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BORIC ACID INHIBITS CELL GROWTH AND INDUCES APOPTOSIS IN BREAST CANCER CELLS

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ABSTRACT

Boric Acid Inhibits Cell Growth and Induces Apoptosis in Breast Cancer Cells

by

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Boric acid (BA), an essential plant micronutrient, has recently been discovered as a chemo-preventive agent in prostate cancer; however, its mechanism of action remains unknown. Our interest is to determine if BA has a preventative role in breast cancer and elucidate its possible mechanism. Integrins are transmembrane proteins responsible for cell growth, survival and adhesion. We hypothesize that BA interacts with integrins to mediate its response. To test this hypothesis we used BA and phenylboronic acid (PBA), a bulkier analog of BA, on breast ductal carcinoma, ZR-75-1, and breast andenocarcinoma, MCF-7.

BA and PBA treatments were tested through proliferation, cytotoxicity and cell cycle analysis experiments. These preliminary results show that BA and PBA effectively inhibit growth in both breast cancer cell lines through dose-dependent cell cycle block and apoptosis. Adhesion and integrin activity assays did not definitively identify which integrins are affected by BA and PBA treatments.

TABLE OF CONTENTS

LIST OF FIGURES

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Finally, I would like to dedicate this thesis to the loving memory of my father, Dr. J. Abiodun Elegbede. He was the best researcher I knew. He enthusiastically nurtured my research interest and encouraged my research efforts, for this I am immensely grateful. You are sorely missed but your legacy lives on. Sun re, Daddy.

CHAPTER 1

INTRODUCTION

1.1 Purpose of the Study

In the United States, breast cancer is the most diagnosed cancer among women and the second leading cause of cancer deaths. In 2007, 178,480 new cases of breast cancer will be diagnosed, and of these diagnosed cases, 40,460 will ultimately die from this disease (1). There are several risk factors which may lead to a higher incidence of breast cancer in women, these include: age, either mid-life (40 – 59 years) or late-life (70+ years) and genetic mutations in breast cancer tumor suppressor genes *brca1* and *brca2* or a family history of breast cancer (1, 2). Treatment options vary between individuals and may consist of one or a combination of the following: surgery (lumpectomy, mastectomy, and/or lymph node removal), chemotherapy, radiation therapy, hormone therapy (i.e. tamoxifen) and biological therapy (i.e. trastuzumab) (1).

Current public health efforts encourage early detection and diagnosis through yearly mammograms especially in women with increased risk factors for breast cancer. Preventive measures promote the need for beneficial changes in lifestyle such as balanced nutrition, increased physical activity and cessation of habits such as smoking. Furthermore, several research efforts are focused on identifying novel, natural chemo-preventative agents as opposed to current synthetic chemotherapeutics, which can introduce harmful side effects in patients and may result in other health problems (3).

Boric Acid (BA), a known essential plant micronutrient, has recently been reported to prevent prostate cancer (4, 5) this addresses the research focus of chemoprevention. The goal of this research is to study the effect of BA in the breast cancer cell lines ZR-75-1 and MCF-7 and to elucidate a possible mechanism of action in these cell lines.

1.2 Research Questions

The recent finding of BA as an anti-cancer agent in prostate cancer highlights a new function for this element; however, the mechanism by which BA performs this function remains unknown. Knowledge of other functions of BA in humans and animals may reveal possible mechanistic sites of action of BA in cancer cells.

Studies have reported that a deficiency in Boric acid in animals negatively affects their growth and reproductive development. Abnormal embryonic growth and oocyte maturation of *Xenopus* have been reported consequences with dietary boron deficiencies (4). The absence of BA is detrimental in animals just as high levels are detrimental in humans resulting in decreased male fertility via decreased semen quality and male reproductive function (6).

BA is also noted to improve the rate of deep wound healing by decreasing the patient healing period by a factor of 3 compared to normal antiseptic wound treatments (7). Possible mechanisms for BA's effect in wound treatment may have to do with its ability to induce angiogenesis and increase the release of extracellular matrix components important for new tissue formation (7, 8).

Applying the knowledge of these available functions of BA, this research work will attempt to elucidate the effect and mechanism of BA action in breast cancer cells. The study will attempt to answer the following research questions:

i) Will BA inhibit breast cancer cell proliferation?

ii) Is the effect of BA a function of cellular toxicity, cell cycle arrest or apoptosis?

iii) Does BA interact with and mediate its effect via extracellular proteins?

1.3 Significance of the Study

Several epidemiology studies detail that a low-fat diet rich in fruits, vegetables, legumes and whole grain consumption, results in a lower incidence of cancers in general and breast cancer specifically (1, 9). These food sources are believed to decrease the incidence of cancer through several methods including the provision of micronutrients such as vitamin A and C and their antioxidant effect which limits the destructive potential of cancerous cells (10 – 12). Encouraging preventive steps that involve natural food products and lifestyle changes is an easy and less harmful way to reduce the chance of a cancer diagnosis and the need for synthetic chemotherapeutic measures.

BA is an essential plant micronutrient, which can be found in several available food and drink sources. The potential of BA to have an anti-cancer effect in breast cancer supports the current trend in prevention research.

CHAPTER 2

LITERATURE REVIEW

2.1 Boron and BA

The fifth element, boron, is a well-known essential micronutrient for higher plant life (13). It is absorbed into the plant via a boron transporter, BOR1, located in root cells or through plant foliage (14, 15), and is required for plant growth as well as the reproductive processes of plant flowering and seed formation (14). Boron is abundant in apples, alfalfa, pears, grapes, avocados, sugar beets, nuts (soybeans), legumes, oil palms and wine, especially when these plants are grown on boron rich soil (15). In the United States, an adult can consume ~1 – 2 mg of boron a day (16) from water and/or food sources, this ingested boron has a mean biological half-life of 21 hours (17); therefore, most consumed boron is excreted in the urine within 72 – 96 hours after intake. This may indicate that boron might also be an essential element in humans; however, this still remains unknown.

Under biological conditions, boron is absorbed in the intestines (18) and reacts with water or hydroxyl containing molecules in the blood to generate the trigonal planar BA ($B(OH)_3$) (Fig. 1), further reaction with water or hydroxyl containing molecules generates the tetrahedral borate ion ($B(OH)_4^-$) (Fig. 2) (5, 15, 19, 20). BA is a weak acid with a pKa of 9.2 and will remain in the uncharged $B(OH)_3$ state at physiological pH of 7.4 (13, 19) the ratio between BA and borate ion at this pH is 98.4% and 1.6% respectively(15).

BA and borate ion can covalently bind to cis-diol molecules via an esterification reaction (19) (Fig. 3). Physiologically, cis-diol molecules include ribose-containing nucleotides (AMP, CMP etc) and co-factors such as NAD^+ and NADH. Deoxyribonucleotides are not reactive with BA or borate ion due to the absence of the ribose 2' hydroxyl group (19, 20).

2.2 BA and Prostate Cancer

BA has recently emerged as a chemo-preventative agent in prostate cancer (5). This discovery is supported by epidemiology studies as well as *in vitro* cell culture and *in vivo* animal studies (4, 21 – 23). An epidemiology study based on data from the third National Health and Nutrition Examination Survey (NHANES III) was interested in the relationship between dietary boron intake and prostate cancer prevalence. This study provided the first evidence for the possibility of boron as an anti-cancer agent. The result identified an inverse relationship between dietary boron intake and the incidence of prostate cancer in spite of other risk factors such as age, smoking, obesity and race (21). The small size of the group of men observed in this study limited the certainty of the findings; however, in a 2007 epidemiology study in Texas, these findings were further supported. This more recent study showed an inverse correlation between the incidence of prostate cancer and high levels of boron in groundwater sources (22). The sample population for this study was obtained from several Texas counties; however, the study was unable to conclude if this inverse trend of boron and prostate cancer can be applied to ethnic minority groups due to the small number of participants from such groups (22).

These studies provided the first evidence of a possible anti-cancer function for BA and also served to establish the need for further study.

Further investigation involved *in vitro* cell culture studies using human prostate cancer cell lines DU-145, PC-3 and LNCaP. Hormone dependent LNCaP and hormone independent DU-145 cells exhibit dose-dependent growth inhibition when treated with BA (4). Hormone independent PC-3 cells required higher concentrations of BA to induce similar inhibitory effects (4). These experiments served to support the finding of BA as an anti-cancer agent in prostate cancer, and to further understand this effect a physiological model study was carried out.

Mouse model studies confirmed the *in vitro* findings in the case of hormone dependent LNCaP cells. Nude mice were orally dosed with BA for two weeks prior to being injected with LNCaP cells. The mice were continually fed BA and tumor size and histology were observed. Treated mice exhibited a decrease in tumor growth as compared to controls. These mice also displayed poor tumor vascularization, which affected tumor morphology and tumor aggressiveness as compared to controls (23). In this study it is believed BA is affecting the serine protease prostate specific antigen (PSA), which is produced by hormone dependent prostate cancers.

PSA catalytically cleaves insulin-like growth factor binding protein-3 (IGFBP-3) producing insulin growth factor-1 (IGF-1), which is found in increasing levels in prostate cancer (Fig. 4). PSA is a serine protease, which contains hydroxyl moieties in its active site. BA can bind to these moieties creating a reversible transition state analog and once this occurs, PSA looses its catalytic activity and therefore causes a reduction in the growth and aggressiveness of prostate cancer (23, 24).

While this *in vivo* study provides a possible mechanism for the role of BA in hormone dependent prostate cancers, it does not offer much insight into the possible mechanistic process of BA in hormone independent prostate cancers, which do not produce PSA. Thus the mechanism by which Boron causes inhibition in hormone independent prostate cancer cells such as DU-145 remains unknown.

This chemo-preventative role of BA in prostate cancers encouraged our efforts to determine if BA has a similar anti-cancer effect on breast cancer by using ZR-75-1 and MCF-7 cell lines.

2.3 Breast Cancer Cell Lines

ZR-75-1 is a non-metastatic, epithelial breast cancer cell line (25, 26). It is characterized as a ductal carcinoma, which implies it originates in cells lining the milk ducts in the breast tissue. This breast cancer cell line is estrogen receptor and progesterone receptor positive and is therefore hormonally regulated. ZR-75-1 expresses low amounts of the tumor suppressor p53 as well as insulin-like growth factor binding proteins 2, 4 and 5 (27). ZR-75-1 cells also produce the cellular product mucin also known as episialin (28). Mucins are transmembrane proteins composed of an apomucin core protein which is heavily glycosylated, ZR-75-1 cells expresses both MUC-1 and MUC-2 proteins (26, 29).

MCF-7 is a metastatic, epithelial breast cancer cell line (25, 26). MCF-7 is an adenocarcinoma and therefore can be found in many different sites in the breast tissue. These cells are estrogen receptor and progesterone receptor positive and also express the tumor suppressor gene p53 (27). Much like ZR-75-1, MCF-7 cells also produces mucin

proteins such as MUC-1 and MUC-3 but in smaller amounts as well as insulin-like growth factor binding proteins-2, -4 and -5 (26, 29). MCF-7 cells also express tissue transglutaminase 2 (TG2), an extracellular, transmembrane enzyme. TG2 catalyzes post-translational protein modifications by introducing inter- or intra-peptide isopeptide bonds via cross-linking reactions (30). This function of TG2 contributes to the ability for MCF-7 cells to metastasize to other tissues in the body (31).

Using these breast cancer cell lines, we hope to investigate whether BA induces an anti-cancer effect. In addition to BA, boron exists in several different chemical analogs one of which is phenylboronic acid (PBA), which is made bulkier by the addition of a phenyl group (Fig. 5). PBA retains similar reactive properties as BA in that it can also complex with cis-diol and hydroxyl moieties which is seen in PBA's functions as a serine protease inhibitor and also as a peptidyl transferase inhibitor (32, 33). In both inhibitory functions, PBA functions by forming a complex with hydroxyl groups in the catalytic site of the enzymes (34). In this research, PBA was used to help study the effects of boron in breast cancer cell lines as it is believed its bulkier shape will induce more dramatic results.

2.4 Integrins

Integrins are a family of glycoproteins which function as heterodimeric, extracellular, transmembrane adhesion receptors (35). These proteins are involved in several key cellular functions including: cell growth, migration (such as chemotaxis, invasion and metastasis), survival, and morphology, as well as wound healing (by inducing blood clotting and inflammation), cell death, cell-to-cell interactions, and cellular attachment to

extracellular matrices (ECM's) (35 – 38). Integrins are composed of combinations of type I transmembrane glycoproteins α and β. The α subunit ranges in size from 120 – 180 kd and non-covalently interacts with the β subunit, which range from 90 – 110 kd (36, 39). The α and β subunits are composed of a large extracellular domain, a transmembrane domain and a relatively short cytosolic domain, the extracellular domain interacts with ECM's and neighboring cell receptors while the cytosolic domain is linked to cytoskeletal elements (37). There are currently 24 known integrin dimers, which are assembled from unique combinations of the known 18 α and 8 β subunits (39, 40).

In order for integrins to function, the extracellular portion of the heterodimer must be activated via a conformational change in its structure (36). The extracellular domains of both α and β subunits have ligand binding sites known as α I and β I domains (36, 41). The α I domain is present in 9 integrin α subunits and is the primary ligand binding site, the β I domain regulates ligand binding to this site; however, in the absence of the α I domain, the β I domain becomes the major ligand binding site (41). Both α and β I domains have metal-ion-dependent adhesion sites (MIDAS) which can bind divalent cations such as Ca^{+2}, Mg^{+2} and Mn^{+2}. These metal cations induce ligand binding and also increase the affinity of an integrins for a ligand (36, 37).

The process of activation begins with integrins in the low affinity state where they are bent at a flexible "knee" joint with their head groups close to the cell plasma membrane (41). In the presence of its ligand, integrins extend from the bent low affinity state into an upright, "closed" state, and once the ligand binds to either the α I or β I domain, the integrin heterodimer converts to the upright, "open" state and is able to mediate its cellular action via outside-in signaling (41) (Fig. 6). It is important to understand that the

process of integrin activation is a dynamic equilibrium process due to the cell responding to its environmental changes (41).

In addition to understanding integrin function and activation, it is also important to be aware that different cell types express different α and β integrin combinations. Furthermore on the surface of one cell type several integrin pairs can be present. For the cell lines used in this research, ZR-75-1 and MCF-7, they have several integrins in common: α_2, α_3, α_5, α_6, α_V, β_1, β_3, β_4 and β_5 (25). MCF-7 cells also express integrin α_4 (42).

2.5 Hypothesis

As BA has emerged with an anti-cancer function in prostate cancer and since boron is common in diet sources and can interact with cis-diol and hydroxyl containing molecules we hypothesize that BA has an inhibitory effect on breast cancer cells and mediates this effect by interacting with integrins. Since integrins are glycoproteins and may contain hydroxyl groups in close proximity to each other, BA may be interacting with integrins and using them to mediate its cellular effect. To test this hypothesis we will look at the effect of BA and PBA on breast cancer cell lines ZR-75-1 and MCF-7. We will also determine how BA and PBA affect integrin function and activation in these breast cancer cell lines.

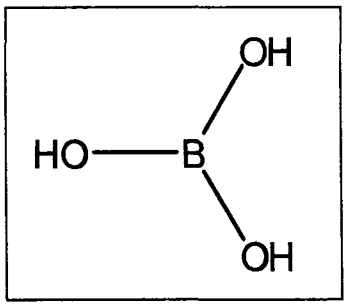

Figure 1: Structure of BA.

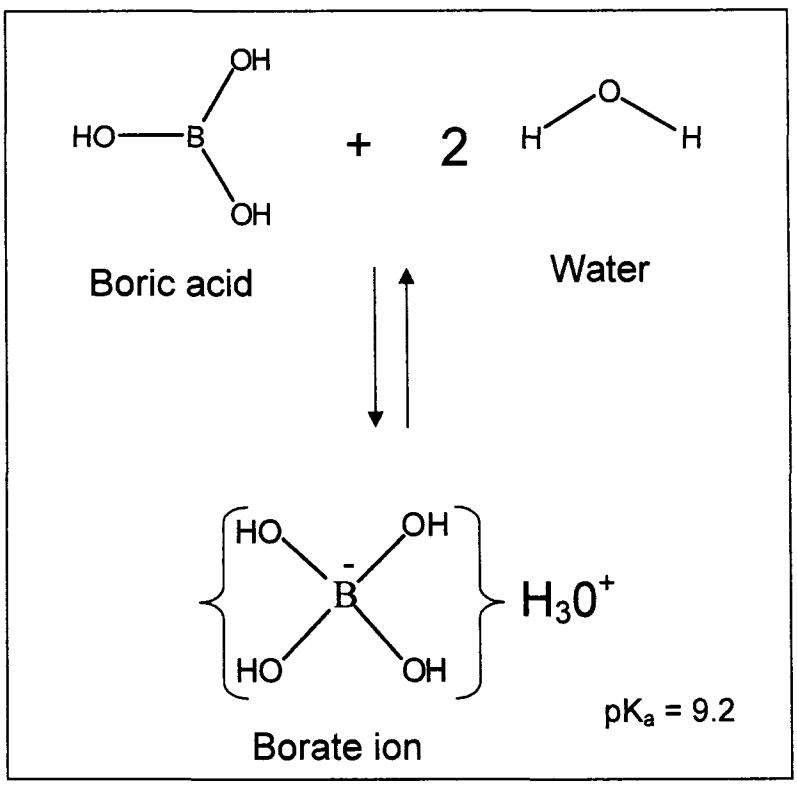

Figure 2: Borate ion synthesis

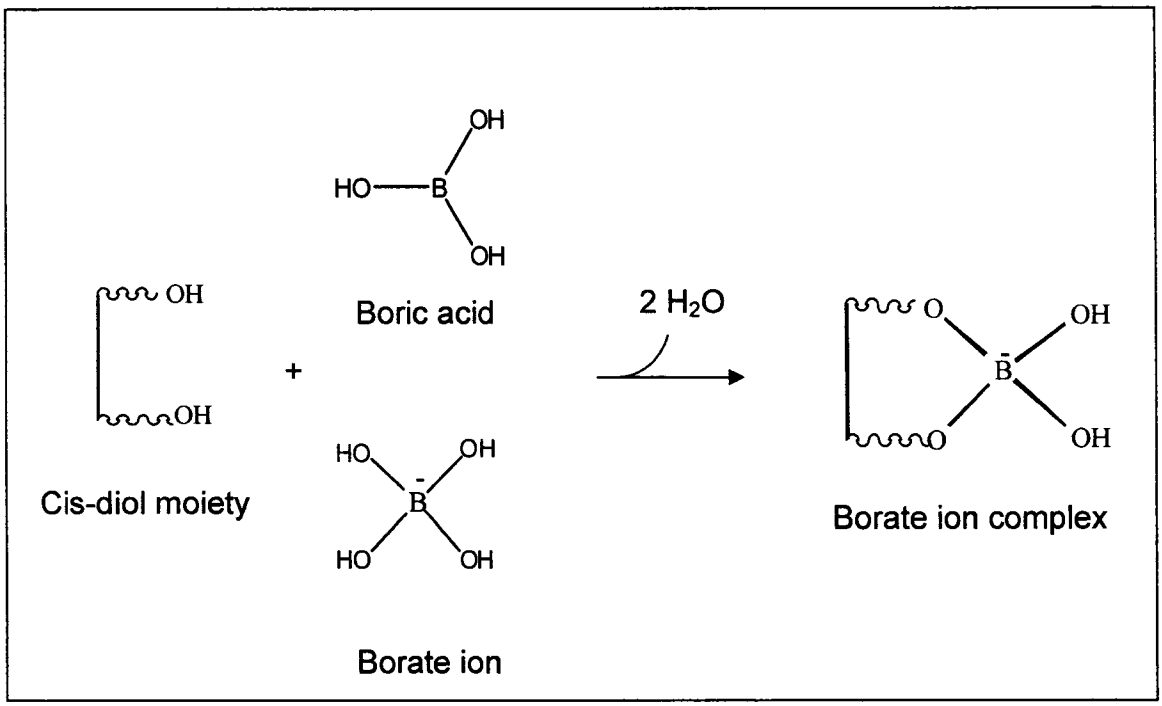

Figure 3: BA and borate ion reaction with cis-diol containing moieties.

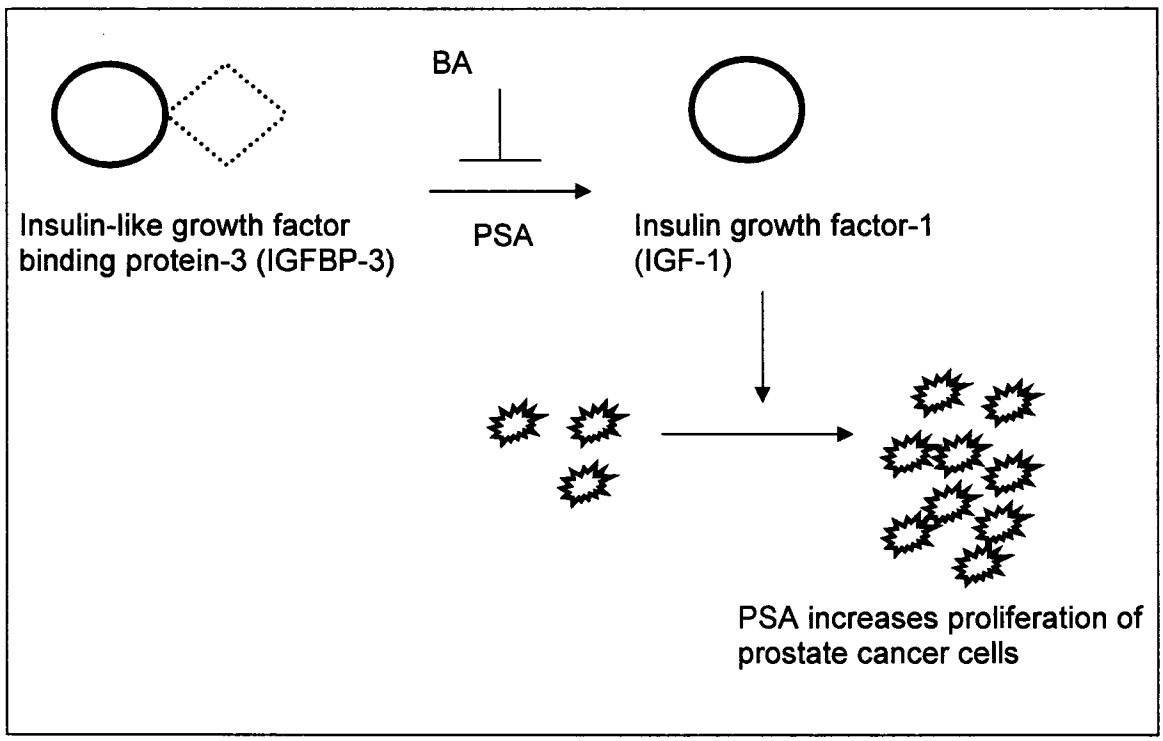

Figure 4: Method of BA inhibition in hormone dependent prostate cancer cells, which produce PSA.

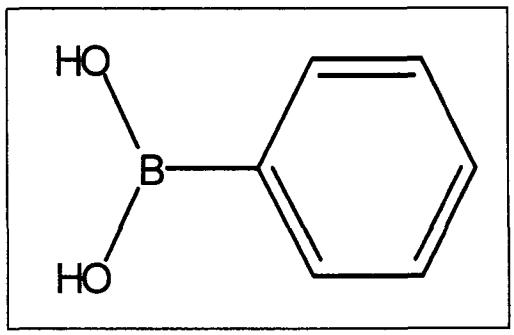

Figure 5: Structure of PBA.

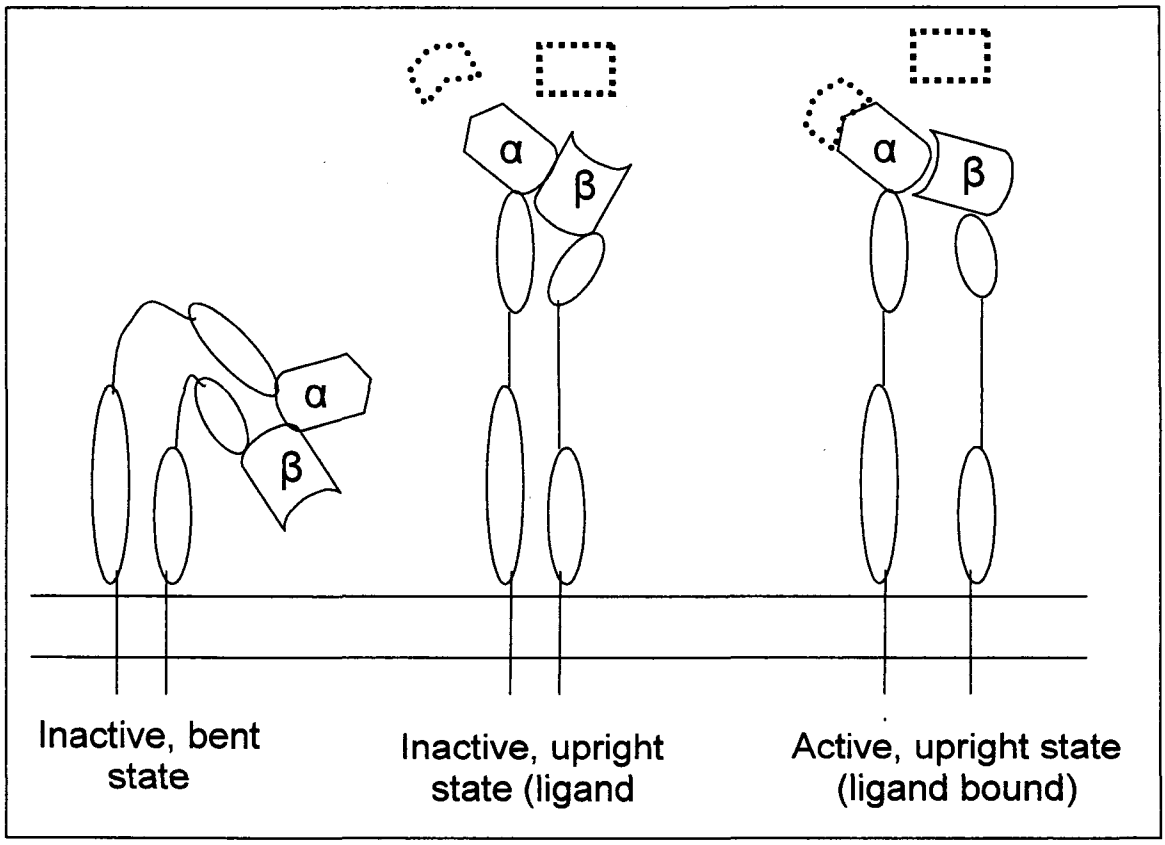

Figure 6: Integrin activation. Integrins dynamically move through all three stages of activation as it responds to the cellular environment.

CHAPTER 3

MATERIALS AND METHODS

3.1 Cells and Cell Culture

Human breast cancer cells ZR-75-1 and MCF-7 were obtained from American Type Culture Collection (Manassas, VA USA). ZR-75-1 cells were cultured in RPMI-1640 medium (10mM HEPES, 1mM Sodium Pyruvate, 2mM L-glutamine, 4500mg glucose 1L, 1500mg Sodium Bicarbonate) (ATCC, Manassas, VA USA) supplemented with 10% FBS (Invitrogen, USA), 25mM HEPES (Sigma-Aldrich, USA) and 1% Penicillin-Streptomycin (Invitrogen, USA). MCF-7 cells were cultured in Minimum Essential Medium (with Earle's Salts and L-glutamine) (Invitrogen, USA) supplemented with 10% FBS, 25mM HEPES pH 7.4 and Penicillin-Streptomycin. Cells were incubated at 37°C and 5% CO_2. Cell harvests were performed by washing cells in Phosphate Buffered Saline (PBS) without $CaCl_2$ or $MgCl_2$ at pH 7.2 (Invitrogen, USA) and then detaching cells from the flask surface with Trypsin-EDTA 1X (0.25% Trypsin with 0.38g EDTA-4Na) (Invitrogen, USA). Cell counts are obtained using a Z1 Beckman Coulter® Particle Counter (Fullerton, CA) and cell treatments were performed using different BA and PBA concentrations from 0.05M stock solutions.

3.2 Z1 Coulter® Particle Counter

Following the harvesting of cells, 0.1 mL of the cell suspension was added to a coulter counter cup containing 9.9 mL of Isotonic Diluent Azide-Free (Isoton) solution (Val-Tech Diagnostics Inc, Pittsburgh, PA). The solution was thoroughly mixed and placed in the Z1 Beckman Coulter® Particle Counter (Fullerton, CA). Three or four measurements within 10% of each other were averaged and used to calculate the amount of cells per 1 mL of solution.

3.3 Agent Preparation

A 0.05M stock solution of BA was prepared by dissolving 0.0309 grams BA (99.5%, Sigma-Aldrich, USA) in 10.0 mL ultra-pure (U.P.) water. A 0.05 M stock solution of PBA was prepared by dissolving 0.0609 grams PBA (99.5%, Sigma-Aldrich, USA) in 10.0 ml UP water. The solutions were transferred to a 20.0 ml syringe (Becton Dickinson, USA) under sterile conditions and filter sterilized with a 0.2 uM polyethersulfone membrane syringe filter (VWR International, USA).

For combination adhesion assays and PBS supplementation in integrin activity assays, 0.05 M stock solutions of $MnCl_2$ (0.0989 g) and $MgCl_2$ (0.101 g) were made in 10.0 mL U.P. water and filter sterilized as described above.

3.4 Proliferation Assay

Eighty thousand cells were seeded into each 25 cm^2 tissue culture flask (Becton Dickinson, USA) and allowed to adhere overnight (MCF-7) or for 3 days (ZR-75-1). Control and treatment experiments were run in duplicates and a 1mM concentration of

BA or PBA was added to respective treatment flasks. Both adhered cells and suspended cells in the medium were counted using the Z1 Beckman Coulter® Particle Counter (Fullerton, CA) during the course of the experiment. The total number of cells was obtained by multiplying the flask volume by the mean of the cell counts.

3.5 Mitochondrial Dehydrogenase Assay

Cellular mitochondrial dehydrogenase function was determined using an alamarBlue assay. On 96-well plates, 5,000 cells/well were seeded in 0.1 mL aliquots and allowed to incubate overnight. Each row of wells was treated with 0, 0.4, 0.8, 1.0, 2.0, 5.0 and 10.0 mM BA or PBA, the first row was the blank and did not contain any cells. Plates were incubated for 24, 48 and 72 hour time points. At each time point, 0.1 mL of cell medium and 0.01 mL alamarBlue (BioSource, Camarillo, CA) was added to each well and the plates were incubated for 4 hours at 37°C and 5%CO_2. The plates were read using the GENios fluorescence plate reader (Tecan Systems Inc. San Jose, CA) at excitation and emission wavelengths of 530nm and 590nm respectively. Percent viability was determined as a factor of controls (n = 4).

3.6 Colony Formation Assay

Tissue culture flasks (25 cm^2) were seeded with 4.0 x 10^5 cells (ZR-75-1) or 2.0 x 10^5 cells (MCF-7) and incubated three days or overnight respectively. Cells were treated with 0, 0.4, 0.8, 1.0, 2.0 and 5.0 mM BA or PBA for 72 hours. Cells were harvested, counted and aliquots containing 600 cells (ZR-75-1) or 200 cells (MCF-7) were then plated in 60mm x 15mm cell culture dishes (Corning Inc., USA) each flask yielded 3

plates. These dishes were incubated at 37°C and 5% CO_2 for 10 – 14 days, dishes were then stained with Crystal violet (0.5% weight/volume crystal violet in 95% ethanol) and colonies, consisting of 50+ cells, were counted. Percent survival was determined compared to controls (n = 3).

3.7 Cell Cycle Analysis

To determine cell cycle phase distribution following treatment, 1.0 x 10^6 ZR-75-1 and 2.5 x 10^5 MCF-7 cells were seeded in each 75 cm^2 tissue culture flask (Becton Dickinson, USA) and incubated overnight. Flasks were treated with 0, 0.4, 0.8, 1.0 and 2.0 mM BA or PBA. After 5 days, attached and suspended cells were harvested, counted and centrifuged at 1800 rpm. Cells were washed with 5.0 mL PBS, centrifuged and resuspended in 1mL cold 95% ethanol to fix cells. To analyze the cells, propidium iodide staining was carried out. Fixed cells were washed with 3.0 mL PBS and incubated with 0.1 mL Triton X 1% buffer and 0.1 mL RNase at 1μg/mL for 10 minutes. Propidium iodide stain (0.2 mL) at 100μg/mL was then added and cells were incubated for 30 minutes in the dark. Samples were analyzed on the Becton Dickinson FACSCalibur (Becton Dickinson, USA) and results evaluated using ModFit LT Versoin 3.0 (Verity Software House, Topsham, ME).

3.8 Florescence Microscopy

Cells were seeded into a 4-well plate, 20,000 cells/well (ZR-75-1) and 10,000 cells/well (MCF-7), and allowed to incubate overnight. Drug concentrations were diluted in medium and 0.5 mL aliquots were placed in wells and the plates were exposed to

treatment for 72 hours. Treatment solution was removed and wells were washed with PBS and then a stain solution consisting of 0.5 mL of Hoechst (2 ug/mL) and 0.5 mL of propidium iodide (10 ug/mL) diluted in PBS was added to each well. The wells were incubated for 15 minutes and the images were acquired.

A bright field image was obtained visualizing cells with a phase lamp. Apoptotic cells were identified using a mercury lamp with an excitation wavelength of 300 - 500 nm and an ultra-violet filter with an emission wavelength of 435-485 nm, a positive finding was stained blue by the Hoechst stain. Necrotic cells were identified using the mercury lamp and a green filter with an emission wavelength of 600 - 660 nm, a positive finding appeared red due to the propidium iodide stain. The images were taken with a Photometrics Cool Snap CCD camera attached to a Nikon Eclipse TE2000-U microscope (Nikon Inc. Melville, NY) and analyzed using MetaVue software (Meta Series 6.0/6.1, Universal Imaging Corporation).

To determine the percent of apoptosis or necrosis, the following equation was used:

$$\frac{\text{Number of positive apoptotic/necrotic cells}}{\text{Total number of cells in the bright field}} \times 100\%$$

The three areas of the wells that were imaged were randomly selected and the same areas were chosen in control and treatment wells (n = 3).

3.9 Adhesion Assay

Time dependent cellular adhesion rates to a plastic flask surface was observed by seeding 25 cm^2 tissue culture flask (Becton Dickinson, USA) with 1.0 mL aliquots containing 1.2×10^5 cells in both cell lines. Controls and treatments were run in triplicate

and a 1 mM concentration of BA, PBA or $MnCl_2$ was added to each flask prior to cells being added, in combination experiments 1 mM of both PBA and $MnCl_2$ were added. Once flasks were seeded with cells, 0.1 mL was promptly removed and counted using the Z1 Beckman Coulter® Particle Counter (Fullerton, CA) to determine the original number of cells seeded. Flasks were then incubated at 37°C and 5% CO_2 for 30, 60, 90 and 120 minutes for both cell lines. At each time point, adhered cells were harvested, counted and the percent adhesion was found using the following equation:

$$\frac{\text{Number of adhered cells harvested}}{\text{Original number of cells seeded}} \times 100\%$$

Average percent adhesion is determined for the three trials at each time point and treatment (n = 3).

3.10 Extracellular Matrix Specific Adhesion Assay

To determine which ECM the breast cancer cells have an affinity towards, a cell dilution was prepared containing either 1.1 x 10^6 cells/mL (ZR-75-1) or 2.0 x 10^6 cells/mL (MCF-7). Aliquots containing 1.65 x 10^5 cells (ZR-75-1) or 3.0 x 10^5 cells MCF-7 of this dilution were placed into each well of a 48-well plate containing different extracellular matrices (Cell Biolabs, USA) and the plate was incubated at 37°C and 5% CO_2 for 90 minutes. The existing medium was aspirated off and the wells washed 4 times with 0.2 mL/well PBS. To visualize the attached cells, 0.2 mL/well cell stain solution (included in the kit) was added to the wells and allowed to develop for 10 minutes at room temperature. The stain solution was then aspirated off and the wells were washed 4 times with 0.5 mL/well deionized water and incubated with 0.2 mL/well

extraction solution (included in the kit) on an orbital shaker for 10 minutes at room temperature. From each well, 150 uL of the extracted stain solution was transferred to a 96-well plate and the absorbance was measured on a Spectramax Plus plate reader (Molecular Devices Corporation Sunnyvale, CA) at 560 nm (n = 2).

For timed adhesion assays on the ECM Fibronectin, cell dilutions of 2.1 x 10^6 cells/mL (ZR-75-1) and 3.5 x 10^6 cells/mL (MCF-7) were prepared. The cell dilutions were aliquoted into three tubes for control, BA treatment and PBA treatment. The BA and PBA treatment tubes were further divided into three tubes each for the 30, 50 and 70 minute time points. BA and PBA treatments were incubated with the cells for 10 minutes before being added to the ECM wells. After 70 minutes, the cells were washed, stained, washed again and the stain was extracted and measured as is described above.

3.11 Integrin Activity Assay

Cells were harvested in unsupplemented PBS by scraping them off the bottom of the culture flask so as not to cleave the integrin proteins with trypsin. Integrin β_1 and $\alpha_v\beta_5$ antibodies as well as a control antibody IgG on 96-well plates were purchased from (Chemicon, Temecula, CA). A single cell suspension of 250,000 cells/mL (ZR-75-1) or 300,000 cells/mL (MCF-7) was suspended in PBS supplemented with 1 mM Mn^{+2} and 1 mM Mg^{+2}. The cells were then aliquoted into separate tubes and incubated with BA (1mM), PBA (1mM) or $MnCl_2$ (1mM) for 10 minutes.

Plate wells were rehydrated with unsupplemented PBS for 10 minutes and poured off before 0.1 mL aliquots of cells were placed in each well and the plate was incubated for 2 hours at 37°C and 5% CO_2. Cells were removed and the wells were washed twice with

PBS supplemented with 1 mM Mg^{+2}. Cells were stained by adding 0.1 mL/well of cell stain solution (included in the kit) for 5 minutes at room temperature. Stain solution was removed and wells were washed 3 times with PBS supplemented with Mg^{+2} and wells were allowed to air dry.

To determine the activity of integrins under control and treatment conditions, 0.1 mL/well extraction buffer (included in the kit) is added and incubated on an orbital shaker for 5 minutes at room temperature, the absorbance of the wells is then read on a Spectramax Plus plate reader (Molecular Devices Corporation Sunnyvale, CA) at 560 nm (n = 3).

3.12 Statistical Analysis

Results were expressed as mean ± standard error (SE). Standard error was obtained by dividing the standard deviation by the square root of the number of runs (n). The efficiency of treatments was determined using one-tailed two-sample t-Test (assuming equal variances), a significant difference was concluded with p values < 0.05 or $p < 0.001$.

CHAPTER 4

FINDINGS OF THE STUDY

4.1 Analysis of Results

To determine the effect of BA and PBA on breast cancer cells a proliferation assay was carried out to monitor cell growth. Cells were continually exposed to 1mM concentration of either BA or PBA and the number of adhered cells was recorded.

BA effectively inhibited growth in ZR-75-1 cells on day 5 by 44%. This level of inhibition was maintained in the cells until the assay was terminated at day 11 (Fig. 7A). However, BA did not have any inhibitive effect on the proliferation of MCF-7 cells. Instead cells displayed a similar amount or superceded the growth of control cells (Fig. 7B).

In PBA treatments, growth in both cell lines was significantly inhibited. ZR-75-1 cells displayed a 32% decrease in growth on day 3 and this inhibition increased to 46% by day 6 and was maintained until the assay was terminated on day 7 (Fig. 8). Growth of MCF-7 cells was inhibited by 43% on day 4 and decreased to 36% on day 5 which was maintained until the assay was terminated on day 7 (Fig. 8B).

After establishing the effect BA and PBA elicit in the respective breast cancer cell lines, the possible avenue by which this growth inhibition is carried out was investigated. Cytotoxicity of 0.4 - 10 mM of BA or PBA after 24, 48 and 72 hour exposure was determined using alamarBlue assay. Mitochondrial dehydrogenase activity is measured

by its ability to reduce a blue dye solution to pink/red in viable cells the absence of the color change indicates cell death. In BA treatments, both ZR-75-1 and MCF-7 cells underwent a time- and dose-dependent decrease in cell viability. Both cell lines exhibited the greatest inhibition after 48 hours and at 10 mM BA with 31% (ZR-75-1) and 60% (MCF-7) inhibition (Fig. 9A and B). Treatment with PBA incurred a more dramatic dose- and time-dependent decrease in cell viability in both cell lines compared to controls. The largest inhibition occurred after 72 hours and at 10 mM PBA with 91% (ZR-75-1) and 97% (MCF-7) inhibition (Figure 10 A and B).

To further test the cytotoxic potential of BA and PBA treatments on ZR-75-1 and MCF-7 cells, long-term treatment effects were investigated via a clonogenic (cell) survival assay. Cells were exposed to 0.4 - 5 mM of each treatment for 72 hours and then cells were allowed to grow for 10 - 14 days, this determines if treatments affect the cells ability to produce viable offspring. In ZR-75-1 cells BA produced the most significant decrease in survival by 40% at 5 mM BA and in MCF-7 cells a 60% decrease in survival was observed at 5 mM BA (Fig. 12A and B) compared to control. Although a decrease in survival is seen at 1 mM BA in MCF-7 cells, it is believed that it does not follow the trend as subsequent trials did not produce the same effect (Fig. 12B). PBA induced a 21% decrease in ZR-75-1 cell survival at 5 mM concentration; however this decrease is not statistically significant (Fig. 13A). In MCF-7 cells, 5 mM PBA resulted in a 78% decrease in cell survival (Fig. 13B), which was significant compared to control.

The experiments in BA and PBA cytotoxicity show that at 1 mM, which is the inhibitory concentration used in the proliferation assays, only $\leq 20\%$ of cells do not survive, this prompted the need to determine what is occurring in cell cycle phases using

flow cytometry and also to evaluate the rate of apoptosis and necrosis in cells using florescence microscopy.

In flow cytometry experiments, cells were exposed to 0.4 - 2 mM BA or PBA for 5 days. This length of exposure was used based on the proliferation assay since the inhibitory effect of BA and PBA has been realized at this point in the cells and also previous trials with shorter exposure times did not produce enough suspended cells for analysis. Attached and suspended cells were analyzed to determine if any dose-dependent trend is observed. Treated attached cells appeared to have the same cell cycle distribution as control cells in both cell lines and with both treatments (Fig. 14A - D).

On the other hand, treated suspended cells displayed an S phase block as is evidenced by a decrease in G0/G1 and subsequent increase in S phase (Fig. 15A - D), the most dramatic effect was seen in suspended ZR-75-1 cells treated with PBA (Fig. 15C) where G0/G1 phase decreased by 36% and S phase increased by 41% as compared to control. This S phase block means that cells have replicated their DNA but cannot undergo cell division and therefore remain in a static state. Unfortunately, the cell cycle analysis did not exhibit a dose-dependent trend in apoptosis, programmed cell death, in either attached or suspended cells at the concentrations used and so to determine if there is a dose-dependent change in apoptosis or if the cells are dying due to necrosis florescence microscopy was used.

Cells were treated with 1, 5 and 10 mM BA or PBA for 72 hours and then Hoechst and propidium iodide stains were added to visualize the apoptotic and necrotic cells respectively. The dramatic increase in treatment concentrations displayed a significant dose-dependent increase in apoptosis in both cell lines and both treatments (Table 1 and

2); however, the increase in necrosis was not as large in both cell lines except for MCF-7 cells treated with PBA (Table 2). This florescence microscopy experiment also provided visual images of the cells after treatment, both ZR-75-1 and MCF-7 cells appeared to have endured morphological changes in cell shape at higher concentrations of BA or PBA compared to control (Fig. 15 – 18).

After establishing that BA effectively inhibits growth of ZR-75-1 but not MCF-7 cells and that PBA inhibits growth in both cell lines, we then focused on possible sites of interaction with these cells. As integrins are extracellular proteins involved in cell growth, survival and morphology, and since BA and PBA treatments affect these cell functions it can be considered that BA and PBA may associate with integrins and induce signaling pathways resulting in cell death or stasis. Our hypothesis is that extracellular proteins such as integrins are a possible target for BA and PBA. Thus ZR-75-1 and MCF-7 cells ability to adhere to different extracellular matrix (ECM) surfaces and the effect of BA and PBA treatments on integrin activity were investigated.

4.2 Treatment Effects on Integrin Function and Activity

To determine if BA or PBA treatments affect the cells ability to adhere to an extracellular matrix (ECM) several different adhesion assays were conducted.

The non-specific adhesion to a plastic flask surface was first investigated in cells that were treated with 1 mM BA or PBA and incubated in a flask for 30 – 120 minutes. Percent adhesion over time was determined from the number of cells adhered at the time point compared to the original number of cells seeded in the flask. In both ZR-75-1 and MCF-7 cells BA did not affect adhesion over time (Fig. 19A and B). When 1 mM PBA

treatment is used, ZR-75-1 cells appear to have decreased binding ability over time (Fig. 20A); however, this decrease is not statistically significant on the other hand MCF-7 cell adhesion is significantly inhibited by 18% at the 60 minute time point (Fig. 20B).

A combination treatment experiment was then carried out by treating cells with 1 mM $MnCl_2$ with or without 1 mM PBA. Metal cations such as Mn^{+2} and Mg^{+2} enhance the ability for integrins to bind to an ECM and so cells treated with $MnCl_2$ should increase adhesion compared to control whereas a combination treatment with PBA may decrease this adhesion. In both ZR-75-1 and MCF-7 cells treatment with $MnCl_2$ significantly increased the cells ability to adhere to the plastic flask surface compared to controls (Fig. 21A and 22A) this difference was observed from 60 – 90 minutes in ZR-75-1 cells and from 30 – 90 minutes in MCF-7 cells. In the combination treatment experiments, 1 mM PBA significantly decreased the effect of $MnCl_2$ at 60 and 90 minutes for ZR-75-1 cells and from 60 - 120 minutes in MCF-7 cells (Figures 21B and 22B). Monitoring adherence rates to a non-specific ECM such as the plastic flask surface appears to be affected by PBA and not by BA treatment; next we investigated the effect of BA and PBA on binding to specific ECM.

First we had to elucidate which ECM ZR-75-1 and MCF-7 cells preferentially bind to, in order to do this we tested untreated cells affinity to Fibronectin, Collagen I and IV, Laminin and Fibrinogen, BSA was a negative ECM control. Cells were allowed to adhere to the ECM for 90 minutes to ensure maximum exposure and adherence. ZR-75-1 cells had a significantly high affinity for Collagen I > Fibronectin = Collagen IV > Fibrinogen compared to BSA (Fig. 23). MCF-7 cells had a significant affinity for only Fibronectin (Fig. 25). Since both cell lines have a high ability to bind to Fibronectin, the

rate of treated cells ability to adhere to this ECM was tested. In addition to deciding which ECM the breast cancer cell lines had an affinity for, a preliminary treatment assay was carried out using ZR-75-1 cells exposed to BA or PBA for 10 minutes and incubated on the ECM surface for 30 minutes. From the results it is determined that treatment with 1 mM BA or 1 mM PBA does not affect ZR-75-1 cells ability to bind to any ECM (Fig. 24). Despite this result we still proceeded to test a timed assay to evaluate if there is a time-dependent affect of BA or PBA treatment on either cell line.

A timed adhesion assay was carried out on the Fibronectin surface. Cells were exposed to 1 mM BA or PBA for 10 minutes and then incubated on the Fibronectin surface for 30, 50 and 70 minutes, the effect of treatment on adhesion is determined by comparing optical density readings at 560 nm to controls. Treatment with BA and PBA both stimulate adhesion in ZR-75-1 cells after 30 minutes but both treatments inhibit adhesion after 50 and 70 minutes compared to controls and PBA inhibition was greater than BA inhibition (Fig. 26). In MCF-7 cells, 1 mM BA decreased adherence to Fibronectin after 30 and 50 minute time points as compared to controls and then stimulated adherence after 70 minutes (Fig. 27). Treatment with 1 mM PBA does not appear to affect adhesion at 30 minutes compared to control, and after 50 minutes, PBA treated cells retain the same amount of adhesion as is seen at 30 minutes while there is an increase in control cell adhesion. After 70 minutes, PBA stimulates adherence to Fibronectin compared to control cells (Fig. 27). BSA functions as the negative control and cells were incubated for the total 70 minutes. The average of three trials of this assay with MCF-7 cells showed that with the presence of error bars there is no significant

difference over time of Fibronectin adhesion in control cells compared to BA or PBA treated cells (Fig. 28).

To further test the affect of BA and PBA on integrins, the effect of treatments on integrin activity was investigated using antibodies to integrins $\alpha_v\beta_5$ and β_1. These integrins were chosen because they are expressed in both cell lines, were commercially available and they also have an affinity for the ECM Fibronectin (25, 43) and could therefore be the possible integrin sites of BA and PBA interaction. Cells were treated with either BA (1 mM) or PBA (1 mM) or $MnCl_2$ (1 mM) for 10 minutes before being incubated with the antibodies for 2 hours. Trials for each treatment were performed in triplicate. IgG is used as a negative control antibody in this experiment.

In ZR-75-1 cells, control cells bind more strongly to $\alpha_v\beta_5$ antibodies compared to β_1 antibodies indicating this integrin is abundant on the cell surface (Fig. 29). In both integrins studied, BA and PBA stimulate integrin activity whereas $MnCl_2$ maintains the same amount of activity ($\alpha_v\beta_5$) or decreases activity (β_1) compared to controls (Fig. 29). Activity of the negative control IgG is the same in all treatments.

MCF-7 control cells display relatively similar binding to β_1 and $\alpha_v\beta_5$ integrin antibodies. In β_1 integrins, BA maintains integrin activity compared to control; however, PBA stimulates activity and $MnCl_2$ decreases activity of this integrin (Fig. 30). In the case of the $\alpha_v\beta_5$ integrins, none of the treatments (BA, PBA or $MnCl_2$) significantly affected integrin activity (Fig. 30).

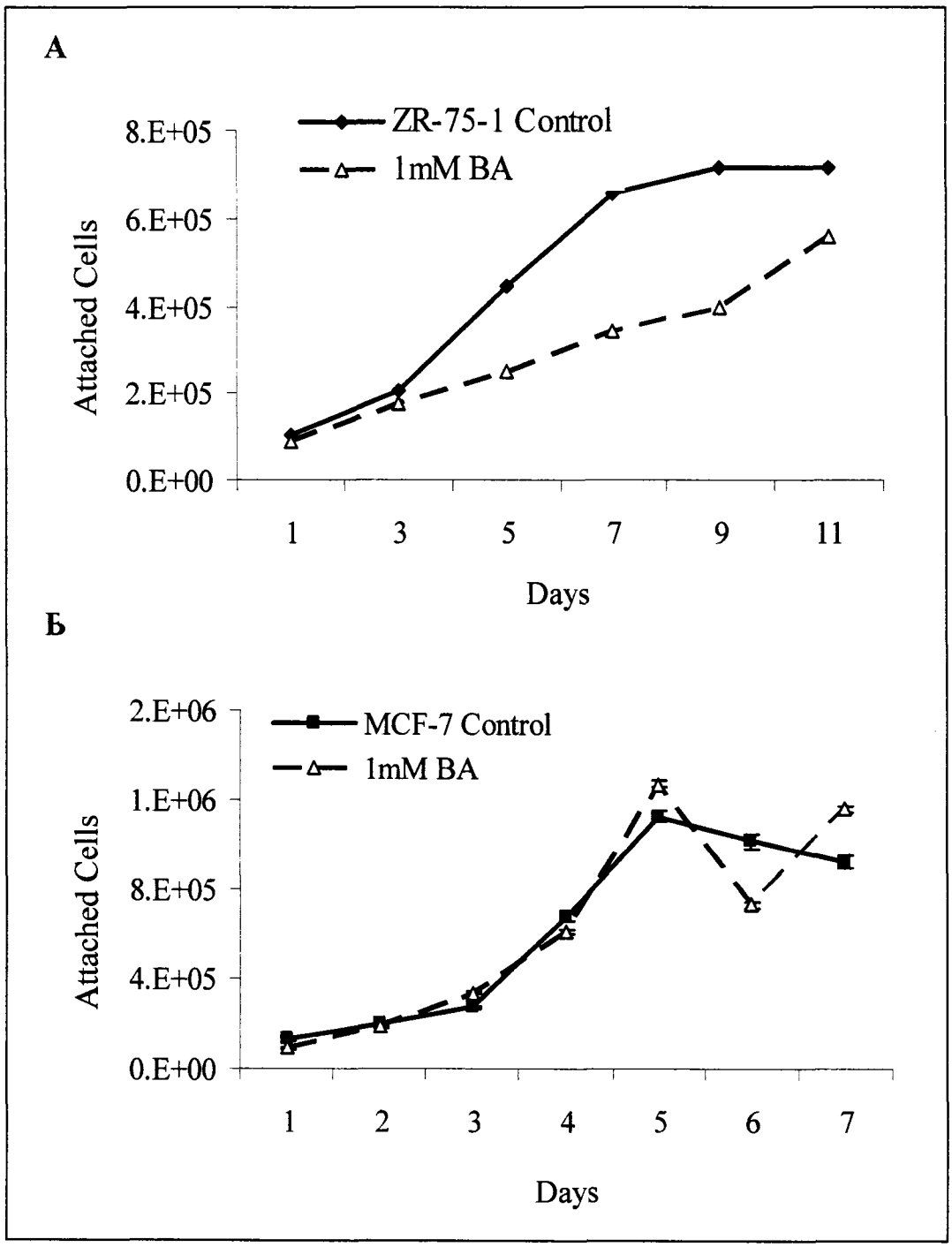

Figure 7. Proliferation assay with BA. A, ZR-75-1 cells continuously exposed to 1 mM BA show growth inhibition at day 5. B, Continuous exposure to 1mM BA does not inhibit MCF-7 cell proliferation.

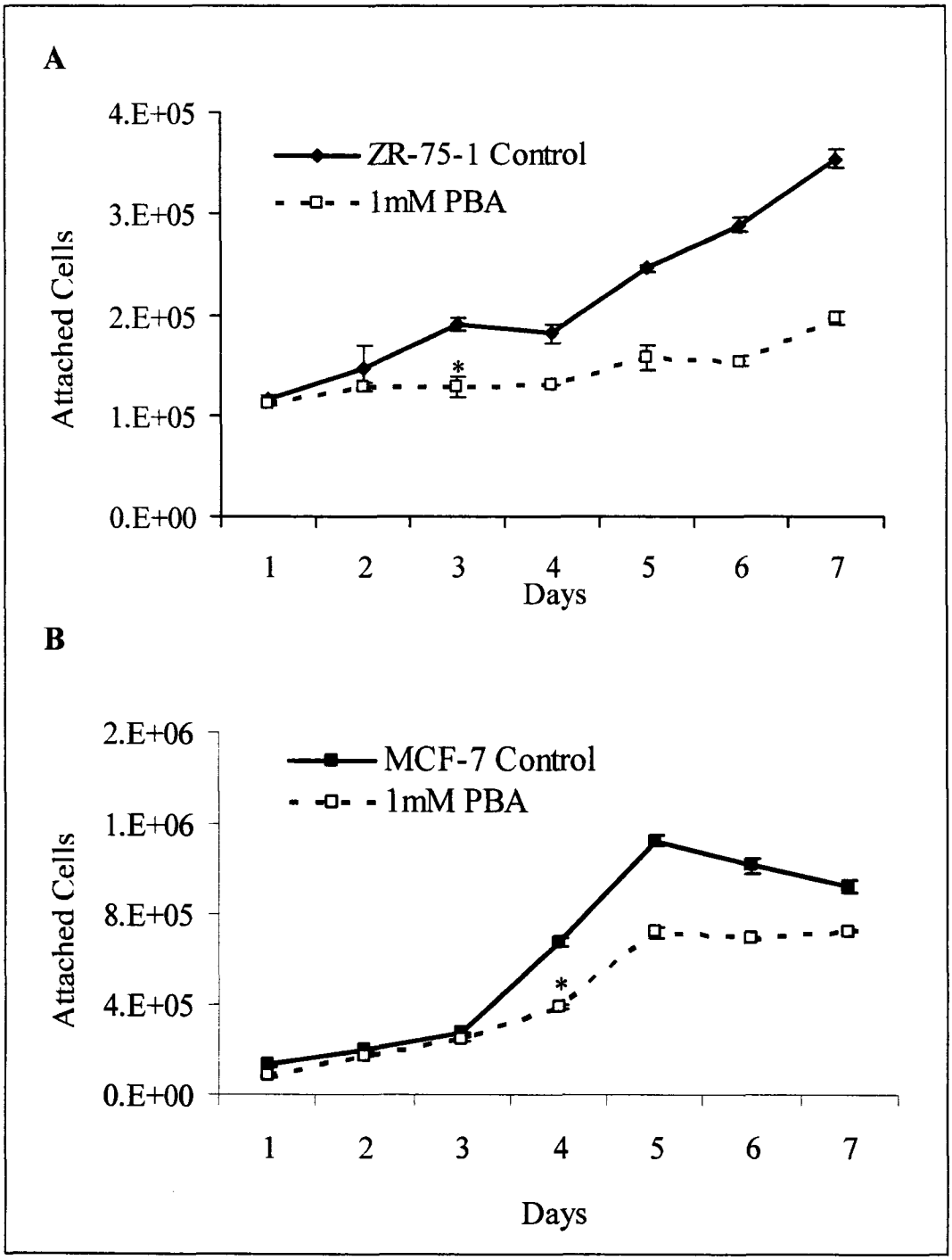

Figure 8. Proliferation assay with PBA. A, Growth is significantly inhibited in ZR-75-1 cells continually exposed to 1 mM PBA after 3 days. B, 1 mM PBA treatment also significantly inhibits growth of MCF-7 cells at day 4. Values represent Mean SE where n = 2. Statistical significance is maintained on subsequent days following growth inhibition (* p-value < 0.05).

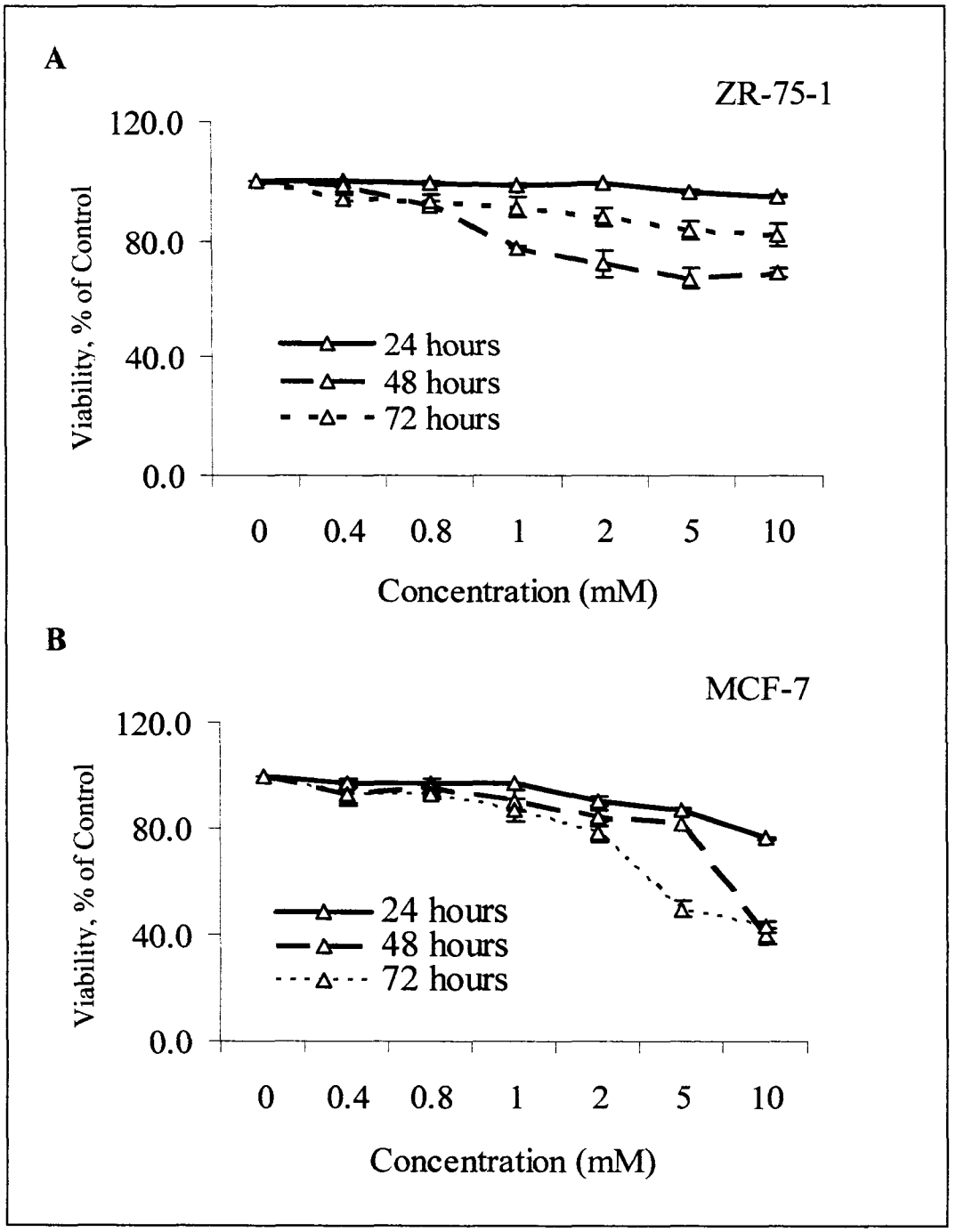

Figure 9. Cell cytotoxicity assay with BA. A, ZR-75-1 and B, MCF-7 cells were exposed to varying concentrations of BA for 24, 48 and 72 hours and cell viability was measured with alamarBlue. Cell viability significantly decreased after 48 and 72 hour exposure to 1.0 – 10 mM in both cell lines with p-value < 0.05 or p-value < 0.001 ($n = 4$).

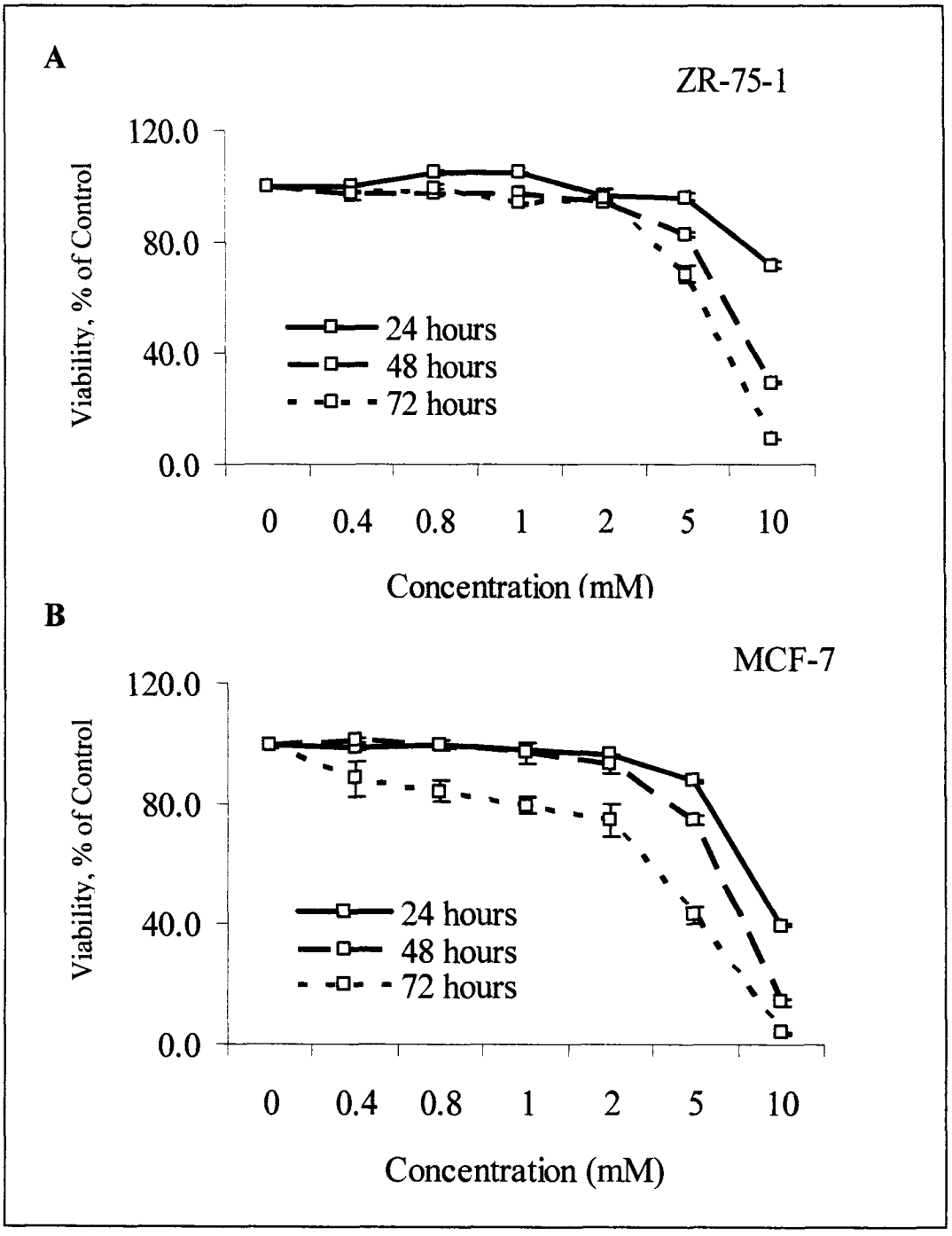

Figure 10. Cell cytotoxicity assay with Phenylboronic acid. Assay procedure is as described in Fig. 9. A, ZR-75-1 cells significantly decreased viability after 24 hour exposure to 5.0 and 10 mM and 48 and 72 hour exposure to 1.0 – 10 mM PBA. B, Significant decrease in cell viability was seen after 24 and 48 hour exposure to 2.0 – 10 mM and 72 hour exposure to 0.4 – 10 mM PBA. Significance is determined as p-value < 0.05 or p-value < 0.001 (n = 4).

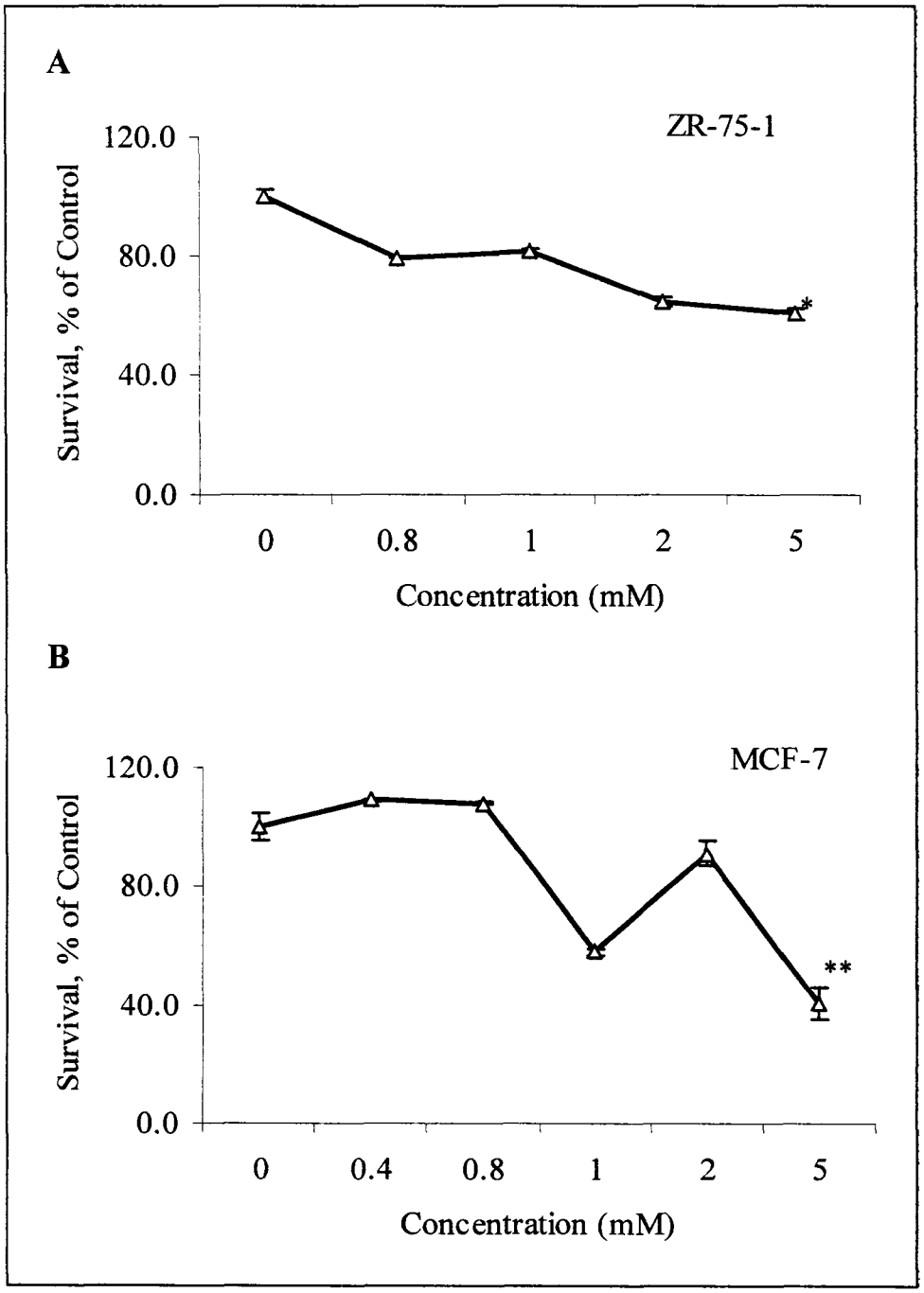

Figure 11. Clonogenic Survival with BA. Cell survival was measured compared to control after 72 hour exposure to BA. A, Cell survival significantly decreased 40% with 5 mM PBA in ZR-75-1 cells. B, MCF-7 cells decreased survival 60% with 5 mM BA, the decrease in cell survival at 1 mM BA does not follow the trend from other trials. Significance is determined as *p-value < 0.05 or **p-value < 0.001 (n = 3).

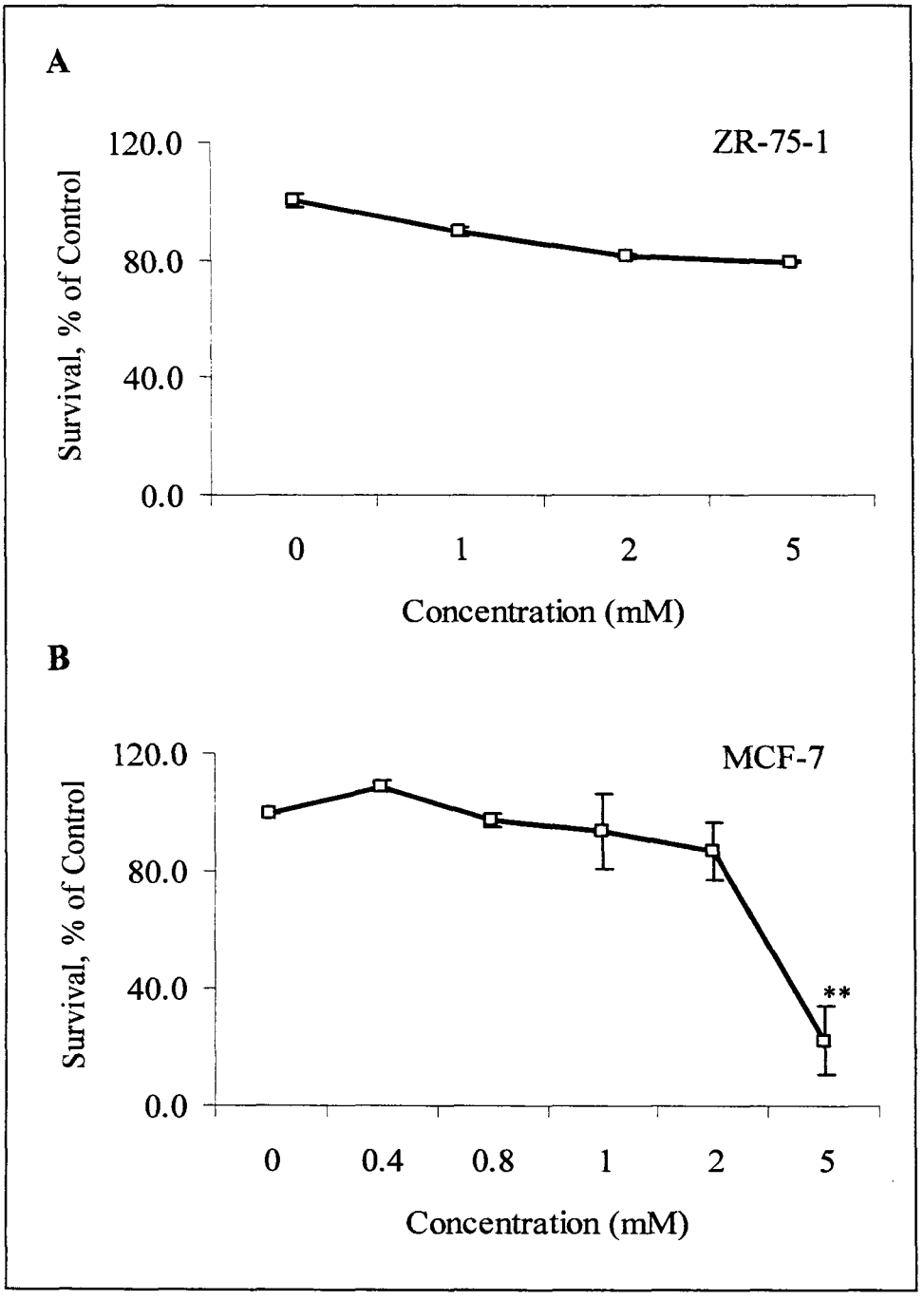

Figure 12. Clonogenic Survival with PBA. A, ZR-75-1 cell survival was decreased by 21% with 5 mM PBA. B, MCF-7 cell survival was significantly decreased by 78% with 5 mM PBA compared to control. Significance is determined as **p-value < 0.001 (n = 3)

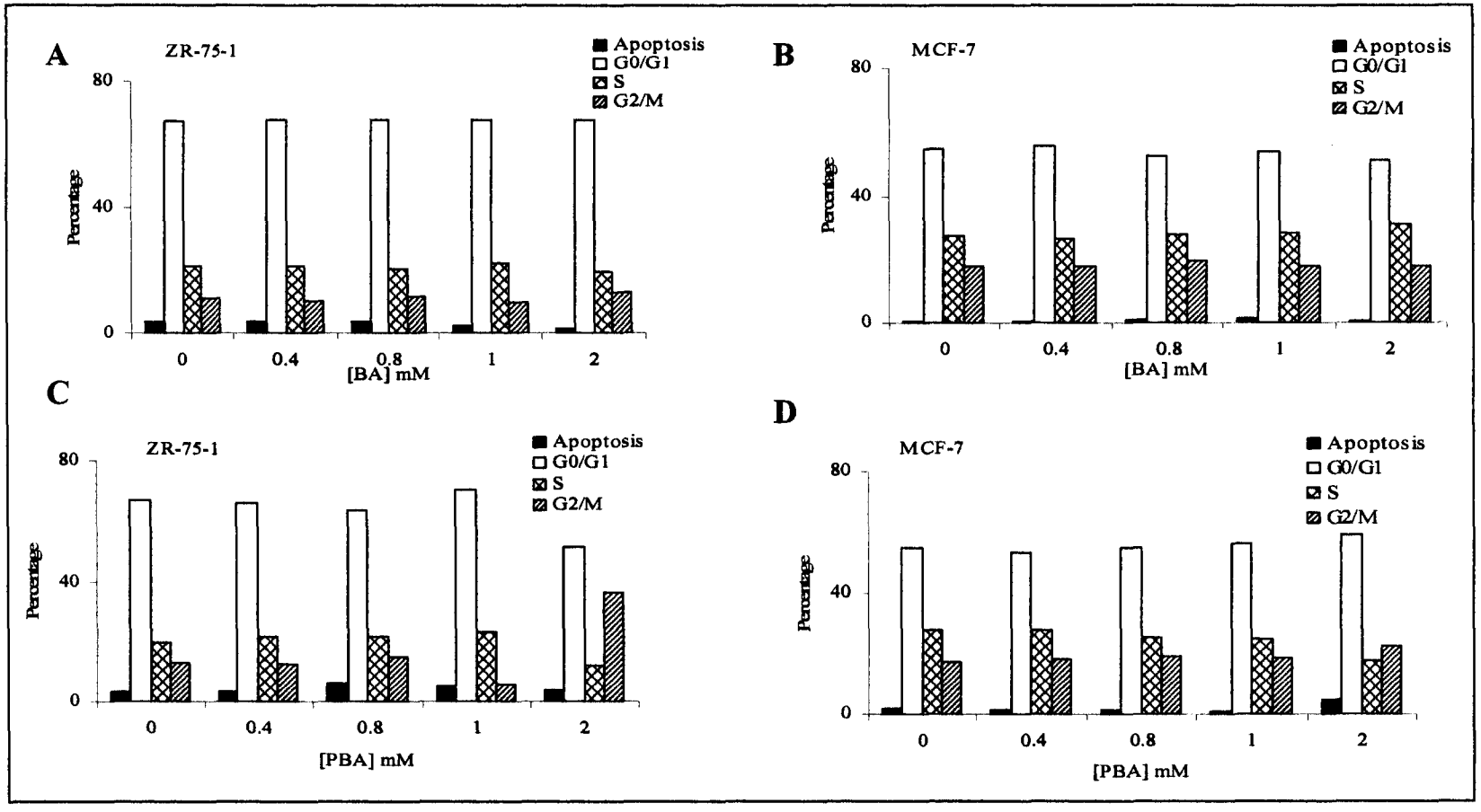

Figure 13. Cell cycle analysis of attached cells treated with BA and PBA. Cells were incubated with BA or PBA for 5 days and cell cycle distribution was determined on attached cells using flow cytometry. A and C, treated ZR-75-1 cells maintain the same cell cycle distribution as controls. B and D, Cell cycle distribution does not change in treated MCF-7 cells compared to control.

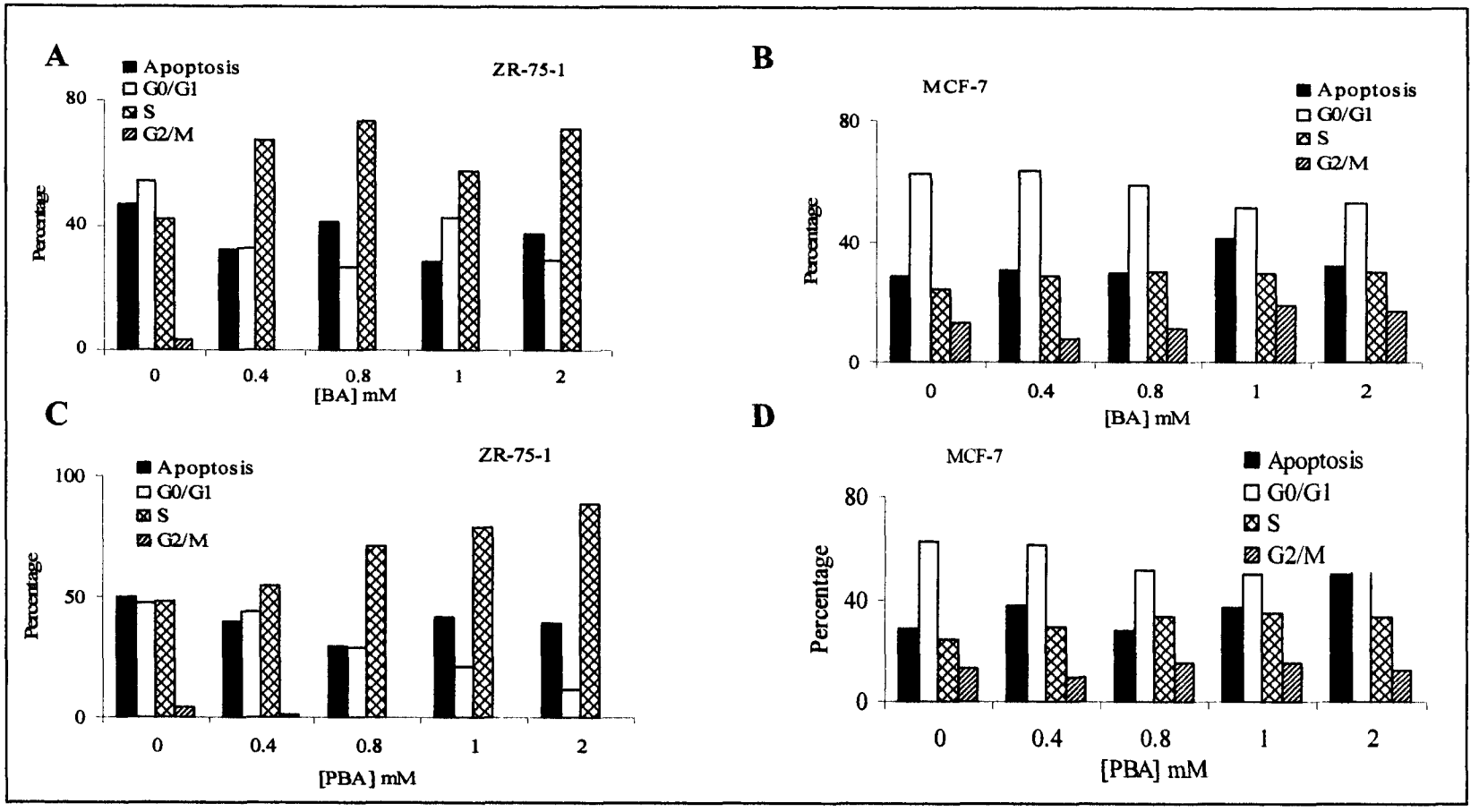

Figure 14. Cell cycle analysis of suspended cells treated with BA and PBA. Cells were exposed to BA or PBA treatments for 5 days and the cells suspended in the medium were collected and cell cycle distribution was determined using flow cytometry. A and C, treated ZR-75-1 cells show a dose-dependent S-phase block compared to controls, treatment with PBA displays the most dramatic effect (C). B and D, MCF-7 cells also show evidence of an S-phase block in the decrease of G0/G1 and increase in S in treated samples compared to controls.

Table 1. ZR-75-1 Florescence Microscopy. Average rate of Apoptosis and Necrosis after 72 hour exposure to BA and PBA from two trials (n = 6).

ZR-75-1		
Treatment	% Apoptosis	% Necrosis
Control	4.0 ± 0.5	0.2 ±0.2
1 mM BA	5.3 ± 1.2	1.8 ± 0.9
5 mM BA	5.8 ± 0.6*	1.1 ± 0.2*
10 mM BA	7.2 ± 1.9	1.5 ± 0.8
Control	4.9 ± 0.7	1.0 ± 0.5
1 mM PBA	7.3 ± 1.4	1.3 ± 0.7
5 mM PBA	10.5 ± 2.8*	4.3 ± 0.5**
10 mM PBA	21.7 ± 6.9*	8.3 ± 2.4*

* $p \leq 0.05$ ** $p \leq 0.001$.

Table 2. MCF-7 Florescence Microscopy. Average rate of Apoptosis and Necrosis after 72 hour exposure to BA and PBA from two trials (n = 6).

MCF-7		
Treatment	% Apoptosis	% Necrosis
Control	1.4 ± 0.4	0.4 ± 0.2
1 mM BA	2.5 ± 0.8	0.1 ± 0.1
5 mM BA	5.9 ± 1.3*	0.6 ± 0.2
10 mM BA	9.2 ± 1.6**	1.6 ± 0.5*
Control	3.2 ± 0.9	0.1 ±0.1
1 mM PBA	3.1 ± 1.0	1.7 ± 0.7*
5 mM PBA	7.5 ± 2.1*	1.7 ± 0.5*
10 mM PBA	10.3 ± 2.0*	19.0 ± 1.6**

* $p \leq 0.05$ ** $p \leq 0.001$.

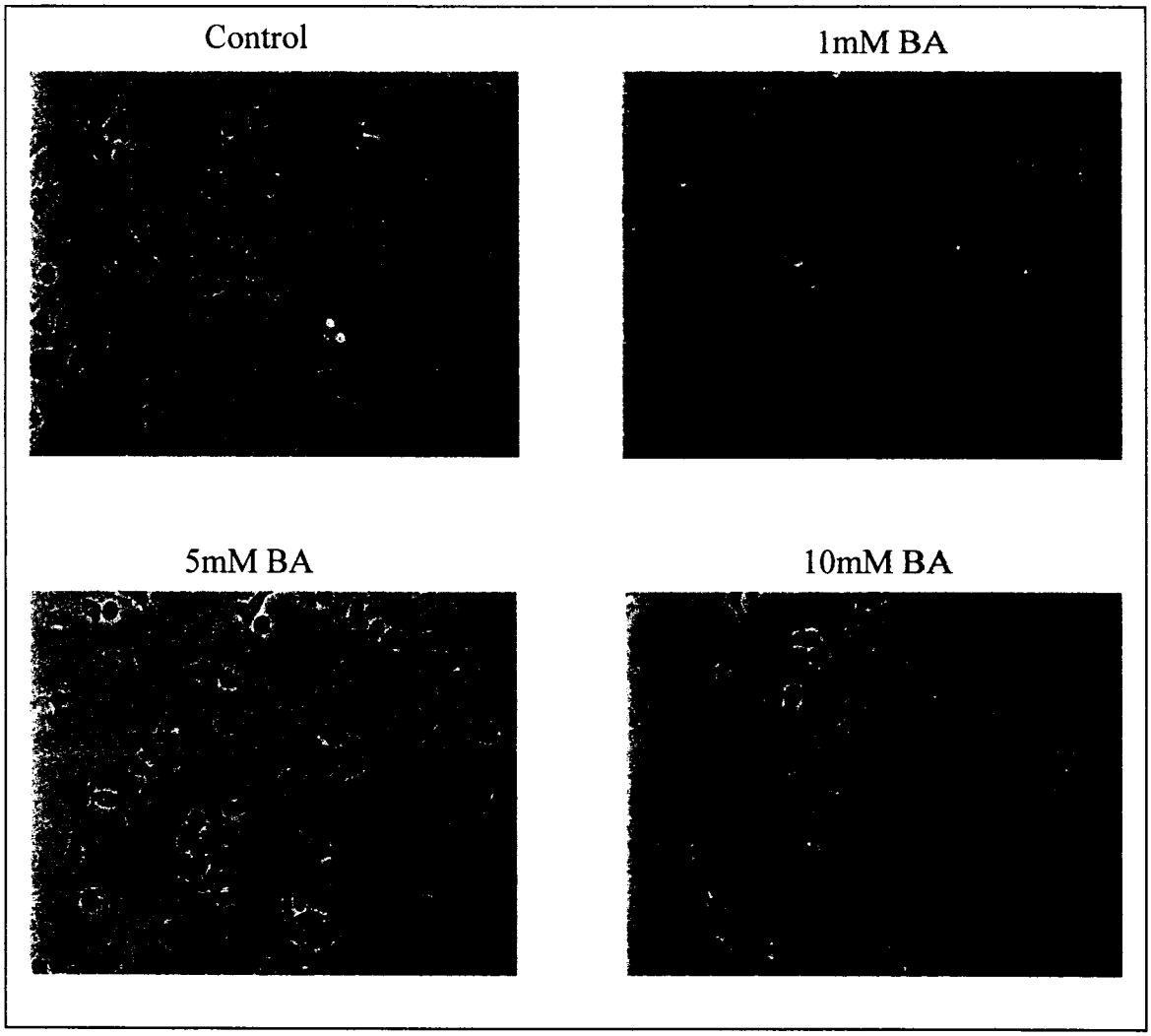

Figure 15. ZR-75-1 treated with BA florescence microscopy images. Cells were treated with increasing concentrations of BA for 72 hours. Hoechst and propidium iodide stains were added and phase contrast microscope images were obtained and then apoptotic (Hoechst) and necrotic (propidium iodide) images were obtained (not shown).

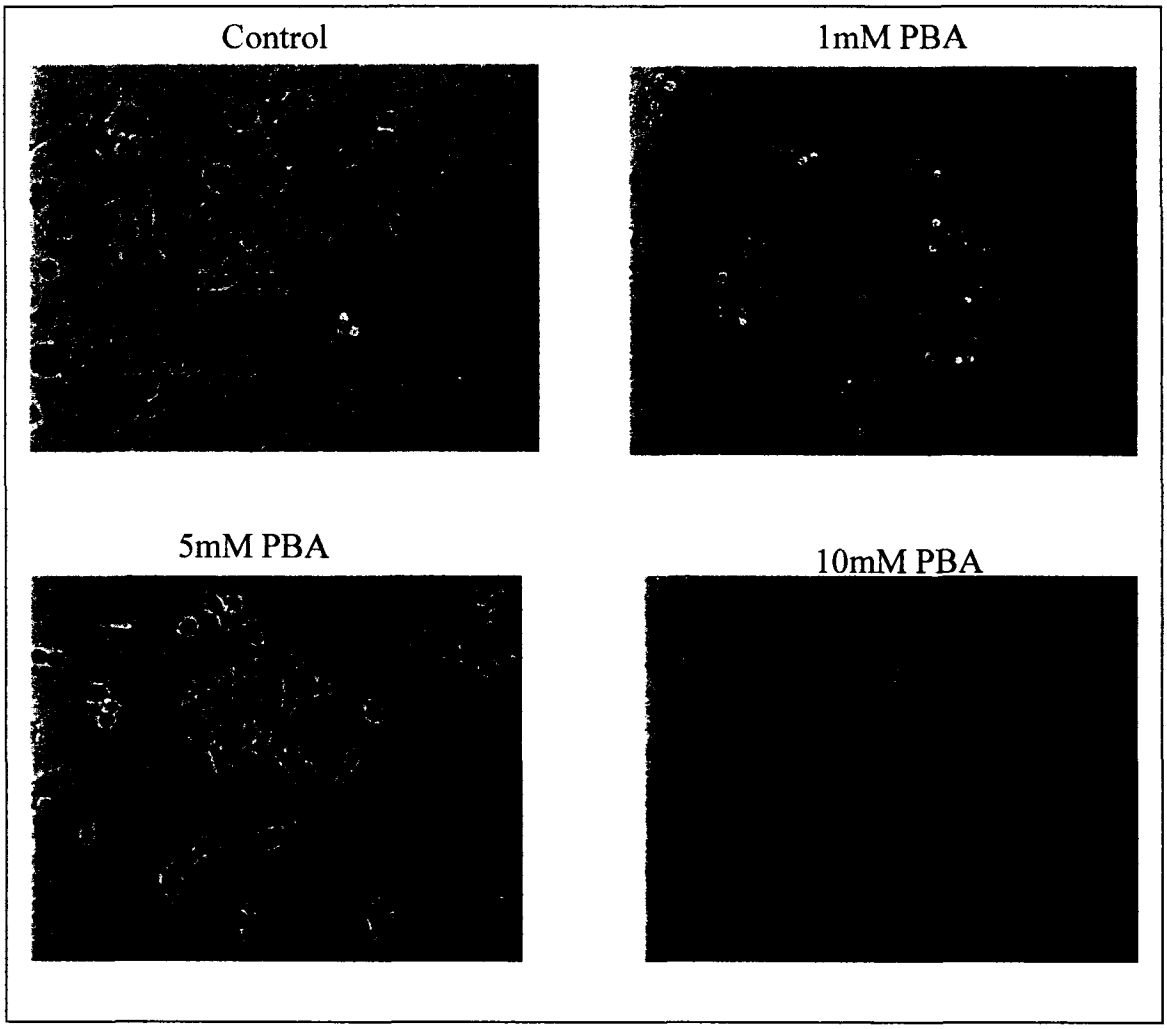

Figure 16. ZR-75-1 treated with PBA florescence microscopy images. Cells were treated with increasing concentrations of PBA for 72 hours. Hoechst and propidium iodide stains were added and phase contrast microscope images were obtained and then apoptotic (Hoechst) and necrotic (propidium iodide) images were obtained (not shown).

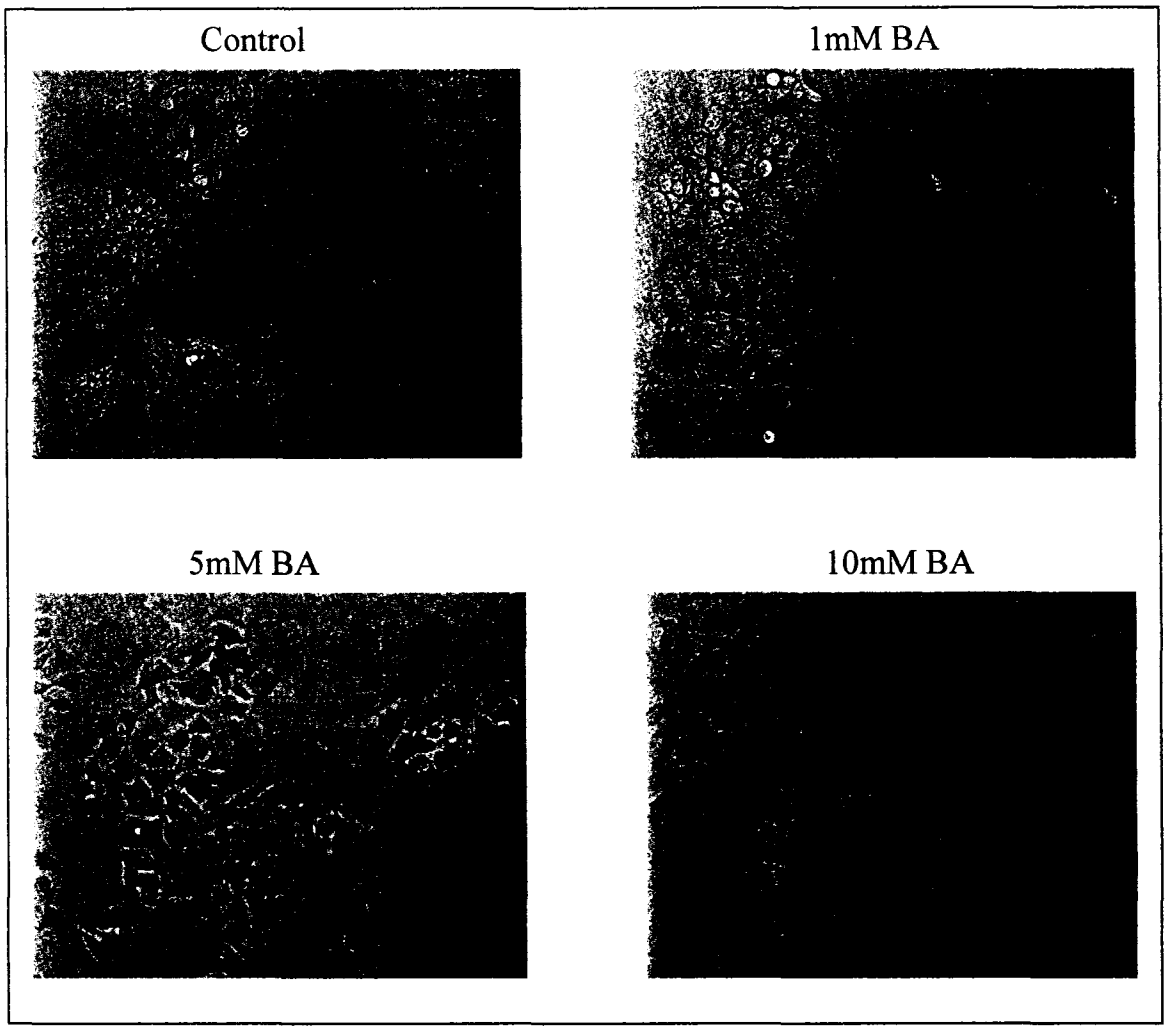

Figure 17. MCF-7 treated with BA florescence microscopy images. Cells were treated with increasing concentrations of BA for 72 hours. Hoechst and propidium iodide stains were added and phase contrast microscope images were obtained and then apoptotic (Hoechst) and necrotic (propidium iodide) images were obtained (not shown).

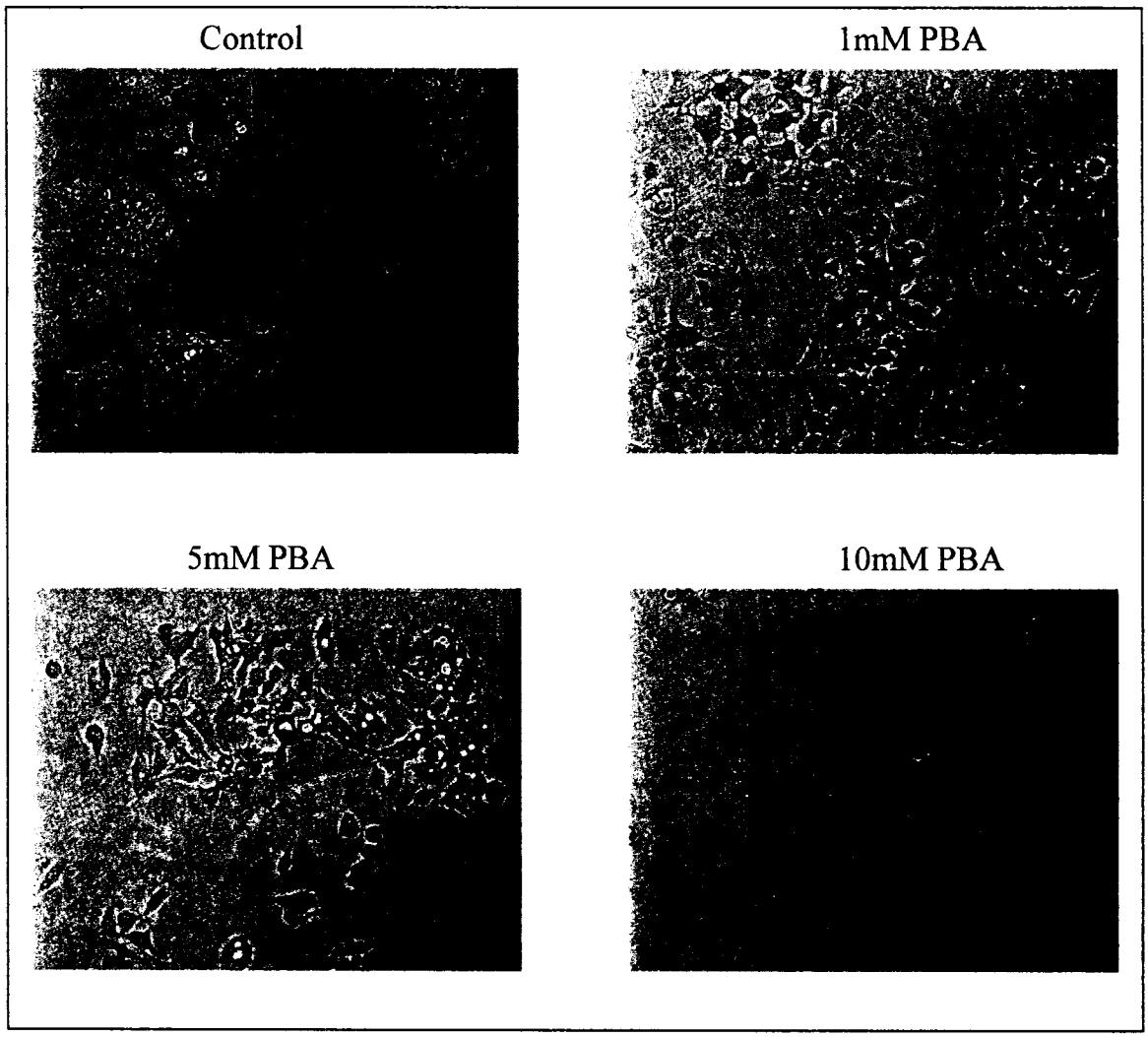

Figure 18. MCF-7 treated with PBA florescence microscopy images. Cells were treated with increasing concentrations of PBA for 72 hours. Hoechst and propidium iodide stains were added and phase contrast microscope images were obtained and then apoptotic (Hoechst) and necrotic (propidium iodide) images were obtained (not shown).

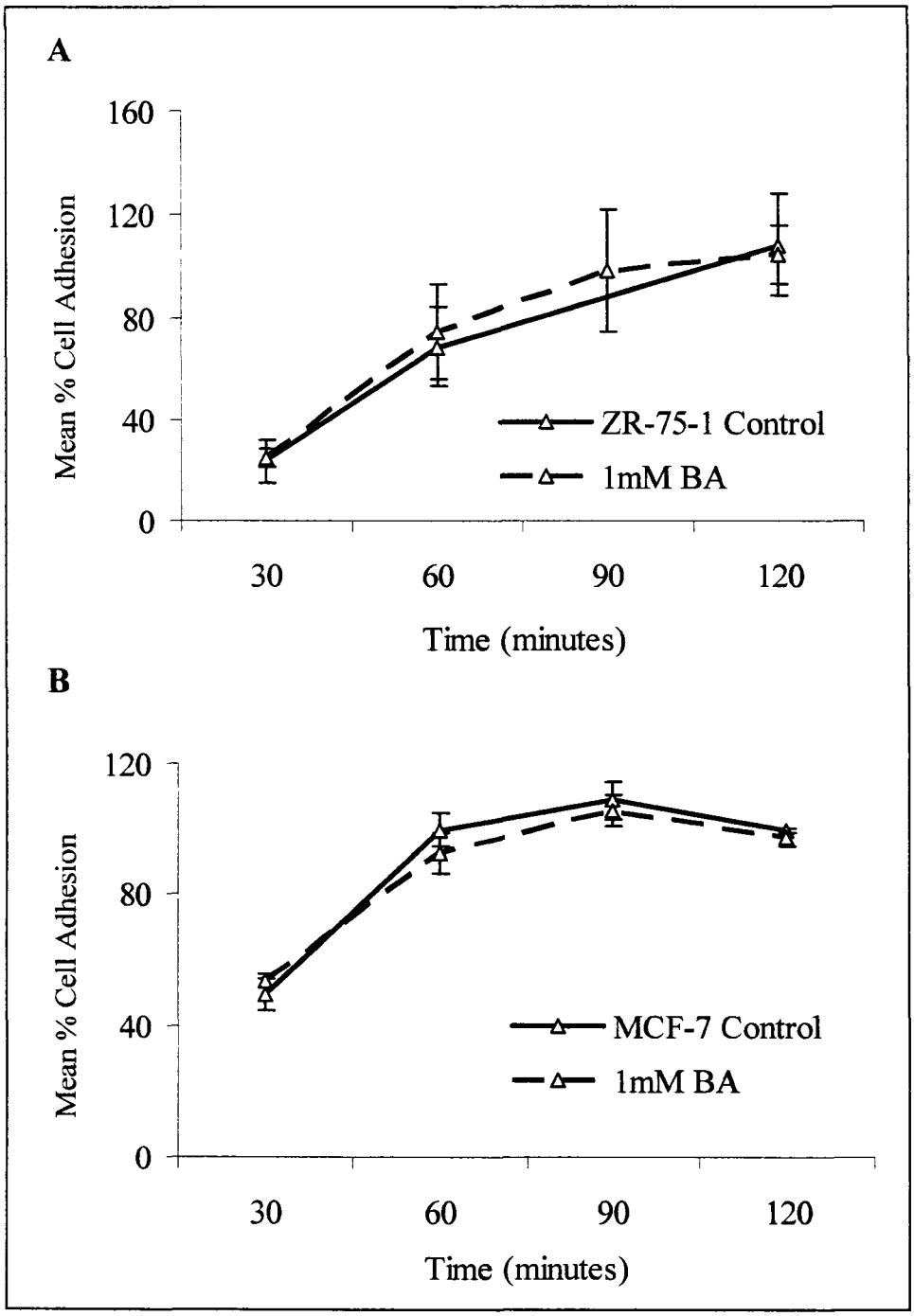

Figure 19.Time-dependent Adhesion assays treated with BA. Cells were treated with 1mM BA for 30 – 120 minutes and cell adhesion rates were determined as a percent of the original number of cells seeded. A, ZR-75-1 and B, MCF-7 cell adhesion was not affected by BA treatment (n = 3).

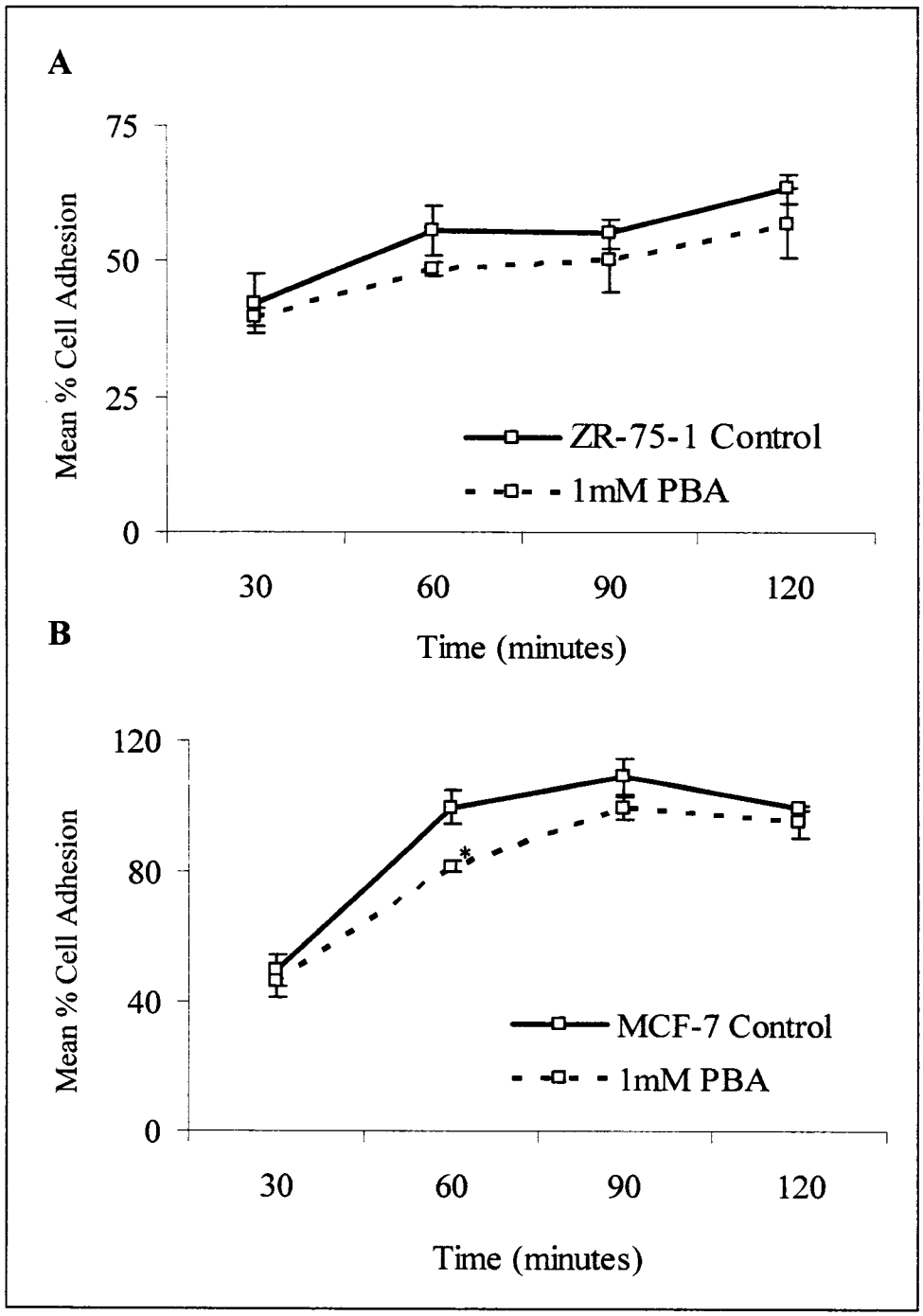

Figure 20. Time-dependent Adhesion assays treated with PBA. Experiment protocol is as described in Fig. 17. A, PBA treatment decreased the rate of adhesion in ZR-75-1 cells at 60 minutes. B, Adhesion in MCF-7 cells was significantly decreased at 60 minutes by PBA treatment. Significance is determined as *p-value < 0.05 (n = 3).

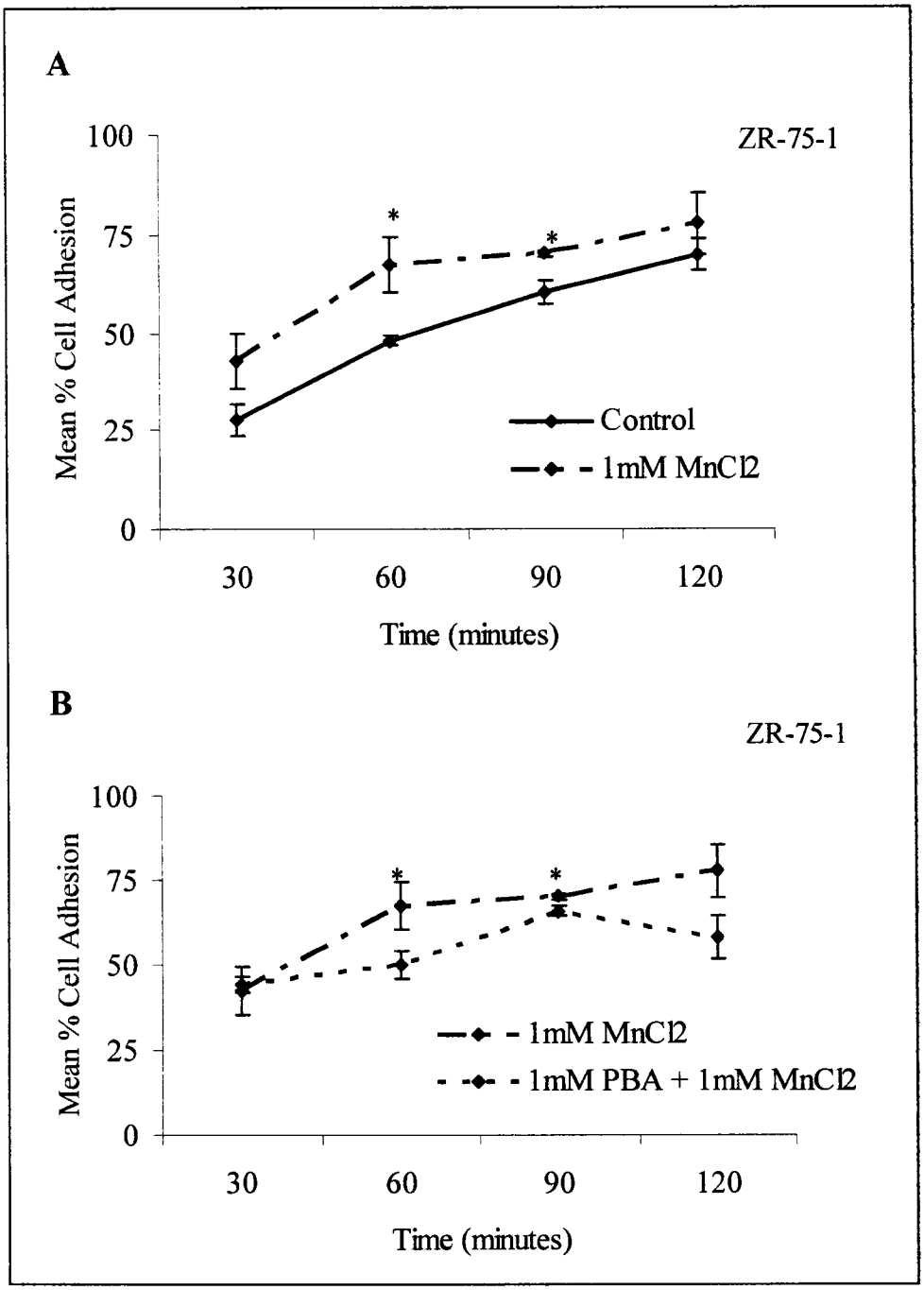

Figure 21. ZR-75-1 combination treatment adhesion assay. Cells were exposed to either 1 mM MnCl2 alone or in combination with 1 mM PBA, attachment is determined compared to original number of cells seeded. A, Treatment with $MnCl_2$ significantly stimulates adhesion at 60 and 90 minutes. B, Combination treatment with PBA significantly inhibits the stimulatory effect of $MnCl_2$ at 60 and 90 minutes. Significance is determined as *p-value < 0.05 (n = 3).

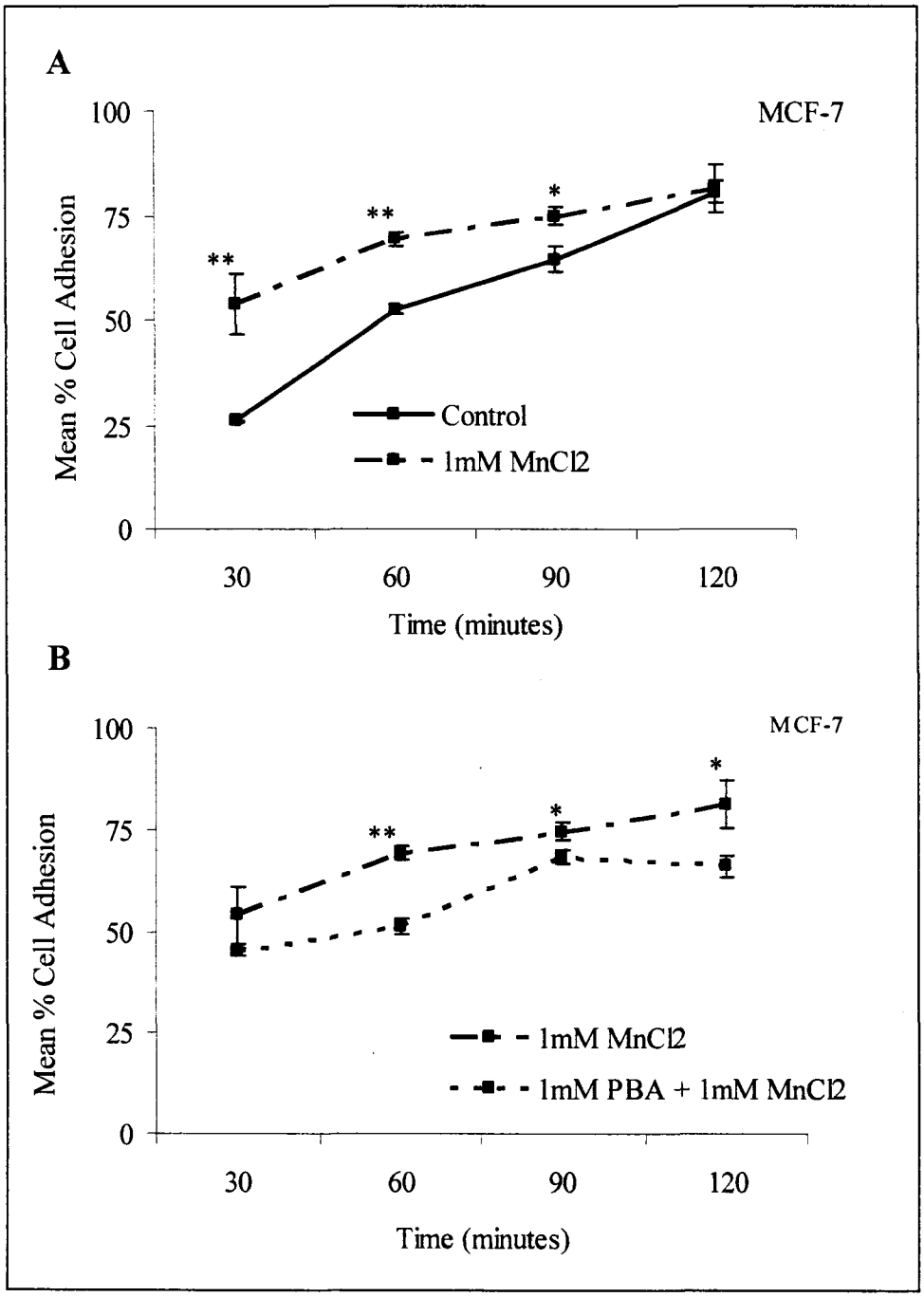

Figure 22. MCF-7 combination treatment adhesion assay. Cells are treated as is described in Fig. 19. A, MnCl2 treatment significantly increased adherence from 30 – 90 minutes. B, In combination with PBA, the stimulatory effect of MnCl2 was significantly inhibited from 60 – 120 minutes. Significance is determined as *p-value < 0.05 or **p-value < 0.001 (n = 3).

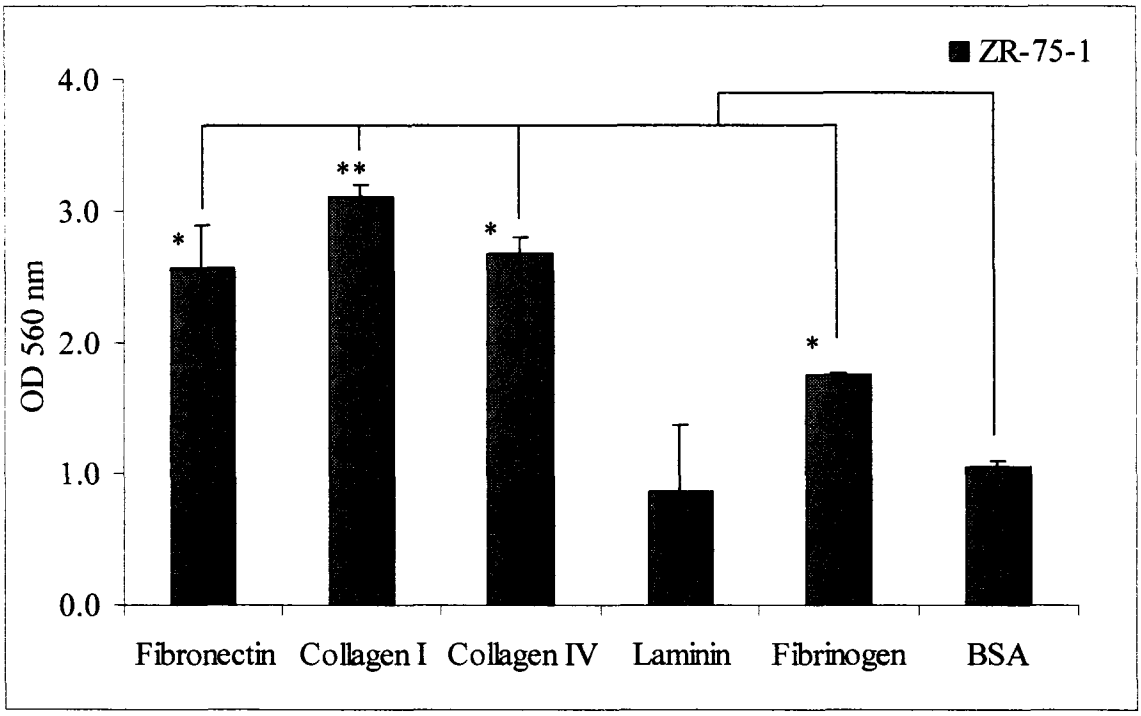

Figure 23. ZR-75-1 ECM affinity assay. Cells were incubated on the represented ECM for 90 minutes and the optical density at 560 nm of a stain solution was measured to determine affinity. ZR-75-1 cells have a significant affinity for Fibronectin, Collagen I, Collagen IV and Fibrinogen as compared to BSA, the negative control. Significance is determined as *p-value < 0.05 or **p-value < 0.001 ($n = 2$).

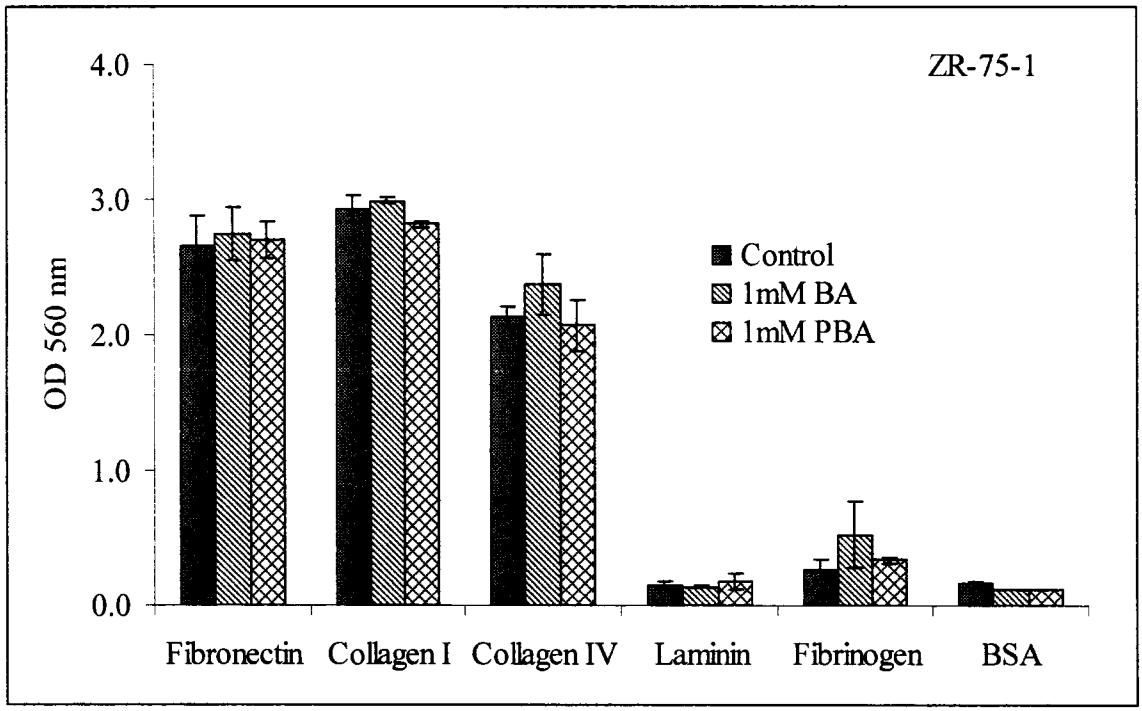

Figure 24. ZR-75-1 BA and PBA treated ECM assay. Cells were pre-treated for 10 minutes with BA or PBA and then incubated for 30 minutes on the represented ECM. Absorbance of the stain solution was determined at 560 nm (n = 2).

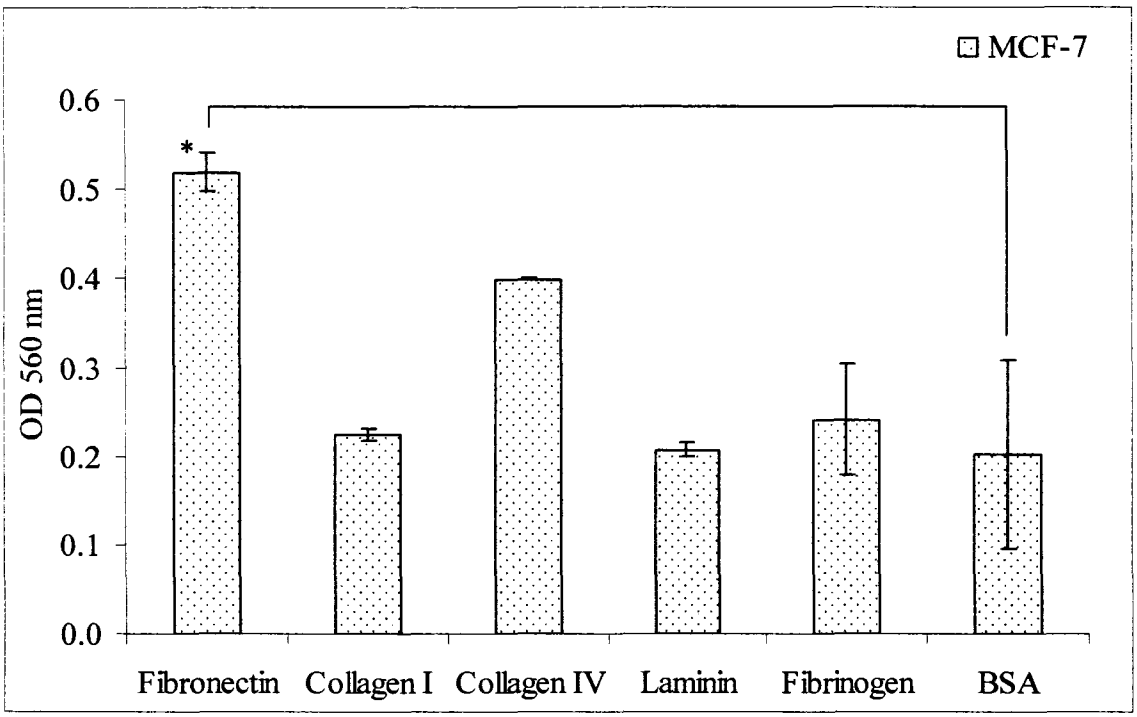

Figure 25. MCF-7 ECM affinity assay. Cells are incubated as described in Fig. 21. MCF-7 cells have a significant affinity for Fibronectin as compared to BSA, the negative control. Significance is determined as *p-value < 0.05 (n = 2).

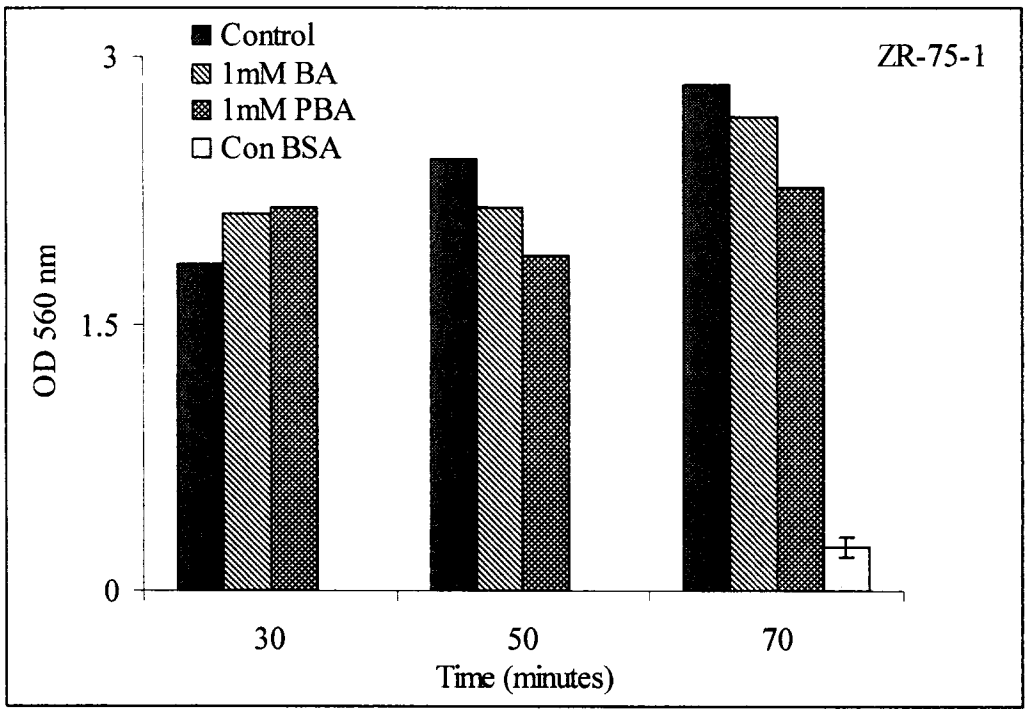

Figure 26. ZR-75-1 time-dependent Fibronectin adhesion assay. Cells are incubated with BA (1 mM) and PBA (1 mM) treatments for 10 minutes and then added onto a fibronectin surface for 30, 50 and 70 minutes. Treatments appear to enhance binding to a fibronectin surface after 30 minutes; however, binding decreases with increased exposure to the fibronectin surface as compared to control. BSA, the negative control, was run in duplicate (n = 2) but for the treatments (n = 1).

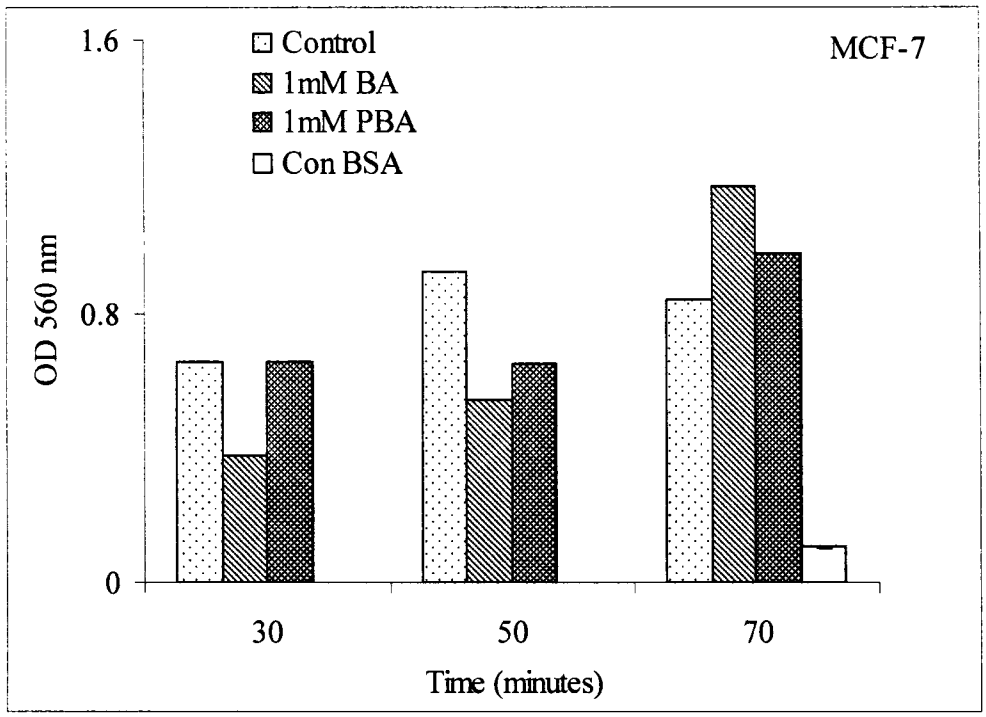

Figure 27. MCF-7 time-dependent Fibronectin adhesion assay. Cells are incubated with BA (1 mM) and PBA (1 mM) treatments for 10 minutes and then added onto a fibronectin surface for 30, 50 and 70 minutes. Treatments appear to decrease binding to the fibronectin surface after 30 – 60 minutes; however, binding is stimulated after 70 minutes as compared to control. BSA, the negative control, was run in duplicate (n = 2) but for the treatments (n = 1).

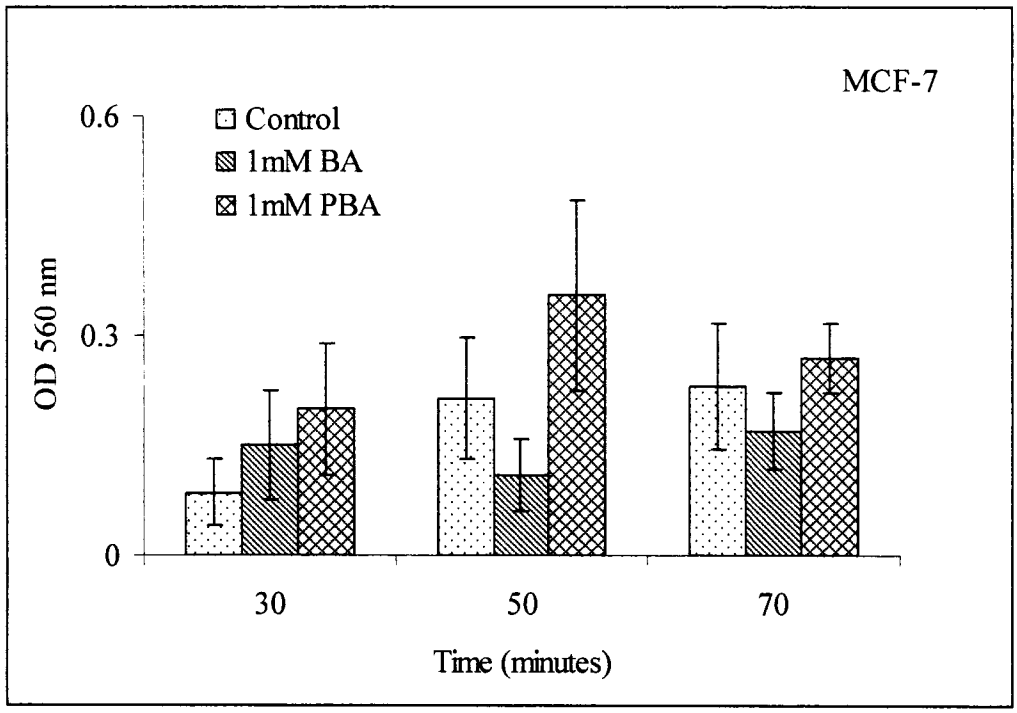

Figure 28. MCF-7 time-dependent Fibronectin adhesion assay (average of three trials). Cells were treated and assay was performed as described in Fig. 27. The mean and SE of data from 3 separate trials were calculated and plotted above (n = 3).

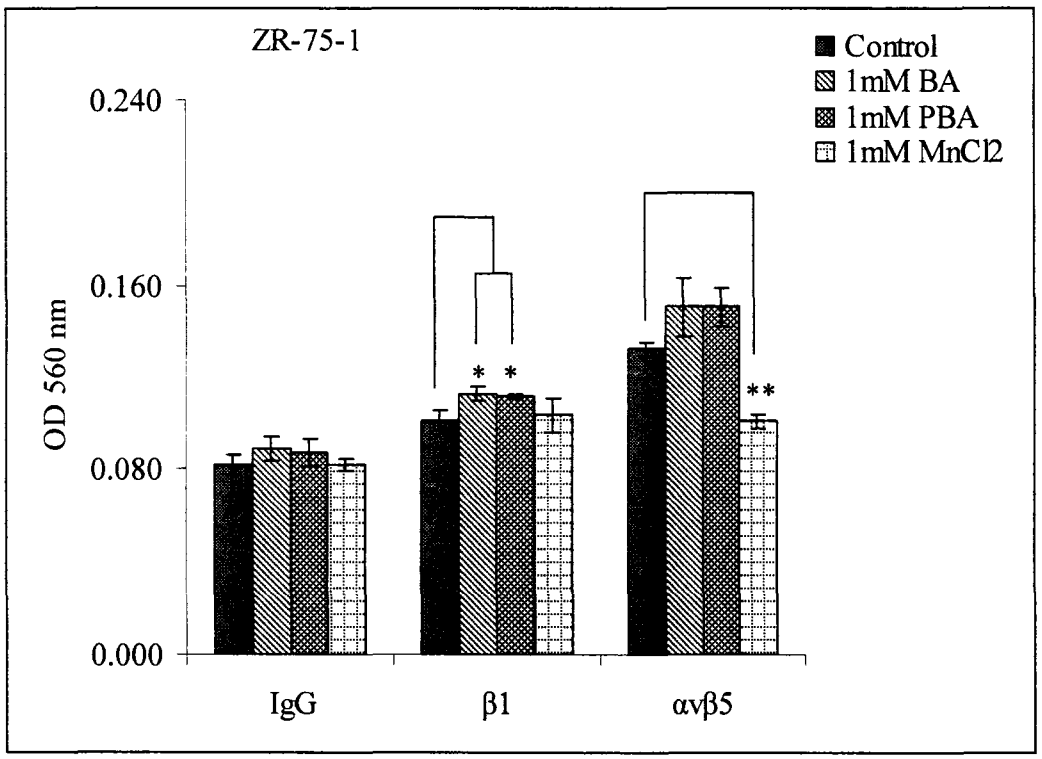

Figure 29. ZR-75-1 integrin activity assay. Cells were exposed to BA (1 mM), PBA (1 mM) or MnCl2 (1 mM) for 10 minutes before being incubated with control (IgG) or integrin $\alpha_v\beta_3$ or β_1 antibodies. Integrin activation is measured as the optical density at 560 nm of a stain solution. The activity of control antibody is maintained constant in spite of treatments. In β_1 integrins activity is significantly stimulated by BA and PBA treatments compared to control. BA and PBA also appear to stimulate activity of $\alpha_v\beta_3$ integrins although it is not significant compared to control. MnCl2 treatments, which were expected to enhance integrin activity either maintained the same activity as controls in β_1 integrins or significantly decreased $\alpha_v\beta_3$ integrin activity. Significance is determined as *p-value < 0.05 or **p-value < 0.001 (n = 3).

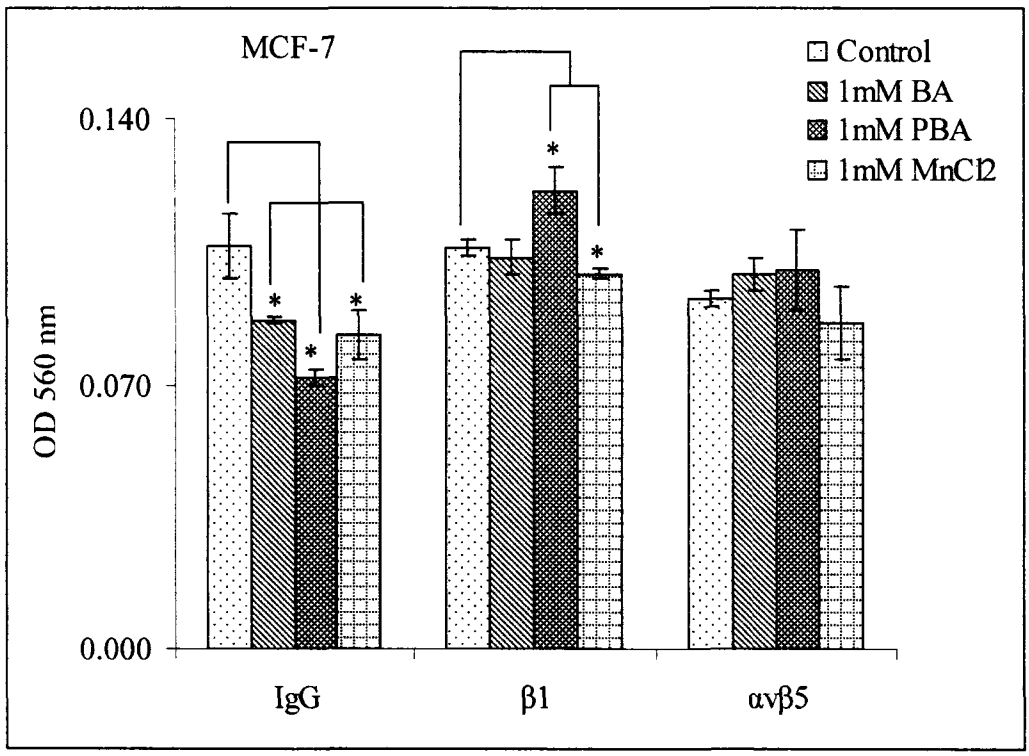

Figure 30. MCF-7 integrin activity assay. Cells were exposed to BA (1 mM), PBA (1 mM) or MnCl2 (1 mM) for 10 minutes before being incubated with control (IgG) or integrin $\alpha_v\beta_3$ or β_1 antibodies. Integrin activation is measured as the optical density at 560 nm of a stain solution. The activity of control antibody significantly decreases compared to control with all 3 treatments. In β_1 integrins activity is maintained by BA treatment and is significantly stimulated by PBA treatment compared to control. In $\alpha_v\beta_3$ integrins, BA and PBA maintain the level of activity compared to control. In this activity assay MnCl2 treatments, which were expected to enhance integrin activity significantly decreased β_1 integrins activity or maintained the same activity as controls in $\alpha_v\beta_3$ integrins. Significance is determined as *p-value < 0.05 ($n = 3$).

CHAPTER 5

SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

5.1 Discussion of Results

The goal of this research was to determine the effect of BA and PBA on breast cancer cells and to elucidate the mechanism of action by answering the following research questions:

i) Will BA and PBA inhibit breast cancer cell proliferation?

Based on experimental evidence it can be concluded that 1 mM BA inhibits growth of ZR-75-1 cells but not MCF-7 and 1 mM PBA can inhibit growth in both cell lines (Fig. 7 and 8). As was assumed, PBA does indeed induce a more dramatic effect than BA and this may be attributed to the presence of the bulky phenyl group and may indicate the site of action is similar in both treatments.

ii) Is the effect of BA and PBA a function of cellular toxicity, cell cycle arrest or apoptosis?

After establishing the effect of BA and PBA on the breast cancer cell lines the next step involved determining which cellular processes are affected to result in proliferation inhibition. From cytotoxicity experiments, alamarBlue and clonogenic survival, it can be concluded that lower concentrations of BA or PBA used (0.4 – 2 mM) only induce $\leq 20\%$ cell death whereas higher concentrations used (5 – 10 mM) can induce higher amounts of cell death (Fig. 9 – 12)

Since the concentration of BA and PBA which caused growth inhibition, 1 mM, is in the lower concentration group and therefore does not induce a large amount of cell death, the focus moved to observing the cells for possible disruption in cell cycle distribution, rates of apoptosis vs. necrosis and morphology changes.

For cell cycle analysis, both adherent and suspended cells were measured to investigate any dose-dependent trends. Cells were also observed after 5 day exposure to treatments, this length of time was chosen as cell proliferation is inhibited at this stage in all the affected cell lines (Fig. 7 and 8) There was no difference in cell cycle distribution of adherent cells for either treatments or cell lines (Fig. 13); however, in the suspended cells there was evidence for an S-phase block which was displayed very prominently in ZR-75-1 cells exposed to PBA (Fig. 14B). To observe apoptosis and necrosis rates as well as cell morphology following treatment, florescence microscopy was utilized.

From florescence microscopy data there appears to be a dose-dependent increase in apoptosis and necrosis in both cell lines with BA and PBA treatments (Table 1 and 2). Cell morphology images reveal changes in cell size at higher concentrations of BA and PBA treatment with the most dramatic difference being seen in ZR-75-1 cells treated with PBA (Fig. 16).

During an S-phase block of the cell cycle, cells have increased in size during G0/G1 phase and have replicated their DNA during S phase; however, these cells cannot undergo mitotic division to produce a daughter cell and so remain enlarged or distorted and unable to grow and may eventually undergo apoptosis, programmed cell death. Cell cycle analysis of an S-phase block is further supported by the microscopy images which display enlarged or distorted cells at high BA and PBA concentrations (Fig. 15 – 18).

Additionally, the fact that ZR-75-1 cells treated with PBA display the most dramatic cell cycle block, supports the proliferation assay results in which PBA treated ZR-75-1 cells have the most static cell growth once inhibition began at day 3 (Fig. 8A). After detailing the effect of BA and PBA and also that the treatments may be influencing cell cycle dynamics and inducing apoptosis to inhibit cell growth, our focus shifted once more to the site of BA and PBA interaction with these cells.

iii) Do BA and PBA interact with and mediate their effects via extracellular proteins?

To test this hypothesis, adhesion of cells was investigated on a plastic flask surface as well as on specific ECM surfaces and also integrins $\alpha_v\beta_5$ and β_1 activity in the presence of BA or PBA treatment was measured.

Adhesion assays on the plastic flask surface concluded that BA does not affect adherence of either cell line and PBA decreases adherence at 60 minutes in both cell lines but not after that time point (Fig. 19 and 20). Combination experiments also showed that PBA in the presence of $MnCl_2$, which stimulates integrin adhesion, can significantly decrease this adhesion at 60 and 90 minutes in both cell lines (Fig. 21B and 22B) indicating PBA and $MnCl_2$ may be interacting with the same substrate such as integrins. This non-specific binding assay does not provide much information on which integrins lead to the decrease in adhesion and so in order to elucidate that information a specific adhesion assay was carried out on the ECM Fibronectin for both cell lines due to their affinity for this matrix (Fig. 23 and 25).

Timed adhesion assays on Fibronectin show a time-dependent decrease in adhesion for both treatments in ZR-75-1 cells (Fig. 26). On the other hand, MCF-7 cells show a time-dependent increase in adhesion to fibronectin in BA and PBA treated cells (Fig. 27).

Several integrins utilize and bind preferentially to fibronectin but for the purpose of this research integrins $\alpha_v\beta_5$ and β_1 were chosen as they bind to the ECM and are also documented as being expressed in both breast cancer cell lines (25).

In both cell lines, BA or PBA treatments either maintained the level of integrin activity seen in controls or significantly increased the level of activity compared to controls (Fig. 29 and 30). Treatment with $MnCl_2$, which stimulates integrin activity, did not induce this effect in the chosen integrins, conversely adhesion was either the same or decreased compared to controls (Fig. 29 and 30). One explanation for the different trends seen is that the studied integrins, $\alpha_v\beta_5$ and β_1, may not be greatly affected by BA or PBA treatments and so do not display a decrease in integrin activity upon treatment additionally, Mn^{+2} may not be the metal cation which stimulates these integrins as there are other divalent cations which perform the same function.

These results have attempted to answer the questions put forth at the beginning of this research. However, there is still need for further research to be carried out to reveal the mechanism of BA and PBA cytotoxicity in breast cancer cells.

5.2 Conclusions and Recommendations for Further Study

From preceding research on BA in prostate cancer cells, growth inhibition occurred following an 8 day exposure to 0.1 – 1.0 mM BA with the level of inhibition ranging from 32 – 98% in DU-145 cells and 40 – 77% in LNCaP cells (4). Further experiments using only the more sensitive DU-145 cell line display that despite the decrease in cell proliferation, there is no significant effect on cell cycle phases in these cells (4); however,

there is a dose-dependent increase in β–galactosidase, which is a marker for cell senescence at pH 4.0 (5).

To further understand the effect of BA on DU-145 cells, researchers measured cell attachment to a plastic flask surface, cell migration and expression of cyclin proteins. Cell attachment and migration experiments revealed a 34% and 89% decrease respectively after 8 day exposure to 1 mM BA (5). When looking at the expression of cyclin proteins, which are needed for cell proliferation and contribute to prostate cancer aggressiveness, researchers noted a decrease in expression levels of cyclins A, B1, C, D1 and E after 7 day exposure to 0.5 and 1.0 mM BA (5). While these experiments expanded on the epidemiology and animal studies (21 – 23) showing BA has some anti-cancer function in prostate cancer, they still do not identify any possible mechanistic site of BA action in these cells.

The present research served to determine the effect of BA in breast cancer cells and compare this to established findings in prostate cancer and additionally compared BA treatment to PBA, a bulkier analog, which was not carried out in prostate cancer cells. From the reported results, both BA and PBA can effectively inhibit growth in breast cancer cell lines ZR-75-1 and MCF-7; however, both treatments induce this effect after 3 – 5 days of exposure to treatments as opposed to the 8 days of treatment needed in prostate cancer experiments. The lack of growth inhibition observed in MCF-7 cells treated with BA maybe due to the presence of a sodium-boron co-transporter (NaBC1) present on the cell surface which can pump out boron molecules from the cell in exchange for Na^+ ions (13), this co-transporter is not present in ZR-75-1 cells.

Upon further investigation into the method by which BA and PBA induce an anti-proliferative function in breast cancer cells, it was determined this is carried out via apoptosis in both cell lines and S-phase block in ZR-75-1 cells treated with BA and PBA, neither of these findings was observed in prostate cancer cells, which highlights the fact that a similar overall finding of growth inhibition can have several underlying factors. Although there is no reported mechanism of BA action in prostate cancer cells, we hypothesized that BA and PBA may interact with and mediate their function via extracellular proteins known as integrins.

Testing this hypothesis resulted in no significant change in cell attachment to a plastic flask surface using BA or PBA treatments; yet, a combination experiment with $MnCl_2$, which stimulates integrin adhesive function, and PBA showed a decrease in the stimulatory effect of $MnCl_2$, indicating $MnCl_2$ and PBA may interact with the same substrates, integrins. Unfortunately preliminary experiments with a timed adhesion assay of both breast cancer cell lines to a Fibronectin surface or ZR-75-1 cells adhesion to different ECM surfaces treated with BA and PBA did not result in any decrease in adhesion. Also experiments specifically measuring activity of integrins $\alpha_v\beta_5$ and β_1 did not display any significant decrease in integrin activity following exposure to 1 mM BA or PBA. While these experiments did not work as expected, they highlight the possibility of BA and PBA having a mechanistic function outside the cell as opposed to only inside the cell.

From the preceding research we can conclude that BA has an anti-cancer function against ZR-75-1 cells and against both ZR-75-1 and MCF-7 cells at higher concentrations. The effect of BA appears to be growth inhibition by affecting cell cycle

progression and inducing apoptosis, programmed cell death. The idea that integrins are the site of action for BA is still an avenue worth pursuing despite the integrin activity results reported here. The fact that combination treatments of $MnCl_2$ and PBA decreased adhesion in both cell lines indicates these treatments are potentially interacting with and affecting cell adhesion molecules such as integrins. The challenge lies in identifying specifically which integrins are affected.

Further experiments can introduce diphenylboronic acid (DPBA) into experiments as it contains two phenyl groups and only one hydroxyl group. Since BA remains in the trigonal planar conformation at physiological pH (13, 19), DPBA may also retain this conformation and so the absence of a second hydroxyl group may prevent DPBA from retaining the same reactivity seen in BA and PBA. Using DPBA in adhesion assays may show that the presence of two hydroxyl groups in BA and PBA is needed to form complexes with cis-diol or hydroxyl containing compounds on the cell surface such as integrins (Figure 3).

Florescence activated cell sorting (FACS) analysis of α and β integrin expression following BA or PBA treatment, may provide a better way to display the effect on integrin activity compared to controls. This may better highlight which integrins decrease activity upon exposure to BA or PBA treatments. In addition, using increasing doses of BA and PBA may produce a more dramatic shift from active to inactive in affected integrins.

Once affected integrins have been identified, their cellular functions can be compared to the cell effects observed and reported here to determine if they are indeed responsible for the growth inhibition and cell cycle block and increase in apoptosis. Furthermore the

levels of proteins such as focal adhesion kinase (FAK), which is involved in the signaling pathway of several integrins (44) and can be measured via western blots after BA and PBA treatment or measured for down- or up-regulation using microarray analysis.

In addition to integrins, other extracellular proteins expressed in the breast cancer cell lines used can be investigated. The effect of BA and PBA treatments on proteins such as MUC-1, MUC-2, MUC-3 and TG2 (26, 29 – 31) which are responsible for cell adhesion and invasiveness (29, 31) in ZR-75-1 and MCF-7 cells can be measured by observing changes in protein expression levels. Both MUC and TG2 proteins have been shown to interact with and affect specific integrin functions (29, 31), further investigation into whether these extracellular proteins affect integrin response in the presence of BA or PBA treatment may help further elucidate the mechanistic site of BA and PBA treatments.

The discovery of the effects of BA and PBA on breast cancer cell lines ZR-75-1 and MCF-7 contribute to the knowledge of BA in its recent anti-cancer role with prostate cancer. The idea that BA and PBA may be interacting with and affecting these cells via their integrin proteins is a novel route for this treatment and requires further study to support this hypothesis. The possibility for BA to be established as an anti-cancer agent in breast cancer may encourage women with increased risk factors for the disease to increase their intake of boron rich foods so as to reduce their chance of developing this disease.

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ABBREVIATIONS

BA	Boric Acid
BSA	Bovine Serum Antigen
DPBA	Diphenylboronic Acid
ECM	Extracellular Matrix
EDTA	Ethylene Diamine Tetracetic Acid
FACS	Florescence Activated Cell Sorting
FBS	Fetal Bovine Serum
IGFBP	Insulin-like Growth Factor Binding Protein
IGF	Insulin Growth Factor
MIDAS	Metal Ion Dependent Adhesion Sites
MUC	Mucin Protein
PBA	Phenylboronic Acid
PBS	Phosphate Buffered Saline
PSA	Prostate Serum Antigen
SE	Standard Error
TG2	Transglutaminase 2

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