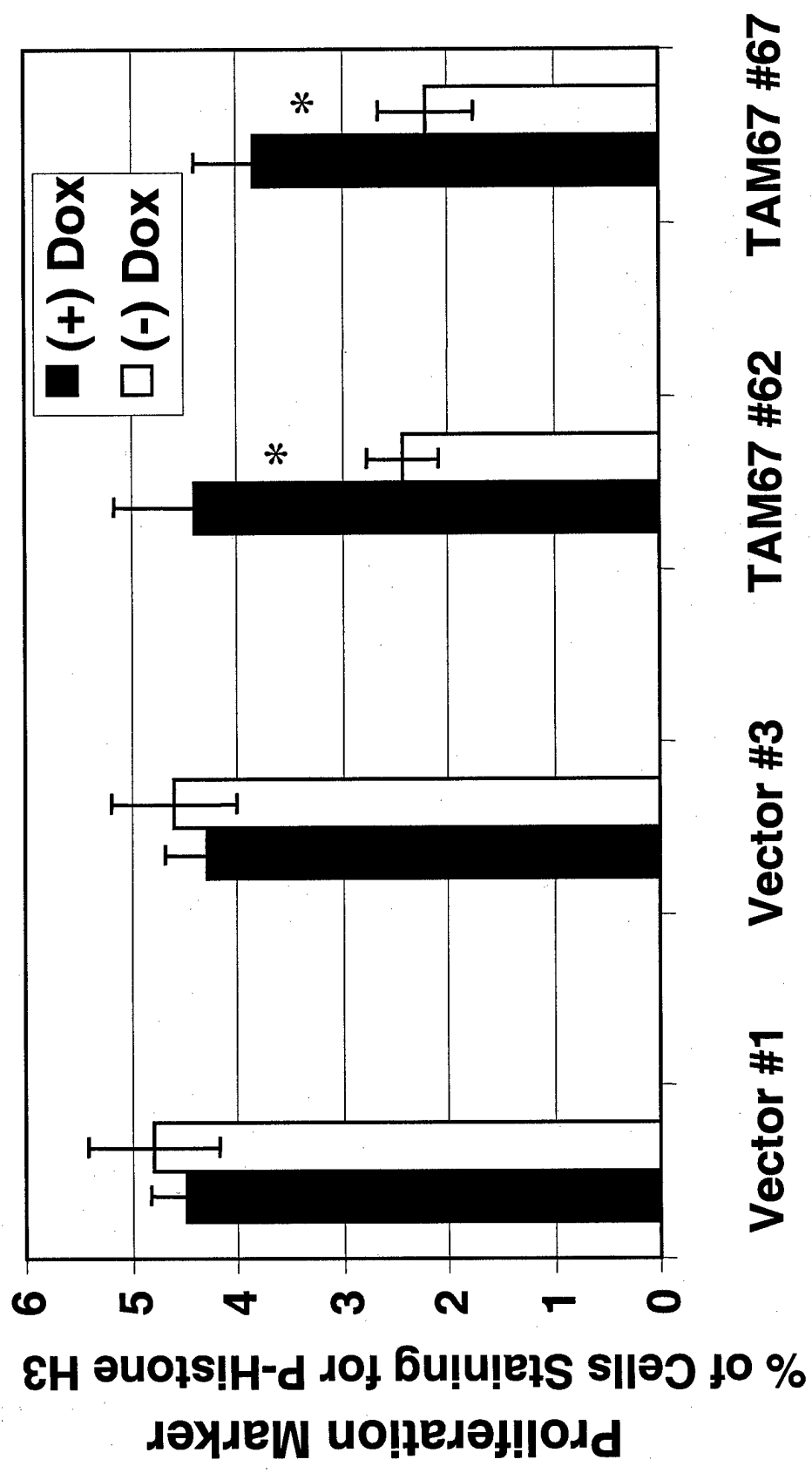


Figure 5C

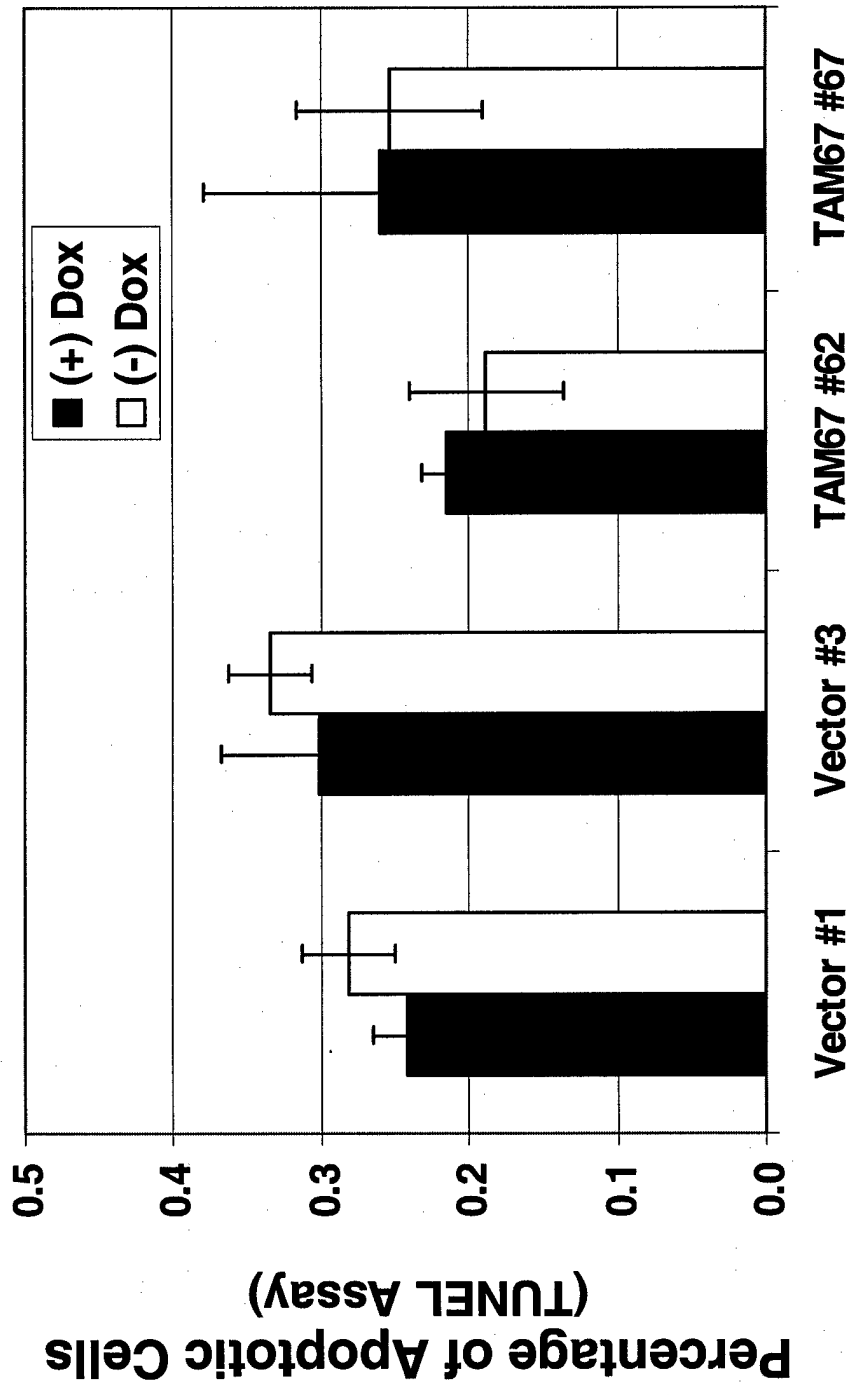


Figure 6

