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Characterization of perillyl alcohol as a cell migration-inhibiting compound

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CHARACTERIZATION OF PERILLYL ALCOHOL AS A CELL MIGRATION INHIBITING COMPOUND

by

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A thesis submitted in partial fulfillment
of the requirements for the

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ABSTRACT

Characterization of Perillyl Alcohol as a Cell Migration Inhibiting Compound

by

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Inhibiting cancer cell migration could greatly improve the prognosis of cancer patients. Prior to commercialization of the Plopper laboratory designed a 96-well plate, we optimized our *in vitro* migration and cell viability assays to meet the standards set by the National Cancer Institute for use in a large-scale drug screen for anti-migration compounds. Using these assays, we measured the cytotoxicity and migration-inhibiting effect of dimethyl sulfuoxide, a drug solvent, in three cancer and one non-cancer cell lines. Next, we investigated plant-derived compounds called monoterpenes, known chemopreventive/ chemotherapeutic agents, for non-cytotoxic, migration-inhibiting properties in MCF-10A normal and MDA-MB 435 cancerous breast cells. Perillyl alcohol at 0.5 mM inhibited normal breast cell migration while it failed to affect cancer cell migration. Finally, we examined the loss of prenylation of RhoA, a

small GTPase protein involved in the formation of focal adhesions and stress fibers, and corresponding morphological changes following exposure to perillyl alcohol.

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ACKNOWLEDGMENTS

Completion of this thesis was made possible by the exceptional assistance I received from my lab members: Dr. Janice Huff, Karl Kingsley and Will Rust. An undergraduate, Kilpatrick Carroll, helped me get my bearings in the lab, for which I am grateful. I would also like to thank Dr. George Plopper, my thesis advisor, for his support and guidance with this project. My thesis committee, Drs. J. Steven De Belle, Stephen Carper and J. Abiodun Elegbede offered welcomed critiques of my research that led to a more complete thesis. Finally, I would like to thank my husband, Peter DeKay, for encouraging me to complete this degree and for unselfishly supporting me in my next endeavor: medical school!

CHAPTER 1

GENERAL INTRODUCTION

Cancer

Cancer progresses in two general stages. Initially, cells fail to respond to controls of cell division and multiply excessively. an abnormal mass of proliferating cells results called a neoplasm. Neoplasms are classified as benign or malignant. A benign neoplasm or tumor is often encapsulated, grows relatively slowly and remains confined to the tissue of origin for several years. In contrast, malignant neoplasms are nonencapsulated, grow rapidly and acquire the ability to invade their surrounding tissues. Malignancy, the second stage of cancer, begins with the cells penetrating the surrounding extracellular matrix (ECM).

The extracellular matrix has two main components--basement membranes and interstitial connective tissue. Basement membranes are continuous extracellular structures, which separate organ parenchyma from the underlying interstitial connective tissue. These membranes are the first extracellular barrier to be crossed by invading tumor cells and consist of a number of proteins and glycoproteins which form a highly cross-linked structure (1). The major constituents of the basement membrane are the protein type IV collagen, laminin and proteoglycans. The interstitial connective tissue is composed of cells

distributed in a meshwork of collagen fibers, glycoproteins (e.g. fibronectin). proteoglycans and hyaluronic acid.

Metastasis requires cancer cells to pass through basement membranes and interstitial connective tissue at least three times. They initially invade the basement membranes during escape from their primary site. Later cancer cells cross basement membranes during both entry into and exit from the blood and/or lymphatic vessels, ultimately disseminating and proliferating into secondary tumors called metastases.

The development of secondary tumors is a sequential process. commonly referred to as a "cascade". and failure to complete any one step prevents metastasis (2). For malignant cells to initiate the metastatic cascade they must detach themselves from neighboring cells and adhere to the ECM, degrade the ECM and migrate through the modified ECM (3). Invading cancer cells must therefore be able to bind to the ECM, to release proteolytic enzymes to catalyze degradation of the ECM and to produce factors to stimulate their migration through the modified extracellular matrix. Many of these chemotactic (motility toward a chemical gradient) factors are degradation products of the ECM components such as fragments derived from laminin, fibronectin and collagen (4). Inhibition of any of these steps could prevent, or at least minimize, the spread of cancer.

Cancer Treatments

Tumor metastasis is the most deadly aspect of the disease. Unlike primary tumors that can be surgically removed and treated with adjuvant chemotherapy and/or radiotherapy, metastases, on the other hand, are difficult to treat and usually fatal (2). The majority of anti-cancer drugs developed target the hyperproliferation of metastatic cells. While many of these drugs are efficacious in treating the beginning stages of cancer, none are curative treatments for metastatic disease. A delay in diagnosis is another contributing factor that renders many drugs ineffective (5). In addition, antiproliferation compounds cause many adverse side effects: nausea, vomiting, suppressed immune system and hair loss. Cancer remains the second leading cause of death in the US (6). It is, therefore, paramount that alternative therapies be developed that treat a greater scope of the disease and with less toxicity to the patient.

Inhibiting invasion and metastasis of cancer cells involves many potential targets for intervention. Tumor cell motility is a salient characteristic contributing to disease progression, but it remains poorly understood. Alterations in the expression of growth factor receptors (7-8), changes in the extracellular matrix and cell interactions (9) and signal transduction pathways (10) are likely to be important. By piecing these factors together in cancer cell migration opportunities for new therapeutic treatments should flourish.

Even though tumor cell migration is a hallmark of metastasis, research since 1978 has yielded fewer than 100 compounds that have some capacity to inhibit tumor cell migration; however, thousands of compounds have yet to be

tested. The reason for the limited number of identified inhibitors of tumor cell migration may be due to the technology used to detect them.

Accurately and rapidly measuring cell migration has been a problem. The majority of *in vitro* cell motility studies are conducted by using modified Boyden chambers in which cells are placed in the upper chamber and stimulated to move across the extracellular-matrix-protein coated filter by haptotaxis (motility toward a bound substrate) (Fig. 1.1); however, the methods by which migration is quantified is more diverse. Fixing, staining and manually counting the migrated cells under a light microscope is a common, time-consuming method (11-14). Binding migrated cells with a fluorescent tag, such as calcein-AM, and then quantifying fluorescence with an inverted microscope and computer program is another method (15-17). The migrated cells can also be removed with trypsin and counted in a Neubauer chamber (18-19). Time-lapsed videomicroscopy is an alternative technique for conducting cell motility assays. Cells are viewed with an inverted microscope, and a high-resolution black and white video camera and recorder record movement (20-21). These existing technologies for measuring cell migration are flawed because they require too much time and money to efficiently screen compounds. One transwell plate, a modified Boyden chamber, can hold 12 samples and costs $25. For example, screening 100 compounds using transwell plates would require several plates per compound with each insert handled separately. Time-lapsed videomicroscopy is also inadequate as it provides a detailed examination of individual cells, but is not an appropriate technology for screening large numbers of compounds.

Our lab has developed an economical, accurate, high-throughput method for measuring *in vitro* cell migration. Our newly designed 96-well migration plate eliminates the handling of individual filters, reduces the number of cells and volume of drug sample used, and allows for many more replicates per treatment condition (Fig. 1.2). The addition of an opaque filter on our plate, in conjunction with innocuous fluorescent cell labeling, provides the ability to quantify the number of migrated at any time point over the 18-hour migration assay (Fig. 1.3). Measuring cell viability with assays such as the MTT test is also possible with our migration plate. Overall, through the application of new technology we should be able to expeditiously identify non-cytotoxic, migration-inhibiting compounds (22).

General Mechanisms of Cell Migration

Cell locomotion is only partly understood. Gaining insight into how cell migration occurs is important for development of anti-migration drugs. Cell migration is regulated by a combination of different processes: forces generated by contraction of actin and myosin, G protein signaling, microtubule dynamics and the turnover of focal adhesions (23). It begins by extending the plasma membrane at the front of the cell into what is called lamellipodia. Polymerization of the actin cytoskeleton drives the extension of the lamellipodia, and it is stabilized by the formation of focal complexes (24). Integrin receptors, actin filaments and associated proteins are found grouped together in these focal complexes. As the cell advances forward, focal complex binding strength and organization increase (24), giving rise to focal adhesions (Fig. 1.4). Focal

adhesions link the cytoskeleton with the extracellular matrix through transmembrane integrin receptors. Through increased strength and organization, focal adhesions provide the cell with points of traction over which it can move. Release of the rear adhesions permits the entire cell to advance forward. Therefore, interfering with the turnover of focal adhesion and focal complexes could impede cell migration. However, the mechanisms that regulate the formation of focal complexes at the cell's leading edge and the release of focal adhesions at the cell's rear remain unclear (23).

Integrin proteins play a key role in the turnover of focal adhesion complexes (24). Integrins are heterodimeric proteins consisting of two transmembrane glycoprotein subunits (α and β) that are noncovalently bound to each other. For adhesion to ligands, the extracellular domain of both integrin subunits is needed, as is the presence of cations. The cytoplasmic domain of integrin α and β subunits anchors the cytoskeleton to the plasma membrane and is required to mediate signaling events. Thus far, the integrin family is composed of 16 α and 8 β subunits that form a combination of α β cell surface receptors with the ability to mediate bidirectional transmembrane signaling from inside to outside and from outside to inside the cell (24).

Cell migration is just one type of cell behavior affected by the activation of these cascades. It is inside-out signaling that regulates integrin affinity for a ligand through the propagation of conformational changes from the cytoplasmic domain to the extracellular binding site (24). This interaction of increased integrin affinity for extracellular matrix proteins that induces cell surface clustering

at focal adhesion sites of the plasma membrane is a process driven from within the cell (25). These focal adhesion complexes are composed of cytoskeletal proteins (actin, talin, vinculin, paxillin and tensin) and signaling molecules [focal adhesion kinase (FAK) cadherin-associated kinase (CAS) and Src tyrosine kinase] (25) (Fig. 1.5). Following inside-out activation, integrin occupation and clustering at focal adhesion complexes elicit outside-in signaling from the extracellular matrix to the cell cytoplasm that leads to cell migration, proliferation, differentiation and survival (25) (Fig. 1.6).

Rho GTPases

To understand how ECM and integrin associations work to control cell behavior, it is necessary to identify proteins that are able to coordinate both cell shape and integrin signaling pathways. A good candidate is the Rho family of proteins. These small GTPases are able to relay integrin-derived signals, as well as organize the actin cytoskeleton (26). Rho GTPases act as molecular switches that cycle between the active GTP- bound and inactive GDP-bound states. This cycling is controlled by GDP/GTP exchange factors, GTPase-activating proteins and guanine nucleotide dissociation inhibitors (26). It is presumed that interactions with these molecules take place at the plasma membrane through geranylgeranylation of the C-terminus on Rho GTPases (27). Protein prenylation modifies certain cellular proteins with either a 15-carbon farnesyl or a 20-carbon geranylgeranyl group generated from the mevalonate pathway.

Microinjection with activated and dominant negative Rho family members leads to a defined hierarchy of GTPase activation; activated Cdc42 activates Rac1, which then activates RhoA (23, 27). Rac appears to drive the development of lamellipodia, whereas Cdc42 stimulates the polymerization of actin at the front to the cell to form long, thin extensions called filopodia (23, 27). RhoA activation leads to the production of large, highly organized focal adhesions and tension (23, 27).

The role of RhoA in stimulating the assembly of stress fibers and focal adhesions is better understood than how Rac1 or Cdc42 induces focal complex assembly (28). Activated RhoA binds to and activates the serine/threonine kinase Rho-kinase (ROKα), which in turn phosphorylates and inhibits myosin light chain phosphatase (23, 28-30). This inhibition results in increased phosphorylation of myosin light chain followed by enhanced myosin filament assembly and actin-activated myosin ATPase activity (28). Overall, through this pathway, RhoA activation results in bundling of actin filament into stress fibers and clustering of integrins into focal adhesions. This membrane-cytoskeleton interaction has been reported to increase invasiveness in tumor cells from liver and breast tissues (29,31). Additionally, the level of RhoA expression has been reported to be several times higher in tumors than in surrounding normal tissue (31). RhoA is also involved in a variety of other biological processes such as cytokinesis, smooth muscle contraction, transformation and apoptosis (31).

GTPases have also been reported to play an important role in the metastatic behavior of tumor cells (32). Therefore, blocking the activation of these proteins by inhibiting their isoprenylation could reduce metastasis (19).

Monoterpenes: Perillyl Alcohol

The monoterpenes limonene and perillyl alcohol (POH) and their metabolites have been shown to inhibit the isoprenylation of protein in cultured 3T3 cells as well as other types of cells (33) (Fig. 1.7). These monoterpenes are plant-derived, inexpensive, low in toxicity and currently in Phase II clinical trials for use as a chemopreventive/chemotherapeutic agent in liver, colon and pancreatic cancer. The cancer suppressing chemopreventive activity of monoterpenes during the promotion phase of carcinogenesis act to prevent the growth of initiated cells. In chemoprevention of mammary and liver carcinogenesis monoterpenes may act by inhibiting tumor cell proliferation or accelerating the rate of tumor cell death (34).

Though its exact mechanism of action is not known, perillyl alcohol is known to inhibit prenyl transferases including geranylgeranyl protein transferase I (GGPTase 1) (35-36). One target of the enzyme GGPTase 1 is the small G protein RhoA: Addition of a geranylgeranyl group to RhoA allows it to become membrane associated upon activation. Activated RhoA in turn stimulates chemical signaling pathways that lead to cell migration and suppression of apoptosis. However, differences have been observed in the inhibition of perillyl alcohol on the activity of type I and type II geranylgeranyl-protein transferases

and fernesyl-protein transferases in different cell lines (37). Thus, in determining the anticancer mechanism of perillyl alcohol, it is important to evaluate this biochemical effect in the particular cell type.

To date there are no published studies examining the effects of perillyl alcohol on cell migration, though I have accumulated data demonstrating that perillyl alcohol inhibits migration of the human breast cell line MCF-10A at sub-lethal doses. Therefore, the central hypothesis of my thesis is that perillyl alcohol inhibits migration of these cells by blocking a RhoA-associated signaling pathway that includes Rho-kinase (ROKα) and myosin light chain.

In chapter 2, I establish the parameters for implementing a large-scale drug screen of anti-cancer agents to identify anti-migration compounds using three human cancer cell lines and one human nontumorigenic breast cell line. Next, in chapter 3, I apply the protocol from chapter 2 to examine what effect perillyl alcohol and related compounds have on cell proliferation, migration and adhesion in human tumor and non-tumorigenic breast cells. Finally, in chapter 4, I investigate the mechanism by which perillyl alcohol inhibits cell migration in human tumor and non-tumorigenic breast cells, specifically, alterations in RhoA protein isoprenylation. Additionally, morphological changes in actin stress fiber and focal adhesion complex formation following treatment with perillyl alcohol are shown. These queries into how signal transduction pathways and cell morphology are affected by perillyl alcohol will aid in refining the search for potential anti-migratory compounds.

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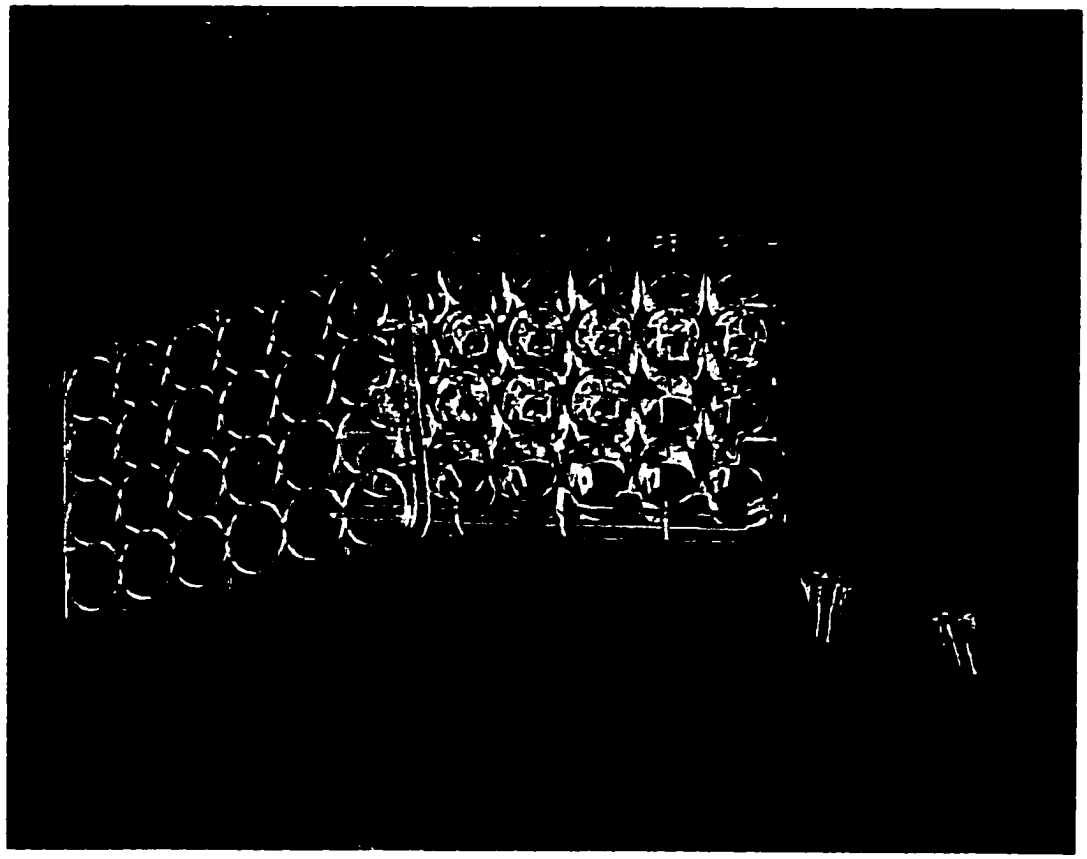


Figure 1.1. A standard Costar transwell 12-filter migration plate showing individual filter inserts.

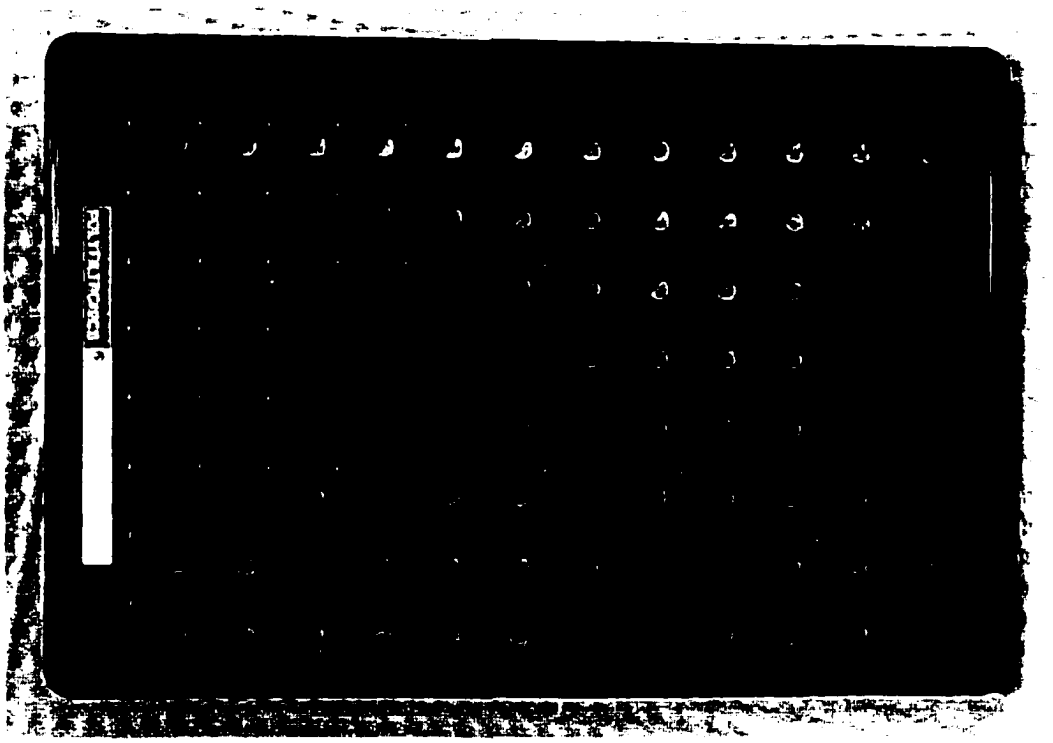


Figure 1.2. 96-well migration plates are designed as a single insert containing all filter wells in one piece.

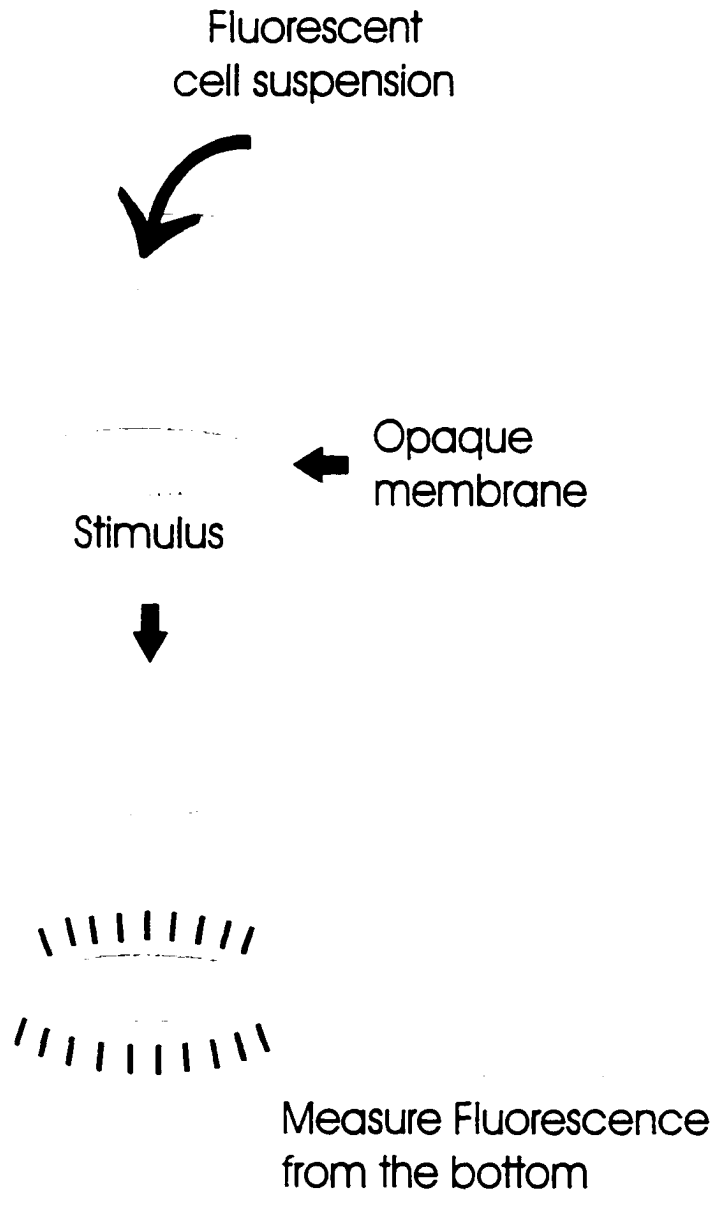


Figure 1.3. Individual filter insert with fluorescence-opaque membrane. Fluorescent-labeled cells migrate across an opaque membrane toward stimulus present in media in feeder well. Emitted fluorescence of migrated cells is measured from underneath the filter.

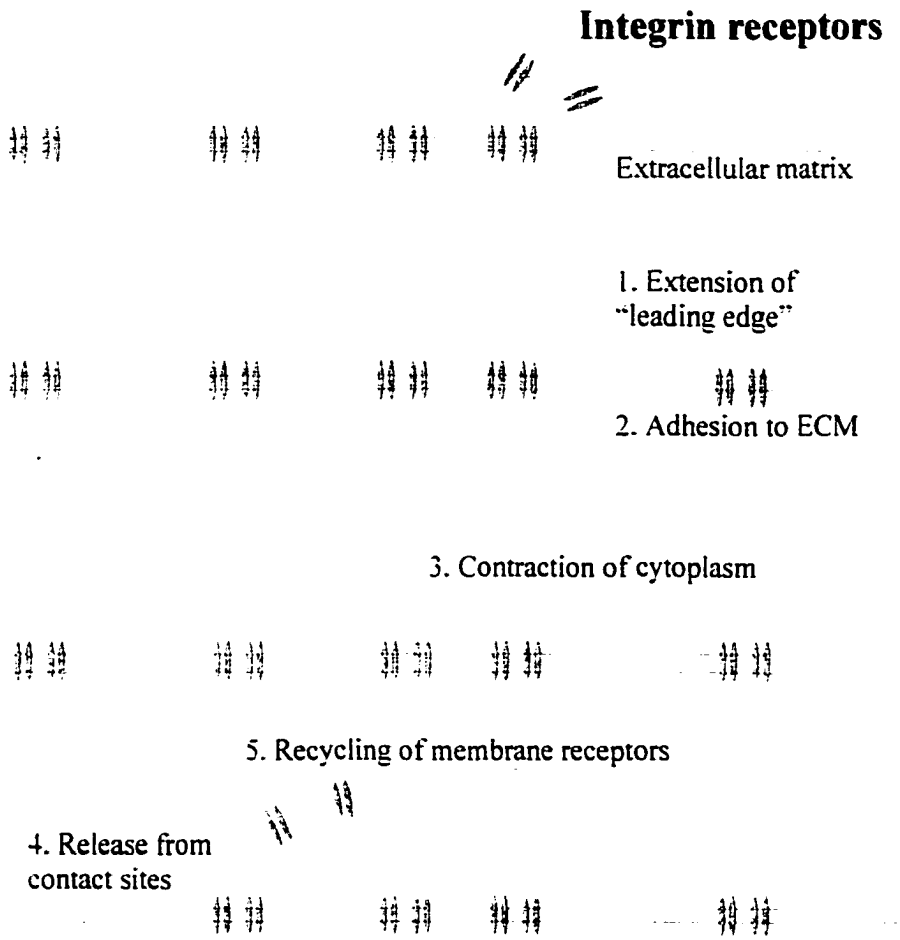


Figure 1.4. Diagram of integrin receptor-ECM interactions in migrating cell. 1. Cell is attached via integrin receptor ligation to ECM proteins on substratum which develops into focal adhesion complexes (blue). 2. Leading edge of cell moves forward forming new integrin-ECM contacts. 3. Contraction of cytoskeletal elements moves cytoplasm forward. 4. Focal adhesion complexes are released at the retracting edge of the cell. 5. Integrin receptors and associated proteins are recycled.

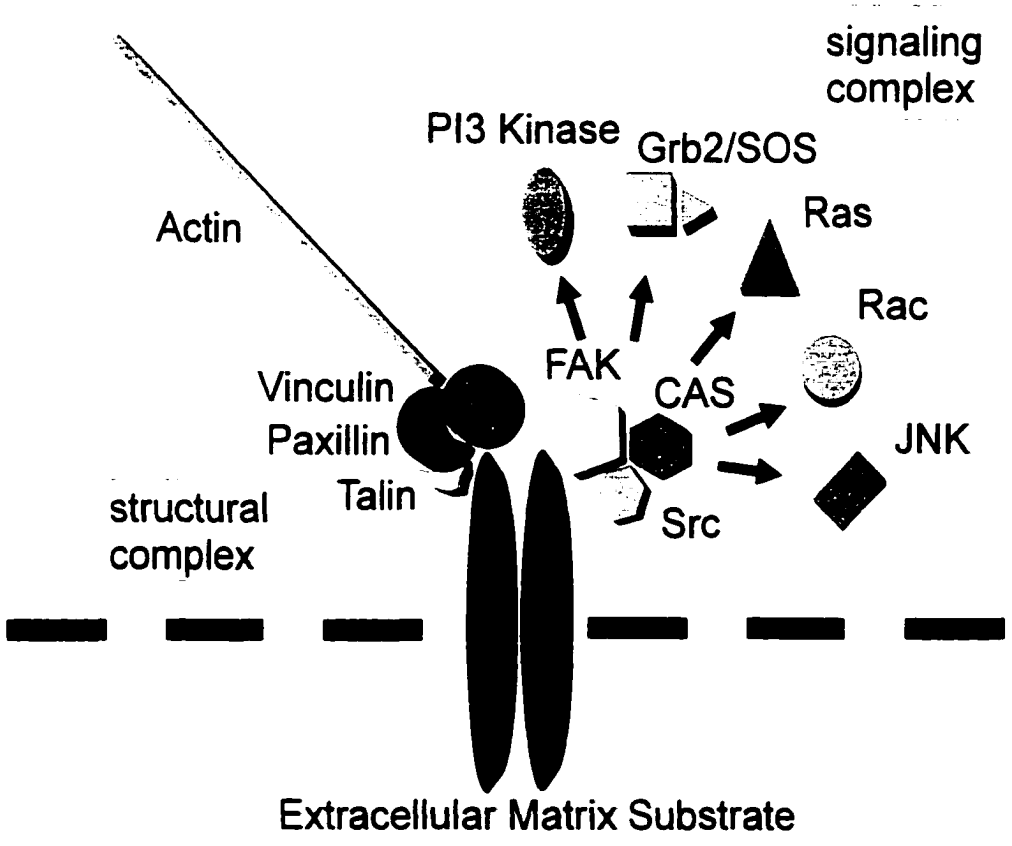


Figure 1.5. Components of a focal adhesion complex assembled at the site of integrin-ECM contact.

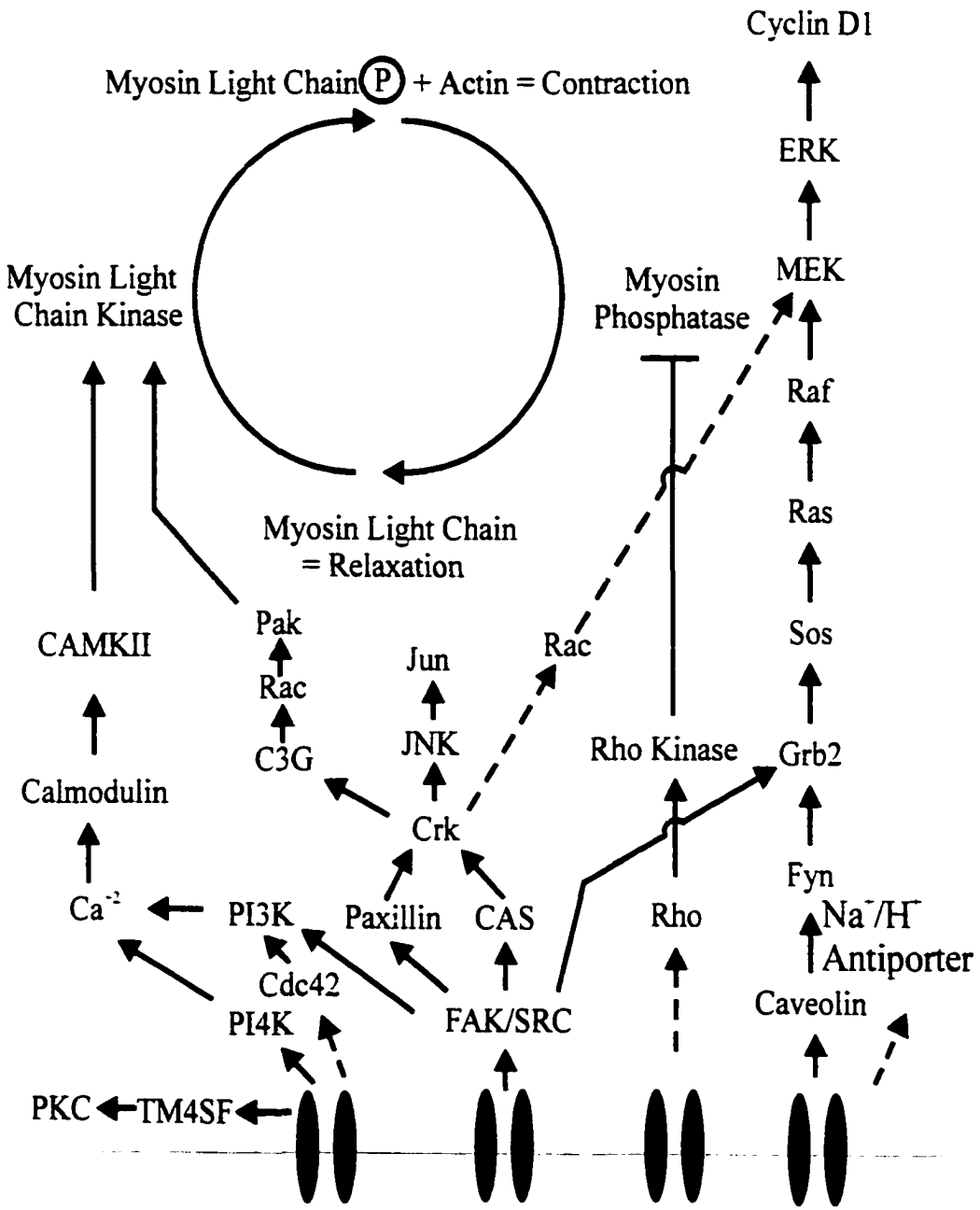
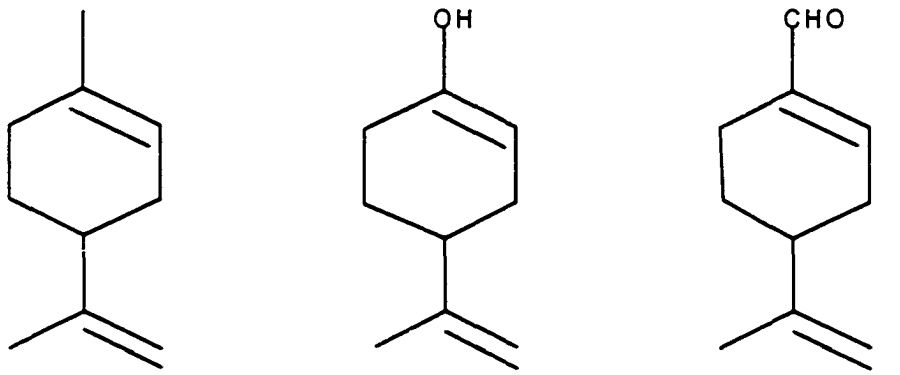


Figure 1.6. Intracellular signaling pathways stimulated by integrin-ECM interactions.



Limonene Perillyl Alcohol Perillaldehyde

Figure 1.7. Oxygenated monocyclic monoterpenes for which limonene serves as a precursor.

CHAPTER 2

CONSTRUCTION OF A DRUG SCREEN FOR ANTI-MIGRATION DRUGS

Abstract

Metastasis of cancer cells greatly increases mortality. Many of the steps involved in cancer spread are potential targets for anti-metastatic treatment. We designed a protocol to screen for drugs with anti-migration effects. This assay required maximizing the migration of four representative cancer and non-cancer cell lines, and eliminating the confounding effects of dimethyl sulfoxide, a solvent used to solubilize the drugs for testing. We determined that all cell lines survived in at least 0.8% dimethyl sulfoxide by using the MTT cell viability assay Cell size was accounted for in making a monolayer of cells on the filter through which they migrate. Selection for extracellular matrix proteins for *in vitro* migration resulted in all four cell lines preferring fibronectin. Finally, migration on fibronectin in the presence of dimethyl sulfoxide was performed to establish the maximal percentage sustained without compromising cell migration. A percentage of 0.2% dimethyl sulfuoxide was innocuous to migration in all four cell lines. These findings support the application of this protocol in a large-scale drug screen for anti-migration compounds.

Introduction

Metastasis is the most deadly aspect of cancer. Localized primary tumors in non-vital organs, such as the skin, are not life threatening (1). Primary tumors can be surgically removed and the remaining local involvement can be treated with adjuvant chemotherapy and/or radiotherapy. However, if the primary tumor acquires the ability to invade its surrounding basement membrane layer of extracellular matrix (ECM) proteins and travels through blood and lymphatic vessels to new areas for colonization, it can then metastasize to form a secondary tumor with a much less certain prognosis for the patient. The progression from a primary to secondary tumor requires the completion of a sequential pathway, commonly referred to as a "cascade", and failure to complete any one step prevents metastasis (1). Metastatic cancer is difficult to treat and is usually fatal (1). Therefore, preventing the development of malignant tumors by inhibiting tumor cell migration is an important area of cancer research.

Understanding how cells migrate is also important for development of anti-migration drugs. Cells move by extending the plasma membrane at the front of the cell into what is called lamellipodia. Polymerization of the actin cytoskeleton drives the extension of the lamellipodia, and it is stabilized by the formation of focal complexes (2). Integrin receptors, actin filaments and associated proteins are found grouped together in these focal complexes. As the cell advances forward, focal complex binding strength and organization increase (2), giving rise to focal adhesion complexes that provide points of traction over which the cell can move. Focal adhesion complexes link the cytoskeleton with the extracellular

matrix through transmembrane integrin receptors to adapter proteins and various signaling molecules. Therefore, interfering with the turnover of focal adhesion and focal complexes could impede cell migration.

Research since 1978 has yielded fewer than 100 compounds that have some capacity to block cancer cell migration; however, thousands of compounds have yet to be tested. The reason for the limited number of identified inhibitors of tumor cell migration may be due to the technology used to detect them.

The majority of *in vitro* cell motility studies are conducted by using modified Boyden chambers in which cells are placed in the upper chamber and stimulated to move across the ECM - protein coated filter by haptotaxis; however, the methods by which migration is quantified is more diverse. Fixing, staining and manually counting the migrated cells under a light microscope is a common method (3-6). Binding migrated cells with a fluorescent tag, such as calcein-AM, and then quantifying fluorescence with an inverted microscope and computer program is another method (7-8). The migrated cells can also be removed with trypsin and counted in a Neubauer chamber (9-10). Time-lapsed videomicroscopy is an alternative technique for conducting cell motility assays. Cells are viewed with an inverted microscope, while a high-resolution black and white video camera and recorder record cell movement (11-12). These existing technologies for measuring cell migration are flawed because they require too much time and money to efficiently screen large numbers of compounds. One transwell plate, a modified-Boyden chamber, can hold 12 samples and costs $25. For example, screening 100 compounds using transwell plates would require

several plates per compound with each insert handled separately. Time-lapsed videomicroscopy is also inadequate; it provides a detailed examination of individual cells, but is not an appropriate technology for screening large numbers of compounds.

We have developed an economical, accurate, high-throughput method for measuring *in vitro* cell migration. Our newly designed 96-well migration plate eliminates the handling of individual filters, reduces the number of cells and volume of drug sample used, and allows for many more replicates per treatment condition. The addition of an opaque filter on our plate, in conjunction with innocuous fluorescent cell labeling, provides the ability to perform quantify the number of migrated at any time point over the 18-hour migration assay. Measuring cell viability with assays such as the MTT test is also possible with our migration plate. Overall, through the application of new technology we should be able to expeditiously identify non-cytotoxic, migration-inhibiting compounds (15).

Prior to the commercialization of our new migration plate, we proceeded to develop a protocol for optimizing the conditions under which to screen for anti-migration drugs. In this study, we established the preferred extacellular matrix protein for maximal cell migration, determined the cytotoxicity of dimethyl sulfoxide (DMSO), delineated the effect of DMSO on migration and measured the number of cells needed to form a monolayer of cells on the migration filter for one non-cancer and three cancer cell lines. DMSO is a commonly used as a drug solvent; therefore, we needed to account for its effect on cytotoxicity and cell migration. The three cell lines representing different types of cancers and the

one non-cancer all preferred to migrate on fibronectin. DMSO cytotoxicity ranged from 0.8% to 2.0%, and migration was unaffected by DMSO in the range of 0.2% to 1.3% for all cell lines, respectively. With these defined parameters, we can apply them to our new migration plate and commence with a large-scale anti-migration drug screen.

Materials and Methods

Materials

SF-268, NCI-H460, MCF-7 and MCF-10A cells were purchased from American Type Culture Collection. Epidermal growth factor, cholera toxin, insulin were obtained from Calbiochem (La Jolla, CA). Horse serum was purchased from Irvine Scientific (Santa Ana, CA). Hydrocortisone, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue) and DMSO (dimethyl sulfoxide) were purchased from Sigma-Aldrich Chemicals (St. Louis, MO). Calcein-AM was purchased from Molecular Probes (Eugene, OR). Transwell migration plates were purchased from Costar (Cambridge, MA). Cell culture flasks and 96-well plates were purchased from VWR (Plainfield, NJ).

Cell Culture

Cells were grown in 75 cm^2 Falcon tissue-culture flasks and maintained at 37° C and 5% CO_2 in humidified chambers. NCI-H460 and SF-268 cells were maintained in Dulbecco's Modified Eagle's Medium High Glucose supplemented with 10% fetal bovine serum purchased from Gemini Bio-Products, Inc. (Calabasas, CA) and 1% L-glutamine (29.2 mg/ml), penicillin G (10,000 units/ml)

and streptomycin sulfate (10,000 mcg/ml) from Irvine Scientific (Santa Ana, CA). MCF-7 cells were maintained in RPMI Medium 1640 supplemented with 10% fetal bovine serum and 1% L-glutamine (29.2 mg/ml), penicillin G (10,000 units/ml) and streptomycin sulfate (10,000 μg/ml) from Irvine Scientific (Santa Ana, CA). MCF-10A cells were maintained in AM media—1:1 mixture of Ham's F-12 medium and Dulbecco's Modified Eagle's Medium High Glucose with 2 mM L-glutamine from Irvine Scientific (Santa Ana, CA) supplemented with the following: epidermal growth factor (20 ng/ml), cholera toxin (100 ng/ml), insulin (0.01 mg/ml), hydrocortisone (500 ng/ml) and 5% horse serum. MDA-MB 435 cells were maintained in RPMI Medium 1640 supplemented with 10% fetal bovine serum and 1% L-glutamine (29.2 mg/ml), penicillin G (10,000 units/ml) and streptomycin sulfate (10,000 μg/ml) from Irvine Scientific (Santa Ana, CA). Cells were routinely passaged using trypsin/EDTA from Irvine Scientific (Santa Ana, CA).

Cell Monolayer

Four replicates of 90-140 x 10^3 cells in a 200 μl volume of media were seeded in a 96-well tissue culture plate. After a four-hour incubation at 37° C, cells were examined under an inverted microscope for evenly covering of the well's 6 mm diameter surface. We extrapolated this number of cells to that needed to cover a 7 mm diameter of the filter on the transwell plate used in the migration assay.

Cell Viability Assay

The MTT assay is based on the cleavage of the yellow dye, 3-(4,5-dimethyl-2-thiazol)-2,5-diphenyl2H-tetrazolium bromide (MTT) to purple formazan crystals by the activity of the enzyme mitochondrial dehydrogenase. In replicates of 12 in a 96-well plate, 6×10^4 cells were plated in 150 µl migration media (Dulbecco's Modified Eagle's Medium High Glucose supplemented with 1% L-glutamine (29.2 mg/ml), penicillin G (10,000 units/ml) and streptomycin sulfate (10,000 mcg/ml) from Irvine Scientific (Santa Ana, CA) and 0.1% bovine serum albumin from Boehringer Mannheim (Indianapolis, IN)) with varying concentrations of dimethyl sulfoxide (DMSO) and incubated for 18 hours at 37° C in a humidified 5% CO_2 atmosphere. Three hours before the termination of the experiment, 50 µl of MTT dye (5 mg/ml) was added to each well and the plate was incubated for three hours. The reaction was stopped by removing 150 µl of the media and replacing it with 150 µl of acidic isopropanol (0.1 N hydrochloric acid in isopropanol) to each well. The plate was protected from light and incubated over night at room temperature. Absorbance was read at 570 nm in plate reader.

Migration Assay

Cell migration assays were performed as previously described in Costar transwell filter plates (15). Filters were either coated with purified ECM (collagen IV, fibronectin, laminin-1 or vitronectin) at a concentration of 20 µg/ml in carbonate buffer (pH 9.3) for 1 hour at room temperature. Filters were then aspirated and blocked in blotto (phosphate-buffered saline with 0.2% Tween-20

(PBST) and 5% skim milk) for 1 hour at room temperature. Next filters were washed in PBST prior to the assay. Thirty minutes prior to starting the assay cells were pre-incubated at 37° C in migration media and varying concentration of DMSO. Cells were seeded at a concentration of either 1.2 or 1.8 x 10^6. depending on cell line, on the transwell filters with and without ECM in the presence or absence of soluble growth factors (serum-enriched media) and varying concentrations of DMSO and allowed to migrate for 18 hours at 37° C in 5% CO_2. Thirty minutes before measuring migration, 5 μM of calcein-AM, a fluorescent dye that chelates calcium, (Molecular Probes; Eugene,OR) was added to the migration wells. To quantify migration, cells were removed from the top of the filter with cotton swabs, filters were washed in phosphate-buffered saline and migrated cells were measured from the bottom of the filter with a plate reader for the incorporation of calcein-AM (14). Relative fluorescence values for each experimental condition were expressed relative to control, untreated samples.

Results

We tested the toxicity of DMSO, due to the use of DMSO in drug packaging for screening, in four representative cell lines. Cell viability was assessed by the ability to metabolize MTT, which was measured by increased absorbance by a plate reader. The NCI-H460 cell line, small cell lung carcinoma tolerated less than 1% DMSO (Fig. 2.1), and the SF-268 cell line, neuronal sarcoma. showed a tolerance for DMSO of 2% or less in concentration (Fig. 2.2).

The third cancer cell line, MCF-7, breast carcinoma, was less tolerant of DMSO with only surviving a concentration of up to 1% or less (Fig. 2.3). The final cell line tested was MCF-10A, a representative of normal non-cancerous, immortalized breast cells, which was the least able to tolerate DMSO at 0.8% or less (Fig. 2.4).

To maximize the migration of these four cell lines we performed *in vitro* migration assays to identify their preference for extracellular matrix protein out of collagen IV, fibronectin, laminin-1 and vitronectin. Blotto was used as a negative control for non-specific binding and media containing serum was used as a positive control. All of the four cell lines migrated most toward fibronectin (Fig. 2.5-2.8).

In order to account for any effect that DMSO may have on migration, cells were migrated on fibronectin with up to the previously determined amount of DMSO that did not affect cell viability. For MCF-10A cells migration was normal in media with DMSO equal to or less than 0.2% (Fig. 2.9). The amount of DMSO that did not interfere with migration was equal to or less than 0.5% for both NCI-H460 (Fig. 2.10) and SF-268 (Fig. 2.11) cell lines, while less than 0.5% had no effect on the MCF-7 cell line's migration (Fig. 2.12).

Additionally, it was necessary to determine the number of cells needed to form a monolayer on the filters used in the migration assay. Three of the cell lines required 120,000 cells to evenly cover the filter. However, the NCI-H460 cells were smaller and took 180,000 cells (Table 2.1).

Discussion

Two important properties of malignant cancer cells are their ability to invade surrounding tissues through a basement membrane (16) and their ability to form metastatic foci at distant sites (17): a process requiring tumor cells to detach from the ECM of the primary tumor and to migrate to other sites. It is supposed that increased cell motility facilitates invasion and metastasis of malignant tumors (16). Inhibiting cancer cell migration and metastasis could effectively eliminate the most deadly aspect of the disease (18).

We previously designed a new 96-well plate for use as a tool for measuring both cell migration inhibition and cell death in cancer cell lines when treated with anti-cancer compounds (14). In conjunction with our new plate, we also developed an economical, accurate method for measuring cell migration (14). In this study, we defined the conditions necessary for optimizing our high-throughput assay to screen for anti-cancer compounds for their ability to stop cell migration of established cell lines *in vitro*.

We determined that the concentration of DMSO that induces cell death in our representative cell lines is greater than the concentration measured for any detectable impairment in cell migration. Although our use of DMSO is only as a probable solvent used for drug packaging and not as an agent for inhibiting cell migration, our results confirm that this assay system is capable of identifying non-cytotoxic, anti-migration compounds (14). We also established that it is possible to use DMSO as a solvent and delineate its effect on migration from those of tested anti-cancer compounds.

Another parameter in question for conducting an anti-migration drug screen is selection of preferred extracellular matrix protein. It is well known that the motility of several normal cells and tumor cells is regulated by ECM proteins such as fibronectin, laminin, vitronectin and type-IV collagen (19). These proteins stimulate chemotaxis (motility toward a chemical gradient) and haptotaxis (motility stimulation toward a bound substrate) (20). Our cell lines represent two carcinomas and one sarcoma, as well as a non-tumorigenic breast cell line. All four cell lines showed a preference for migrating on fibronectin, which aids in simplifying the migration assay for a drug screen.

Determining cell size is also of importance for performing the migration assay. Plating the cells to form a monolayer on the top of the filter for the migration assay is necessary to mimic *in vivo* conditions. Among our cell lines the small cell carcinoma (NCI-H460) cells are approximately two thirds the size of the other cell lines.

In summary, we demonstrated that it is possible to optimize our high-throughput assay for screening potential antimigratory compounds. Using four cell lines designated by the National Cancer Institute, we were able to discern between the cytotoxicity of DSMO and its effect on migration in our assay, which is necessary due to the use of DMSO as a drug solvent. According to our results from this study we would be able to proceed with screening anticancer compounds held by such institutions as the National Cancer Institute when our 96-well migration plate becomes commercially available.

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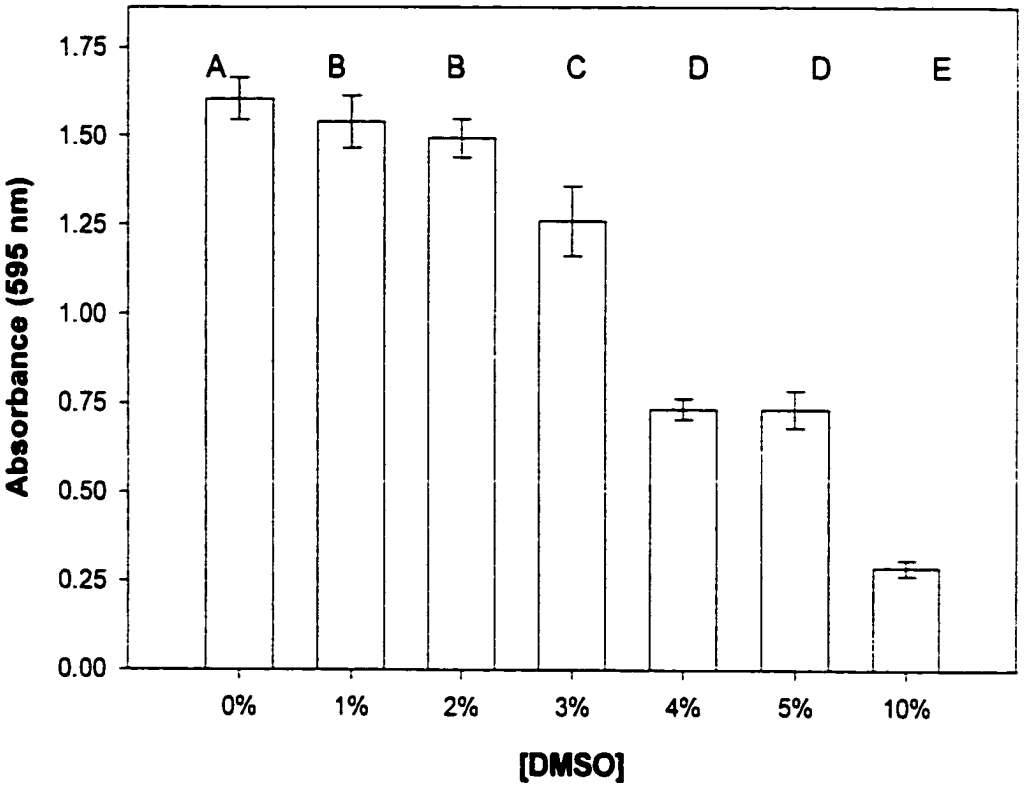

Figure 2.1. NCI-H460 cells survived 18 hours of exposure to < 1% DMSO. Bars are mean ± SD absorbance. Bars with the same letter were not significantly different from each other. In DMSO, differences were significant [analysis of variance (ANOVA) $F[6,41] = 556.37$, $P < 0.0001$, $n = 7$/bar]. A student-Numan-Keuls test (SNK) identified four groups ($P \leq 0.05$).

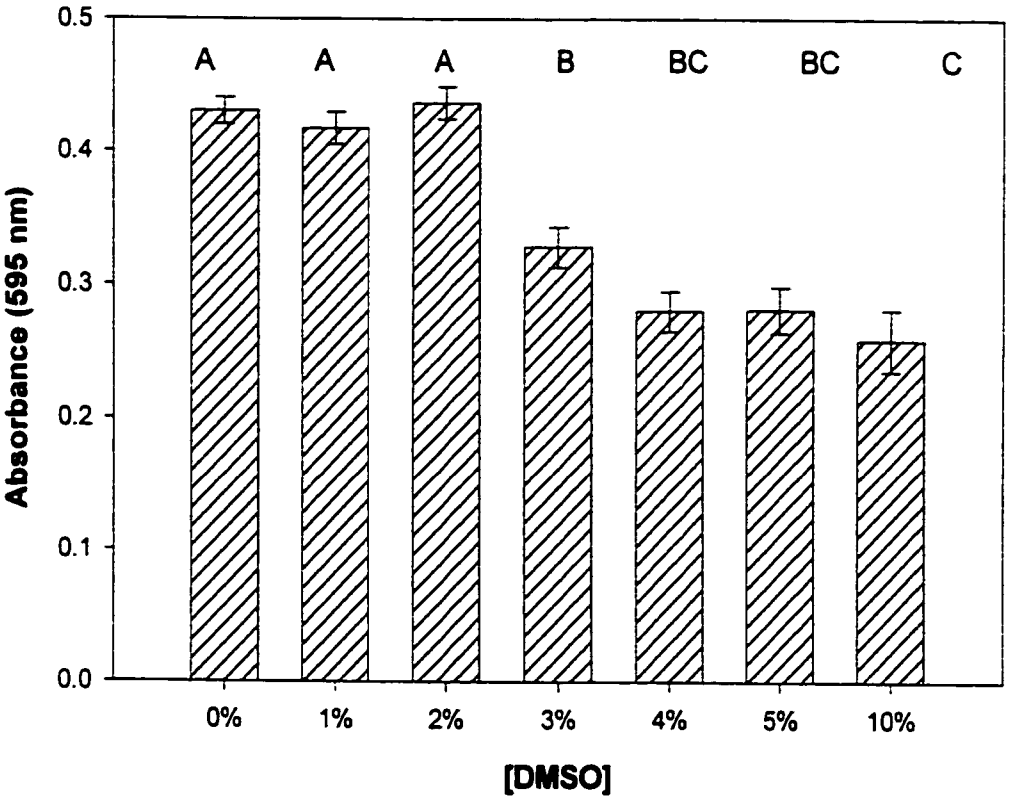

Figure 2.2. SF-268 cells survived 18 hours of exposure to ≤ 2% DMSO. Bars are mean ± SD absorbance. Bars with the same letter or letters were not significantly different from each other. In DMSO, differences were significant [analysis of variance (ANOVA) $F[6,27] = 19.18$, $P < 0.0001$, $n = 4$/bar]. A student-Numan-Keuls test (SNK) identified two partly overlapping groups and one other group ($P \leq 0.05$).

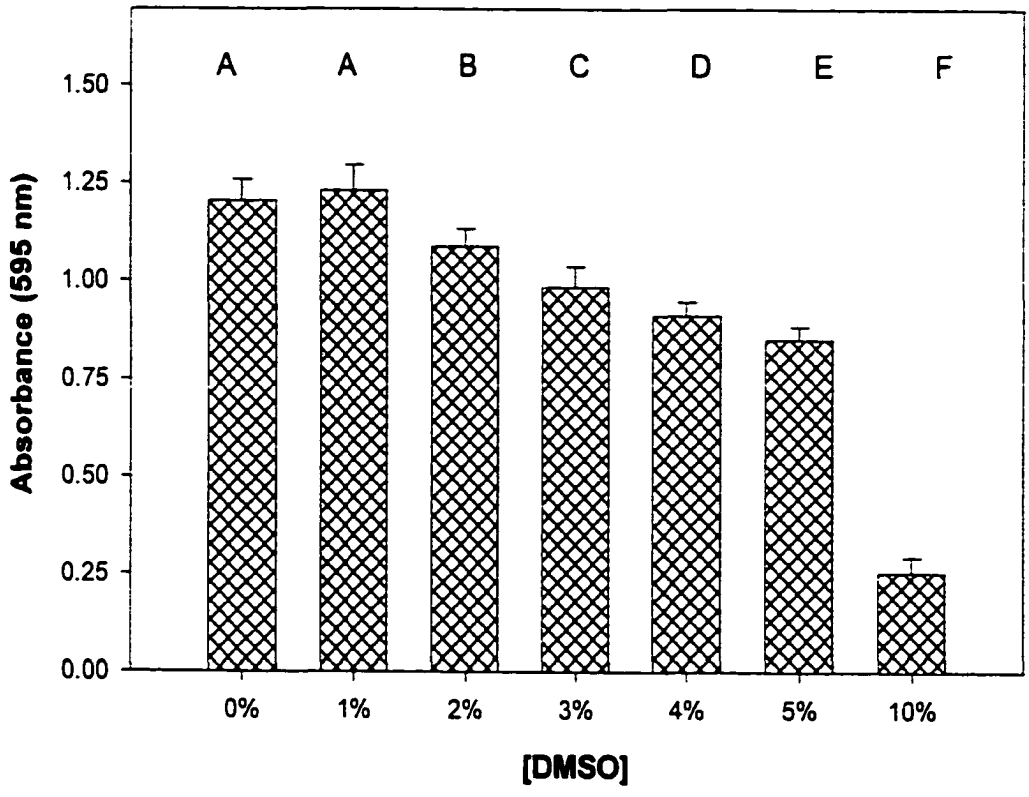

Figure 2.3. MCF-7 cells survived 18 hours of exposure to ≤ 1% DMSO. Bars are mean ± SD absorbance. Bars with the same letter were not significantly different from each other. In DMSO, differences were significant [analysis of variance (ANOVA) $F[6,54] = 381.60$, $P < 0.0001$, $n = 8$/bar]. A student-Numan-Keuls test (SNK) identified six groups ($P \leq 0.05$).

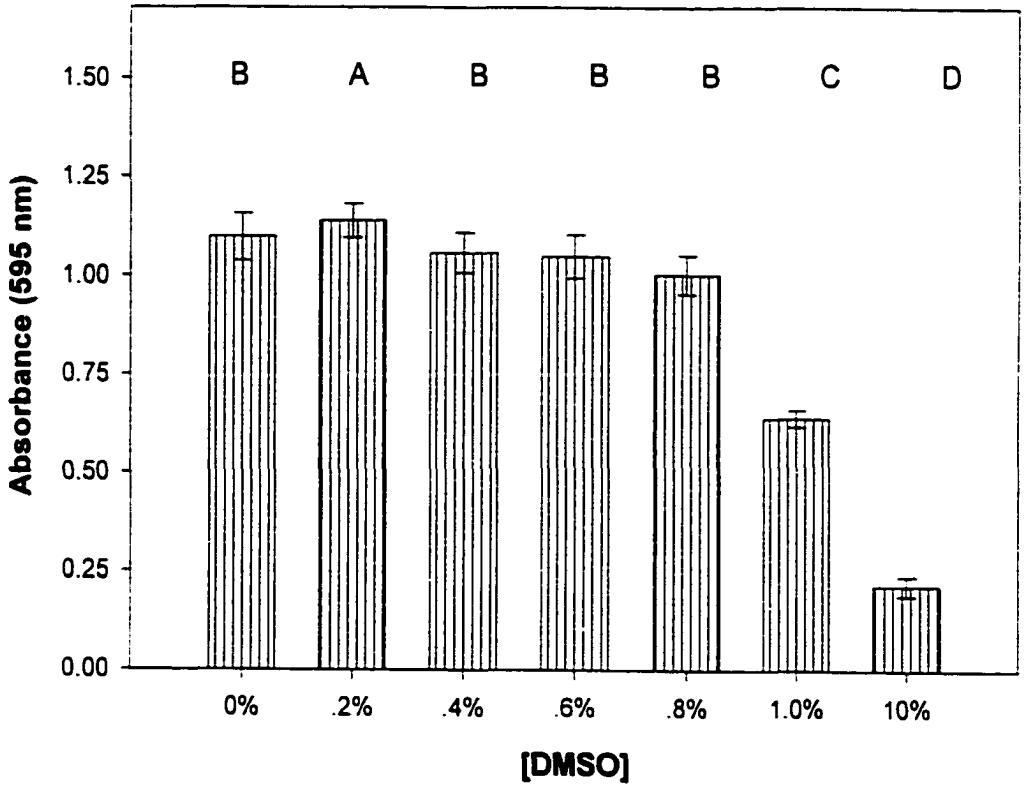

Figure 2.4. MCF-10A cells survived 18 hours of exposure to ≤ 0.8% DMSO. Bars are mean ± SD absorbance. Bars with the same letter were not significantly different from each other. In DMSO, differences were significant [analysis of variance (ANOVA) $F[6,55] = 436.37$, $P < 0.0001$, n = 8/bar]. A student-Numan-Keuls test (SNK) identified four groups ($P \leq 0.05$).

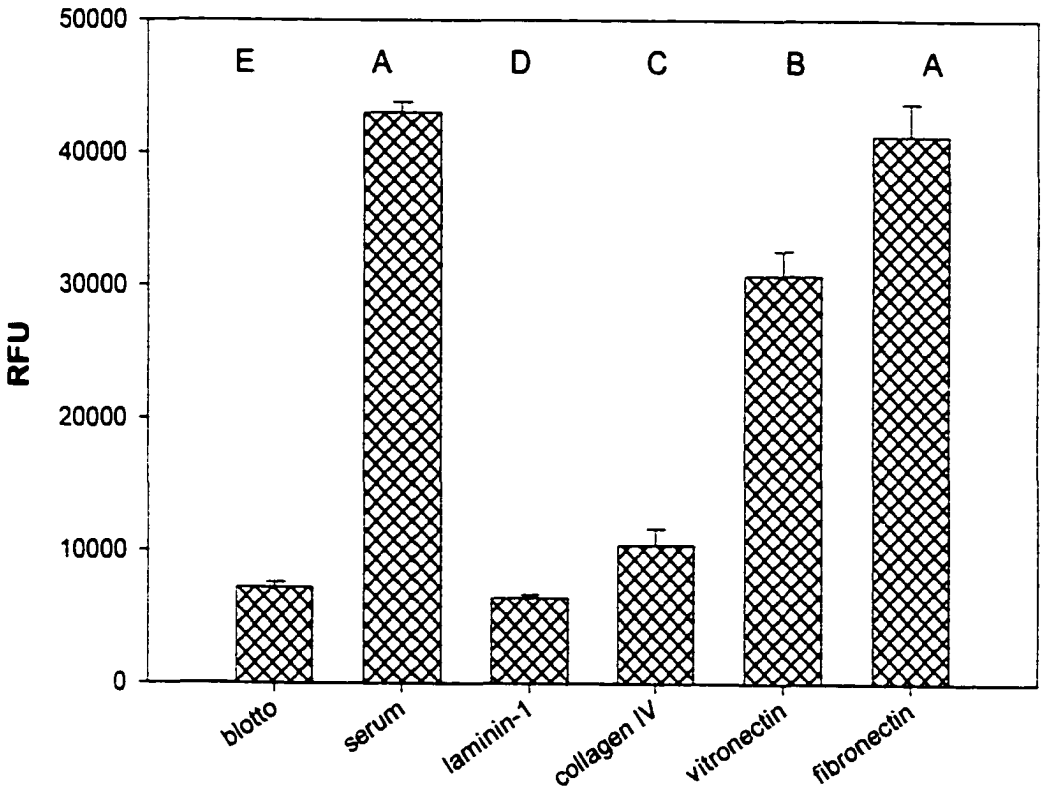

Figure 2.5. MCF-7 cells preferentially migrated on fibronectin. Fluorescence was measured at 485 nm for excitation and at 530 nm for emission. Bars are mean ± SD relative fluorescent units (RFU). Bars with the same letter were not significantly different from each other. On extracellular matrix proteins, differences were significant [analysis of variance (ANOVA) $F[5, 23] = 459.93$, $P < 0.0001$, $n = 4$/bar]. A student-Numan-Keuls test (SNK) identified five groups ($P \leq 0.05$).

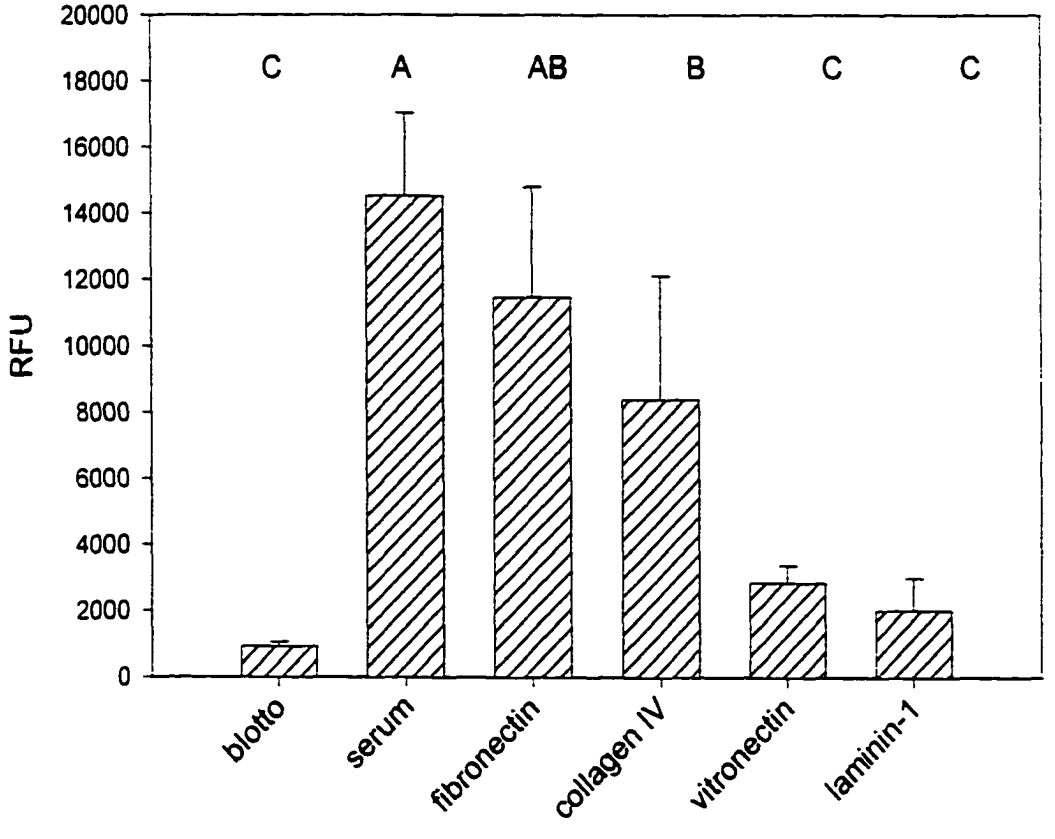

Figure 2.6. SF-268 cells preferentially migrated on fibronectin and collagen IV. Fluorescence was measured at 485 nm for excitation and at 530 nm for emission. Bars are mean ± SD relative fluorescent units (RFU). Bars with the same letter or letters were not significantly different from each other. On extracellular matrix proteins, differences were significant [analysis of variance (ANOVA) $F[5, 23] = 23.11$, $P < 0.0001$, $n = 4$/bar]. A student-Numan-Keuls test (SNK) identified two partly overlapping groups and one other group ($P \leq 0.05$).

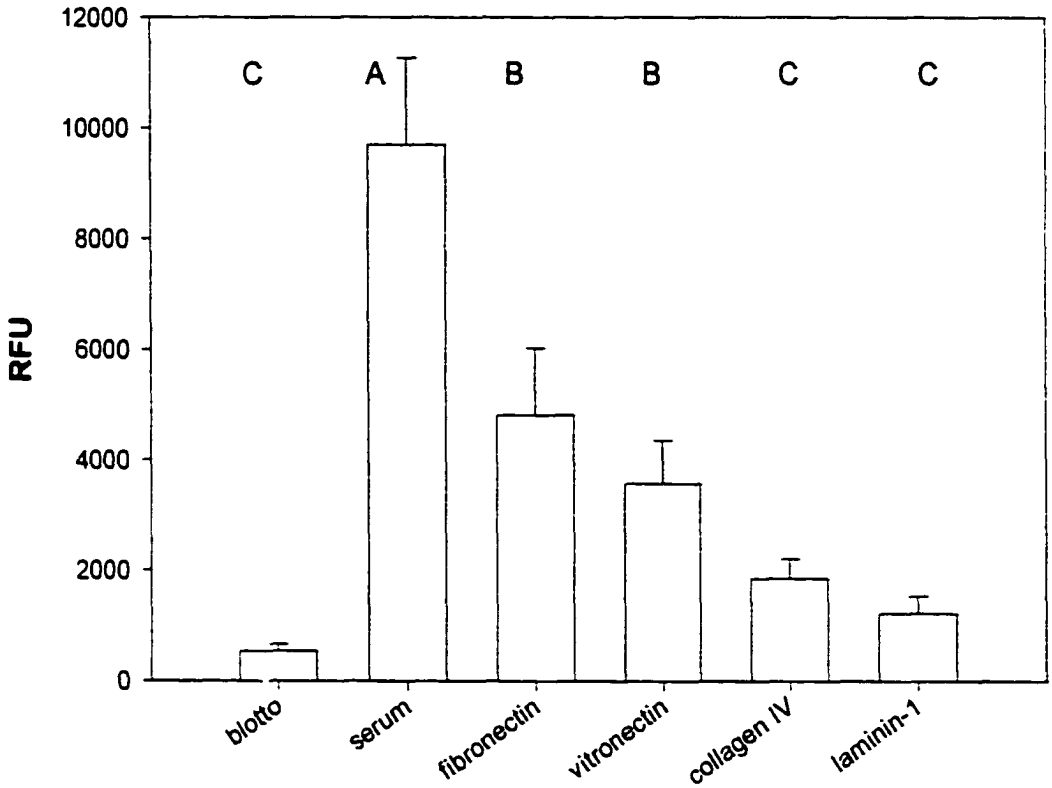

Figure 2.7. NCI-H460 cells preferentially migrated on both fibronectin and vitronectin. Fluorescence was measured at 485 nm for excitation and at 530 nm for emission. Bars are mean ± SD relative fluorescent units (RFU). Bars with the same letter were not significantly different from each other. On extracellular matrix proteins, differences were significant [analysis of variance (ANOVA) $F[5, 23] = 57.15$, $P < 0.0001$, $n = 4$/bar]. A student-Numan-Keuls test (SNK) identified three groups ($P \leq 0.05$).

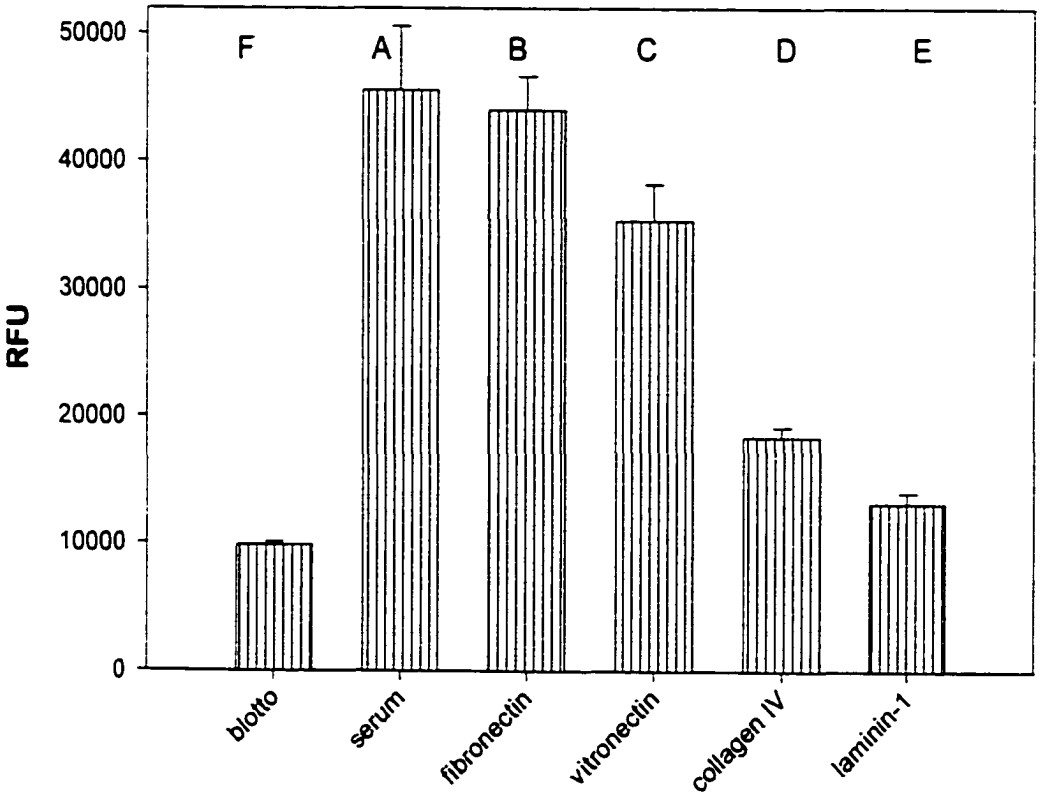

Figure 2.8. MCF-10A cells preferentially migrated on fibronectin. Fluorescence was measured at 485 nm for excitation and at 530 nm for emission. Bars are mean ± SD relative fluorescent units (RFU). Bars with the same letter were not significantly different from each other. On extracellular matrix proteins, differences were significant [analysis of variance (ANOVA) $F[5, 23] = 591.80$, $P < 0.0001$, $n = 4$/bar]. A student-Numan-Keuls test (SNK) identified six groups ($P \leq 0.05$).

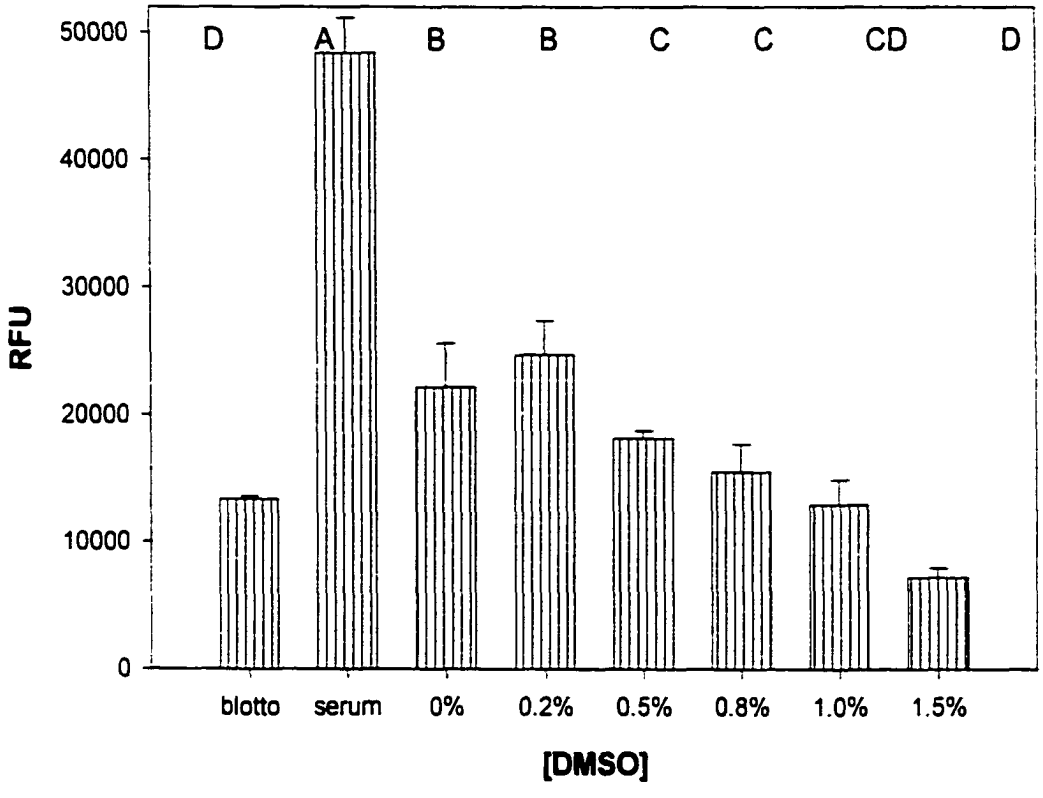

Figure 2.9. Migration of MCF-10A cells was unaffected by ≤ 0.2% DMSO on fibronectin. Fluorescence was measured at 485 nm for excitation and at 530 nm for emission. Bars are mean ± SD relative fluorescent units (RFU). Bars with the same letter or letters were not significantly different from each other. In DMSO and on fibronectin, differences were significant [analysis of variance (ANOVA) $F[7, 23] = 104.42$, $P < 0.0001$, n = 3/bar]. A student-Numan-Keuls test (SNK) identified two partly overlapping groups and two other groups ($P \leq 0.05$).

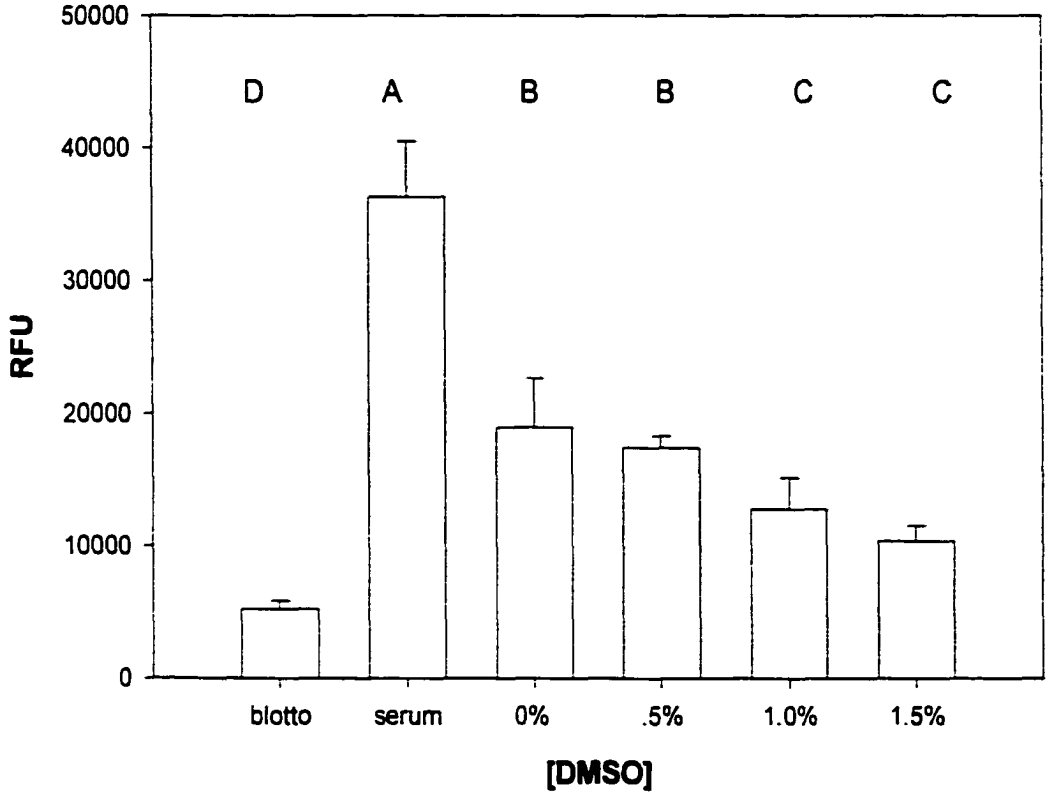

Figure 2.10. Migration of NCI-H460 cells was unaffected by ≤ 0.5% DMSO on fibronectin. Fluorescence was measured at 485 nm for excitation and at 530 nm for emission. Bars are mean ± SD relative fluorescent units (RFU). Bars with the same letter were not significantly different from each other. In DMSO and on fibronectin, differences were significant [analysis of variance (ANOVA) $F[5, 23] = 69.59$, $P < 0.0001$, $n = 4$/bar]. A student-Numan-Keuls test (SNK) identified four groups ($P \leq 0.05$).

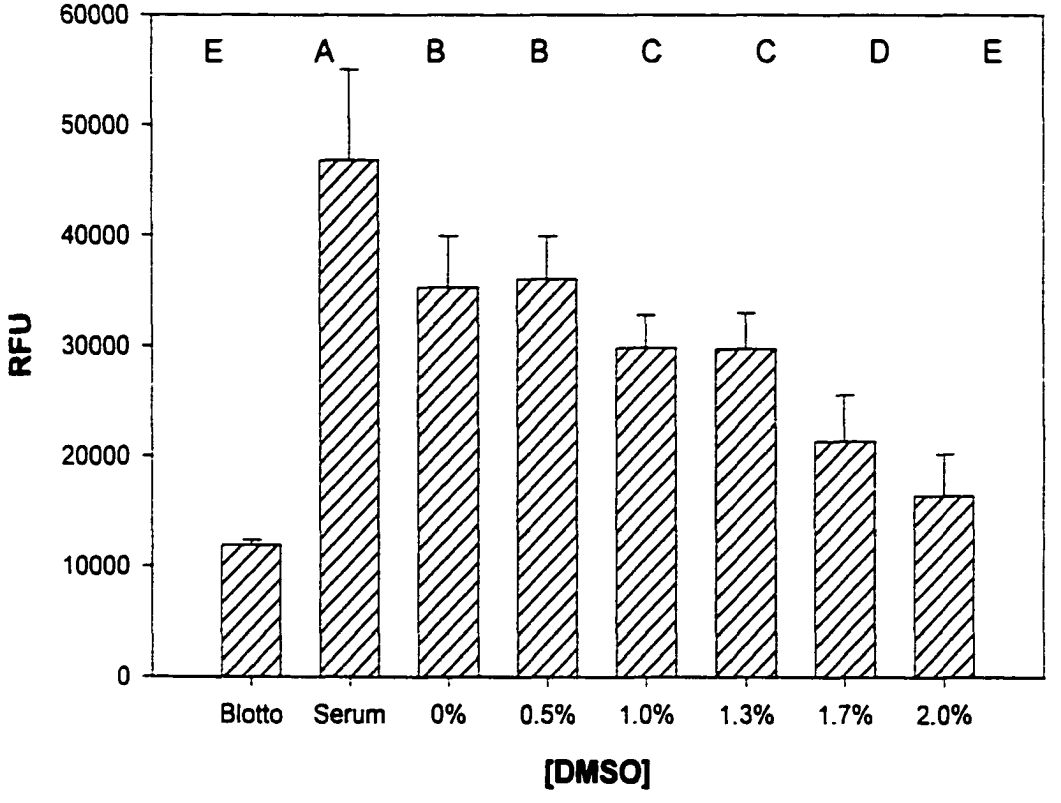

Figure 2.11. Migration of SF-268 cells was unaffected by ≤ 0.5% DMSO on fibronectin. Fluorescence was measured at 485 nm for excitation and at 530 nm for emission. Bars are mean ± SD relative fluorescent units (RFU). Bars with the same letter were not significantly different from each other. In DMSO and on fibronectin, differences were significant [analysis of variance (ANOVA) $F[7, 23] = 55.55$, $P < 0.0001$, $n = 3$/bar]. A student-Numan-Keuls test (SNK) identified five groups ($P \leq 0.05$).

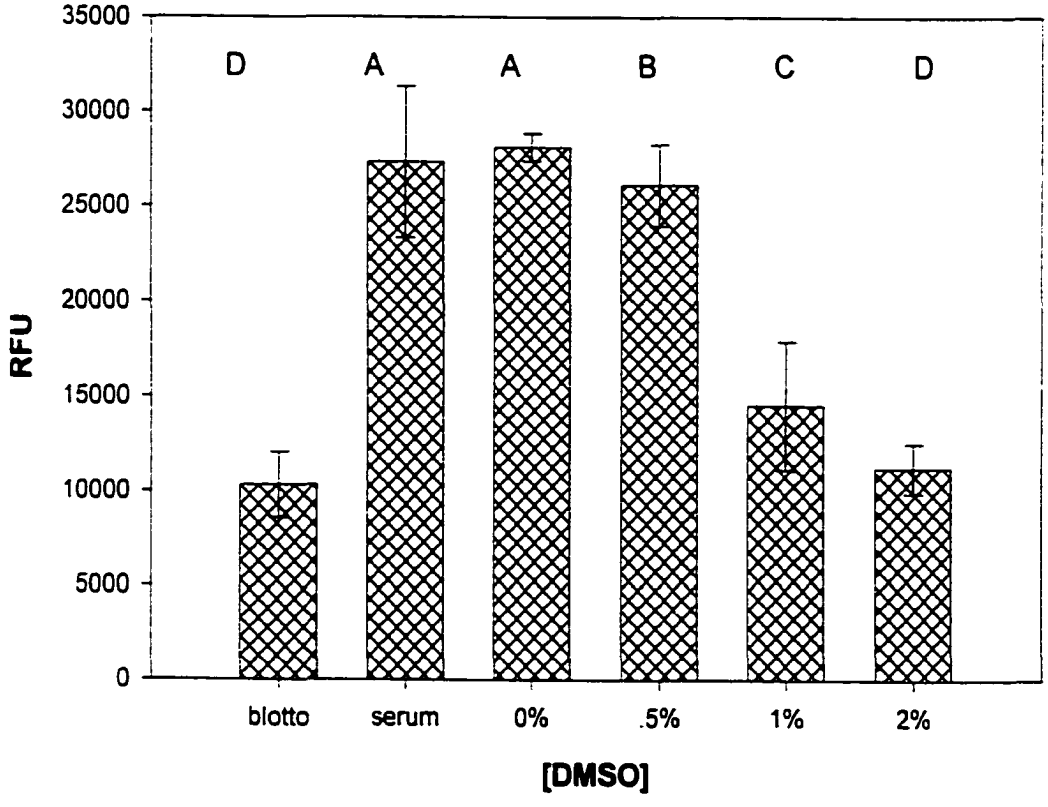

Figure 2.12. Migration of MCF-7 cells was unaffected by < 0.5% DMSO on fibronectin. Fluorescence was measured at 485 nm for excitation and at 530 nm for emission. Bars are mean ± SD relative fluorescent units (RFU). Bars with the same letter were not significantly different from each other. In DMSO and on fibronectin, differences were significant [analysis of variance (ANOVA) $F[5, 23] = 76.91$, $P < 0.0001$, $n = 4$/bar]. A student-Numan-Keuls test (SNK) identified four groups ($P \leq 0.05$).

condition	tests	MCF-10A	MCF-7	SF-268	NCI-H460
DMSO%	cell viability	≤ 0.8%	≤ 1%	≤ 2%	< 1%
DMSO%	migration	≤ 0.2%	< 0.5%	≤ 0.5%	≤ 0.5%
ECM selection	migration	fibronectin	fibronectin	fibronectin collagen IV	fibronectin vitronectin
monolayer		120,000	120,000	120,000	180,000

Table 2.1. Summary of optimized parameters for a large-scale drug screen for anti-migration compounds: All cell lines were viable in ≤ 2% DMSO for 18 hours, preferentially or equally on migrated on fibronectin as compared to other ECM proteins and their migration was unaffected in ≤ 0.5% DMSO. The number of cells needed to form a monolayer that evenly covers the migration filter was greater for the NCI-H460 cell line compared to the other cell lines.

CHAPTER 3

IDENTIFICATION OF PERILLYL ALCOHOL AS A CELL-MIGRATION-INHIBITING DRUG

Abstract

Chemotherapeutic and chemopreventive effects of limonene have been demonstrated in breast cancer with tumor regression and low toxicity. The monoterpene d-limonene and its related compounds, perillyl alcohol and perillaldehyde were chosen as candidate drugs for application in the anti-migration drug screen assay. Using a human breast non-tumorigenic cell line MCF-10A, we investigated the toxicity of these compounds in a 24-hour cell growth assay. Results delineated toxicity as greatest for perillaldehyde, intermediate for perillyl alcohol and least for limonene. A haptotactic *in vitro* migration assay of MCF-10A cells with limonene and perillyl alcohol revealed that a non-cytotoxic concentration of 0.5 mM perillyl alcohol inhibited migration, while the same concentration of limonene failed to do so. Adhesion in the MCF-10A cell line and a human breast cancer cell line MDA-MB 435 was unaffected by 1.5 mM perillyl alcohol. Growth was inhibited in MDA-MB 435 cells at a concentration of 0.4 mM perillyl alcohol. A non-cytotoxic and migration-inhibiting concentration of perillyl alcohol was undetected for MDA-MB 435 cells. Our results for

cytotoxicity in cancer and non-cancer cells corroborate findings of prior research. The novel observation that perillyl alcohol inhibits cell migration at a non-toxic dose may bear further fruit in deciphering an underlying mechanism for impeding cell migration.

Introduction

Identifying drugs that inhibit tumor cell migration has been limited by the lack of available technology. To date fewer than 100 compounds have been shown to possess anti-migration properties against cancer cells, while researchers have discovered thousands of anti-proliferative drugs. Current methods of measuring cell migration employ techniques that require copious amounts of time and money to generate effective data. The most popular device used is the "Transwell" or "modified Boyden chamber" that consists of 24 individual filter inserts and costs $25 per plate. Our lab has developed an automated, high-throughput in vitro cell migration/cytotoxicity assay to improve efficiency and costs involved in screening drugs for potential anti-migration effects.

Our migration assay is based on a common technique of measuring cells that move through a porous membrane. However, instead of counting stained cells manually under a microscope, which is time consuming and less precise, we label the migrated cells with a fluorescent tag, such as calcein-AM (Molecular Probes; Eugene, OR) and a plate reader accurately measures the level of fluorescence in seconds. Another novel feature of our assay is that it can be

used with the 96-well migration plate designed by our lab. Multiple replicates can be screened in much less time because our plate does not have individual filters but rather a single mold with 96-wells. The opaque filter on our plate is also unique. Being able to measure fluorescence from the top and bottom of the filter will allow us to test for cytoxicity with a compound such as MTT and cell migration with calcein-AM in the same well (8).

Prior to the manufacturing of our new plate, we used Transwell plates to complete the optimization of the conditions for cancer and non-cancer cell migration with our new protocol. These conditions include identifying which extracellular matrix protein to coat the filters with to maximize migration for different cell lines, measuring cytotoxicity due to dimethyl sulfoxide (DMSO) exposure, and accounting for the effect of DMSO on migration. DMSO is a common solvent used to solubilize compounds.

In this study, we applied the cytotoxcity and migration assays to screen drugs, already known to have anti-cancer effects, for anti-migration properties. The chemotherapeutic and chemopreventative effects of d-limonene, a monoterpene found in the essential oils of citrus fruits, spices and herbs, have been studied extensively in spontaneous and chemically induced rodent tumors (1). Limonene serves as a precursor to other oxygenated monocyclic monoterpenes such as carveol, carvone, menthol, perillyl alcohol and perillaldehyde (2). Due to success in tumor regression in various rodent cancer types, clinical testing of the cancer chemotherapeutic activity of limonene and

perillyl alcohol is in progress (3-4), though their exact mechanism of action remains unknown.

We chose to investigate three related compounds, limonene, perillyl alcohol and perilladehyde, for possible anti-migration effects in human breast cancerous (MDA-MB 435) and non-cancerous (MCF-10A) cells. Prior to assessing inhibition of migration, we measured the cytotoxicity of these compounds, finding that perillaldehyde was lethal at all tested concentrations. We proceeded next to investigate limonene and perillyl alcohol's effect on MCF-10A cell migration. At a concentration of 1.0 mM, limonene did not affect migration. However, a non-cytotoxic concentration of perillyl alcohol decreased MCF-10A cell migration. Unfortunately, migration of MDA-MB 435 cells was not inhibited without compromising cell viability. Additionally, adhesion in both cell lines was unaffected by concentrations of perillyl alcohol relevant to the measured anti-migration activity of MCF-10A cells. The results of this investigation suggest that perillyl alcohol has diverse effects on migration in different cell lines. One possible mechanism of action of perillyl alcohol may involve reduction in the isoprenylation of G proteins such as RhoA (5), which may lead to inhibition of cell migration.

Materials and Methods

Materials

MCF-10A and MDA-MB 435 cells were purchased from American Type Culture Collection. Epidermal growth factor, cholera toxin, insulin were obtained

from Calbiochem (La Jolla, CA). Hydrocortisone was purchased from Sigma-Aldrich Chemicals (St. Louis, MO). Horse serum was purchased from Irvine Scientific (Santa Ana, CA). Calcein-AM was purchased from Molecular Probes (Eugene, OR). Transwell migration plates were purchased from Costar (Cambridge, MA). Cell culture flasks and 96-well plates were purchased from VWR (Plainfield, NJ). Limonene, perillyl alcohol, perillyl aldehyde were purchased from Aldrich Chemical Co. (Milwaukee, WI).

Cell Culture

Cells were grown in 75 cm^2 Falcon tissue-culture flasks and maintained at 37° C and 5% CO_2 in humidified chambers. MCF-10A cells were maintained in AM media—1:1 mixture of Ham's F-12 medium and Dulbecco's Modified Eagle's Medium High Glucose with 2 mM L-glutamine from Irvine Scientific (Santa Ana, CA) supplemented with the following: epidermal growth factor (20 ng/ml), cholera toxin (100 ng/ml), insulin (0.01 mg/ml), hydrocortisone (500 ng/ml) and 5% horse serum. MDA-MB 435 cells were maintained in RPMI Medium 1640 supplemented with 10% fetal bovine serum and 1% L-glutamine (29.2 mg/ml), penicillin G (10,000 units/ml) and streptomycin sulfate (10,000 μg/ml) from Irvine Scientific (Santa Ana, CA). Cells were routinely passaged using trypsin/EDTA from Irvine Scientific (Santa Ana, CA).

Proliferation Assay

In 96-well plates 3 x 10^3 cells were seeded in the appropriate serum-containing media and incubated for 2 hours at 37° C and 5% CO_2 in humidified chambers. Cells were then washed twice with PBS and grown in migration

media (MCF-10A: DMEM and 0.1% BSA; MDA-MB 435: RPMI 1640 and 0.1% BSA) with indicated concentrations of limonene, perillyl alcohol or perillaldehyde for 24 hours. Cells were washed twice with PBS, fixed in 3.7% formaldehyde for 15 minutes, stained with crystal violet and lysed with 1% sodium docecyl sulfate (SDS). Absorbance was read at 595 nm using a plate reader. Growth was measured as compared to a standard curve of cells grown in serum-containing media.

Adhesion Assay

Cell adhesion assays were performed as previously described in Costar 96-well Cell Culture Cluster plates (6). Tissue culture plates were either coated with purified fibronectin at a concentration of 20 μg/mL for 1 hour at room temperature. Wells were then washed with phosphate-buffered saline with 0.2 % Tween-20 (PBST), blocked with 5 % blotto (PBST with 5% skim milk) for one hour at 25^0 C. Wells were then washed twice with PBST prior to assay. Cells were seeded at a concentration of 1×10^6 in cell culture plates in migration media (for MCF-10A: DMEM and 0.1% BSA; for MDA-MB 435: RPMI 1640 and 0.1% BSA) with or without perillyl alcohol and were allowed to attach for eighteen hours at 37^0 C in a humidified incubator containing 5% CO_2. Unbound cells were dislodged by inverting, submerging and rocking the plate in PBS for 15 minutes. Cells were then fixed in 3.7% formaldehyde, stained with crystal violet and lysed with 1% SDS. Absorbance was measured at 595 nm in a plate reader.

Migration Assay

Cell migration assays were performed as previously described in Costar transwell filter plates (7). All filters except for the negative control,blotto, were coated with purified fibronectin at a concentration of 20 µg/ml in carbonate buffer (pH 9.3) for 1 hour at room temperature. Filters were then aspirated and blocked in blotto (phosphate-buffered saline with 0.2% Tween-20 (PBST) and 5% skim milk) for 1 hour at room temperature. Next filters were washed in PBST prior to the assay. Thirty minutes prior to starting the assay cells were pre-incubated at 37° C in migration media and varying concentrations of perillyl alcohol. Cells were seeded at a concentration of either 1.2×10^6 on the transwell filters with and without ECM in the presence or absence of soluble growth factors (serum-enriched media) and varying concentrations of POH and allowed to migrate for 18 hours at 37°C in 5% CO_2. Thirty minutes before measuring migration, 5 µM of calcein-AM, a fluorescent dye that chelates calcium, (Molecular Probes; Eugene,OR) was added to the migration wells. To quantify migration, cells that did not migrate were removed from the top of the filter with cotton swabs, filters were washed in phosphate-buffered saline and migrated cells were measured from the bottom of the filter with a plate reader for the incorporation of calcein-AM (8). Fluorescence values for each experimental condition were expressed relative to control, untreated samples.

Results

The monoterpene d-limonene and its related compounds, perillyl alcohol and perillaldehyde, showed substantial differences in a 24-growth assay. Cytotoxicity was determined by comparing absorbance readings of crystal violet as related to cell number for a standard curve of cells grown in migration media alone. MCF-10A cells exposed to 0.5 mM perillaldehyde and 1.0 mM perillyl alcohol could not grow or survive (Fig. 3.1). However, migration media with limonene tested up to 1.0 mM was innocuous, allowing the cells to replicate as normal.

Haptotactic migration assays using perillyl alcohol and limonene with MCF-10A cells on fibronectin to determine if either compound could inhibit cell migration established perillyl alcohol as such a compound. Limonene was unable to affect migration at the 1.0 mM maximum concentration tested, while perillyl alcohol decreased migration at 0.5 mM, a non-cytotoxic dose (Fig. 3.2).

To test the effect of perillyl alcohol on adhesion both non-cancerous (MCF-10A) cells and cancerous (MDA-MB 435) cells were used. Over an 18-hour exposure to increasing concentrations of perillyl alcohol, both MCF-10A and MDA-MB 435 cell lines tolerated up to 1.5 mM without a significant difference compared to 0 mM (Fig. 3.3).

Growth and migration assays were performed for both cell lines. In MCF-10A cells migration was inhibited at a concentration of 0.5 mM of perillyl alcohol. Cell viability for MCF-10A cells, as measured as not less than baseline of 30 x 10^3 cells, was stable in 0.75 mM of perillyl alcohol (Fig. 3.4). For MDA-MB 435

cells, migration was not inhibited by perillyl alcohol at a concentration that was also not cytotoxic (Fig. 3.5).

Discussion

We have shown that the monoterpene perillyl alcohol (POH), unlike its other related compounds limonene and perillaldehyde, can inhibit migration in the nontumorigenic MCF-10A human breast cell line. This inhibition is induced by a concentration of POH that is not cytotoxic or cytostatic. Our results from a 24-hour growth assay demonstrate that perillaldehyde is considerably more toxic than either perillyl alcohol or limonene. This may be due to increased affinity between perillyl aldehyde and the enzymes farnesyl transferase (FT) and geranylgeranyl transferase (GGT) as has been shown for the minor metabolite, perillic acid methyl ester, of limonene and POH (9). These enzymes are involved in post-translational modification of small G-proteins that are involved in a myriad of cell activities such as growth and migration. Inhibition of FT and GGT is suspected to be the basis of the anti-tumor effects of perillyl alcohol and limonene (9). However, as shown by our results and in accord with others, an equal concentration of limonene is not as effective in blocking cell proliferation as perillyl alcohol (2,9).

Migration of MCF-10A cells was also unaffected by treatment with 1.0 mM limonene whereas 0.5 mM perillyl alcohol markedly reduced it. Again, this difference is most likely attributable to the limonene metabolites and perillyl alcohol having a greater potency than limonene in the inhibition of small G-

protein isoprenylation (1,10). A probable mediator of this inhibited migration is the small G-protein RhoA, a member of the Rho family of small GTPases that is involved in a signaling pathway for cell migration (5,11).

Adhesion in MCF-10A cells is more tolerant of perillyl alcohol treatment. At 1.5 mM, adhesion was unaffected unlike the decreased migration measured at 0.5 mM POH. Even in the cancer cell line, adhesion was maintained. The requirements for adhering to a surface are much less demanding on the cell than migrating. Adhesion is a static activity and migration is a highly coordinated kinetic action of many structural and signaling proteins.

To further assess POH as a possible inhibitor of cancer cell migration we tested it in the human breast cancer cell line MDA-MB 435. Our results show that POH is unable to block migration at a non-cytotoxic level in this cell line. This may be due to this cell line expressing a higher level of RhoA as has been reported in other tumor cells as compared to nontumorigenic cells (12). An increased invasive phenotype is also characteristic of some liver and breast tumor cells in response to RhoA activation (12-13). Additionally, research has shown that perillyl alcohol treatment significantly increased apoptosis in pancreatic cancer cells relative to non-malignant pancreas cells (14). Thus, our results with POH treatment in MDA-MB 435 cells may not be attributable to the same mechanism as that in nontumorigenic MCF-10A cells.

In conclusion, we have shown that perillyl alcohol is a non-cytotoxic, anti-migration agent in the human breast cell line MCF-10A. This inhibition was not seen in the cancer cell line MDA-MB 435, which may be due to greater

expression of RhoA and increased activation/deactivation of other signaling pathways sensitive to perillyl alcohol treatment. From this point. it seems reasonable to further investigate the involvement of RhoA protein in the POH-treated MCF-10A decreased cell migration.

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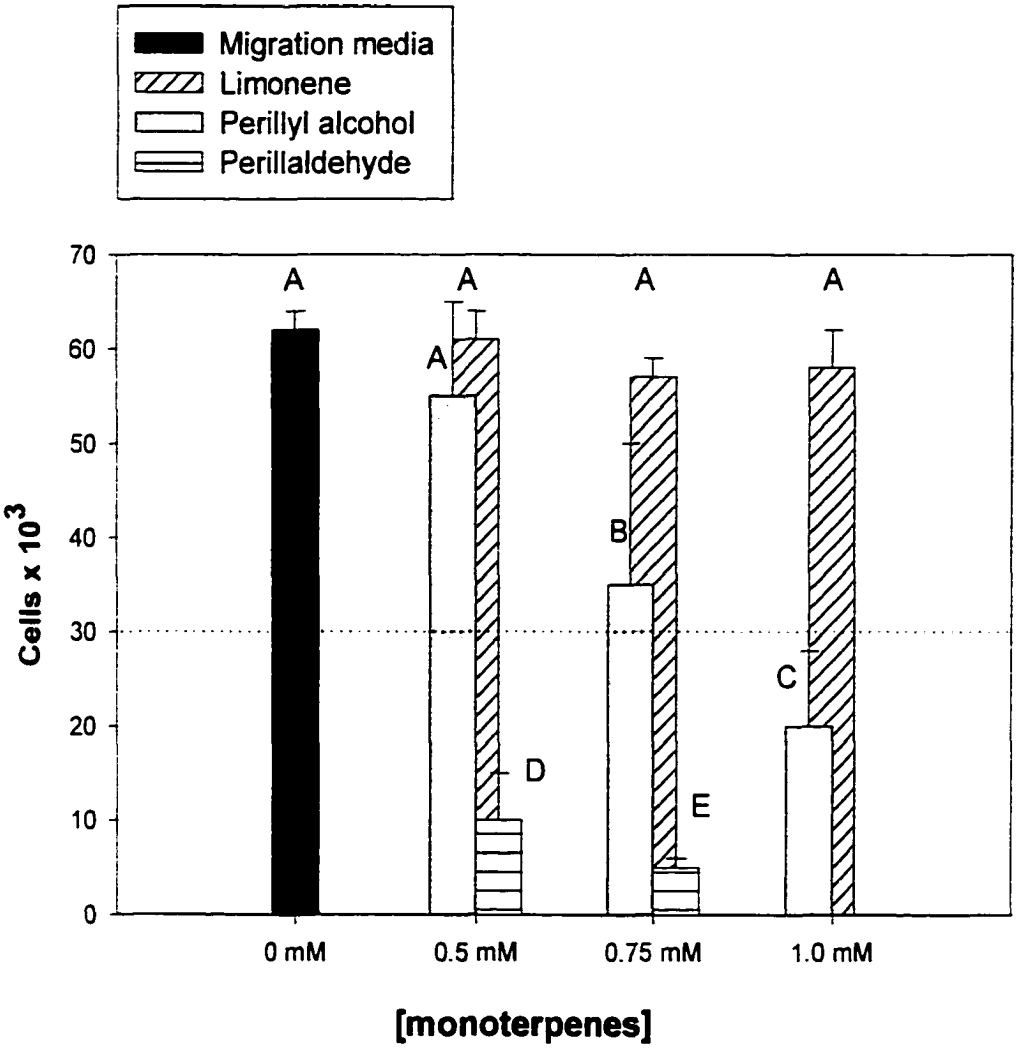

Figure 3.1. MCF-10A cells replicated as normal when exposed to 1.0 mM limonene and 0.5 mM perillyl alcohol. All tested concentrations of perillaldehyde showed a dose-dependent increase in cytotoxicity; 1.0 mM killed all of the plated cells. Migration media represents untreated condition. Dotted line represents the number of cells initially plated. Bars are mean ± SD number of cells. Bars with the same letter were not significantly different from each other. In the various treatment conditions, differences were significant [analysis of variance (ANOVA) $F[9, 98] = 121.63$, $P < 0.0001$, $n = 10$/bar]. A student-Numan-Keuls test (SNK) identified five groups ($P \leq 0.05$).

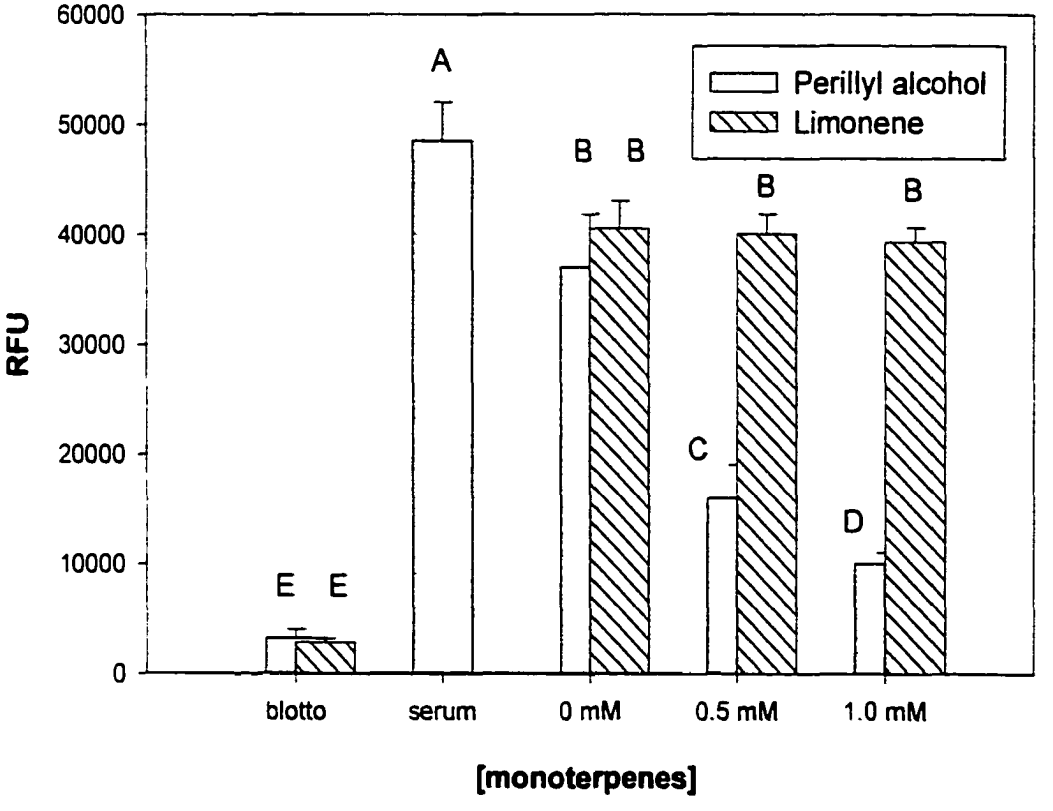

Figure 3.2. Migration of MCF-10A cells was inhibited by 0.5 mM perillyl alcohol on fibronectin, whereas 1.0 mM limonene failed to slow cell migration under the same conditions. Fluorescence was measured at 485 nm for excitation and at 530 nm for emission. Bars are mean ± SD relative fluorescent units (RFU). Bars with the same letter were not significantly different from each other. In treatment conditions and on fibronectin, differences were significant [analysis of variance (ANOVA) $F[7, 23] = 231.76$, $P < 0.0001$, n = 3/bar]. A student-Numan-Keuls test (SNK) identified five groups ($P \leq 0.05$).

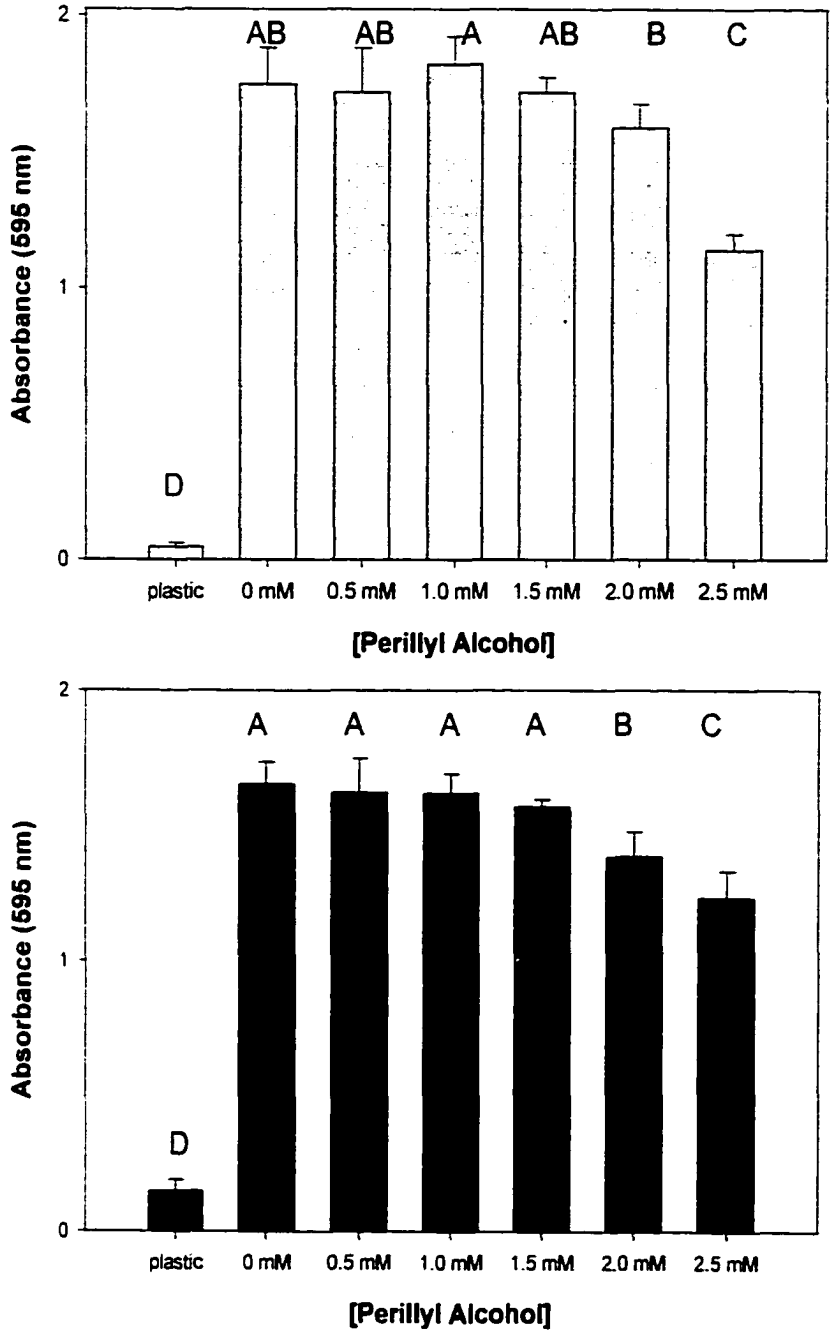

Figure 3.3. Adhesion on fibronectin of MCF-10A cells (gray bars) and MDA-MB 435 cells (black bars) was unaffected by 1.5 mM perillyl alcohol in migration media after 18 hours of exposure. Bars are mean ± SD absorbance. Bars with the same letter or letters were not significantly different from each other. In treatment conditions and on fibronectin, differences were significant [analysis of variance (ANOVA) F: (MCF-10A) [6, 55] = 144.88, $P < 0.0001$, n = 8/bar; (MDA-MB 435) [6,41] = 252.45, $P < 0.0001$, n = 7/bar]. A student-Numan-Keuls test (SNK) identified: (MCF-10A) two partly overlapping groups and two other groups; (MDA-MB 435) four groups ($P \leq 0.05$).

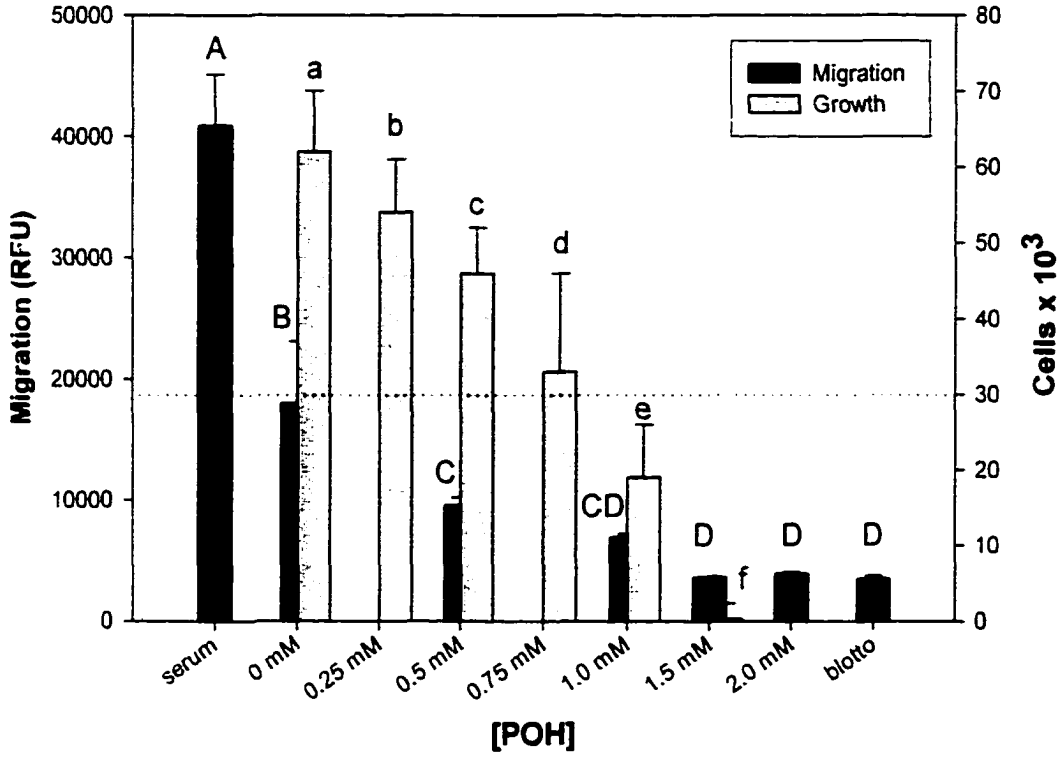

Figure 3.4. Migration and growth assays with MCF-10A cells exposed to increasing concentrations of perillyl alcohol (POH) established that at 0.5 mM cells were still replicating while their migration was inhibited. Blotto is a negative control. Dotted line represents the initial number of cells plated for the growth assay. Migration assay did not include POH concentrations of 0.25 mM and 0.75 mM. Fluorescence was measured at 485 nm for excitation and at 530 nm for emission. Migration: Bars are mean ± SD relative fluorescent units (RFU). Bars with the same upper case letter or letters were not significantly different from each other. In treatment conditions and on fibronectin, differences were significant [analysis of variance (ANOVA) F: [6, 20] = 33.80, P < 0.0001, n = 4 or 2/bar. A student-Numan-Keuls test (SNK) identified two partly overlapping groups and two other groups ($P \leq 0.05$). Growth assay for 1.5 mM and 2.0 mM POH severely reduced the number of viable cells. Cell number was determined using a standard curve and measured by absorbance at 595 nm. Growth: Bars are mean ± SD number of cells. Bars with the same lower case letter were not significantly different from each other. In treatment conditions, differences were significant [analysis of variance (ANOVA) F: [7, 79] = 253.58, P < 0.0001, n = 10/bar. A student-Numan-Keuls test (SNK) identified six groups ($P \leq 0.05$)

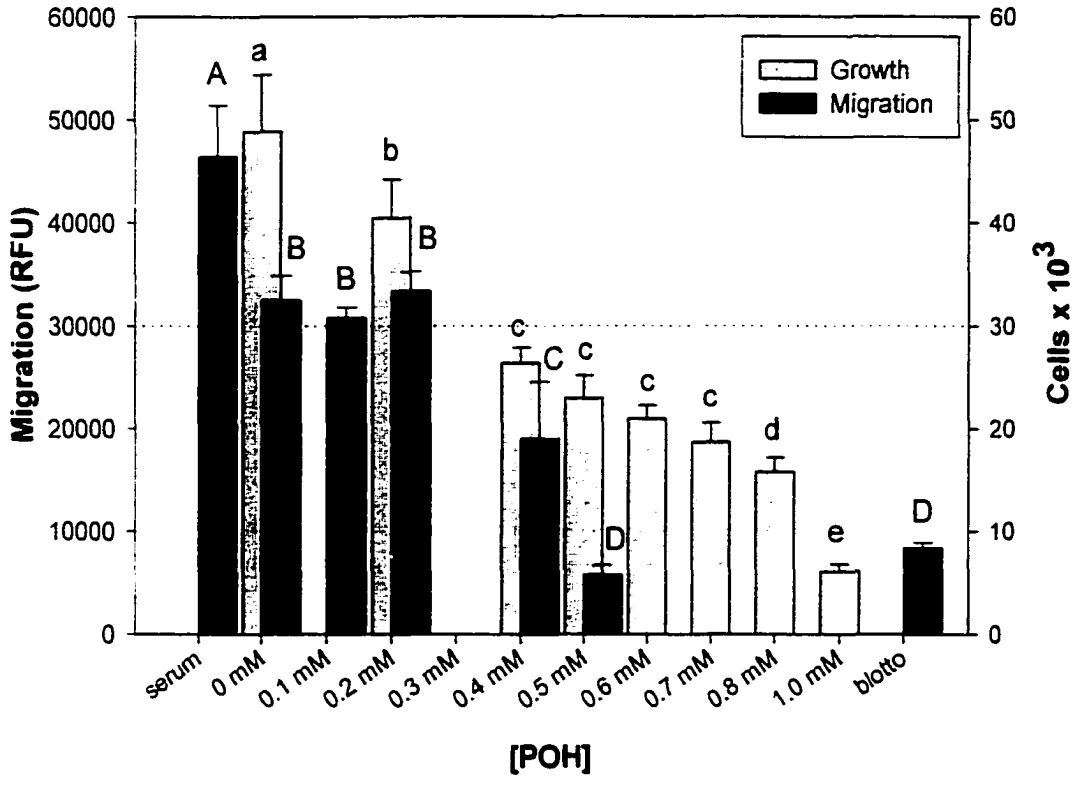

Figure 3.5. Migration and growth assays with MDA-MB 435 cells exposed to various concentrations of perillyl alcohol (POH) established that there was not a non-cytotoxic concentration of POH that could inhibit migration. Blotto is a negative control for the migration assay. Dotted line represents the initial number of cells plated for the growth assay. Migration assay did not include POH concentrations greater than 0.5 mM. Fluorescence was measured at 485 for excitation and at 530 nm for emission. Migration: Bars are mean ± SD relative fluorescent units (RFU). Bars with the same upper case letter were not significantly from each other different. In treatment conditions and on fibronectin, differences were significant [analysis of variance (ANOVA) F: [6, 23] = 33.80, $P < 0.0001$, n = 4 or 2/bar. A student-Numan-Keuls test (SNK) identified four groups ($P \leq 0.05$). Growth assay does not include a 0.1 mM POH concentration. For the growth assay, the number of cells was determined by a standard curve using absorbance measured at 595 nm. Growth: Bars are mean ± SD number of cells. Bars with the same lower case letter were not significantly different from each other. In treatment conditions, differences were significant [analysis of variance (ANOVA) F: [7, 80] = 56.08, $P < 0.0001$, n = 10/bar. A student-Numan-Keuls test (SNK) identified five groups ($P \leq 0.05$).

CHAPTER 4

DETERMINATION OF THE MECHANISM BY WHICH PERILLYL ALCOHOL INHIBITS BREAST CELL MIGRATION

Abstract

Perillyl alcohol is currently in clinical trials for prevention and treatment of various cancers. One of the identified cellular activities of perillyl alcohol is the inhibition of the enzyme type 1 geranylgeranyl transferase. RhoA is a protein that requires prenylation to be active and to initiate a pathway involved in cell migration. We hypothesized that the decreased migration observed in POH-treated MCF-10A and MDA-MB 435 cells would be the result of the translocation of RhoA to the cytosol, thus impairing its migration signaling pathway. In this study, we first measured by western blot whether exposure to perillyl alcohol down-regulated the production of RhoA protein in human breast non-tumorigenic MCF-10A and MDA-MB 435 breast cancer cells and found that it did not. We also found that RhoA protein expression was greater in the non-cancerous MCF-10A cells than the MDA-MB 435 cells. Next the distribution of RhoA protein between the aqueous and detergent-enriched phases was analyzed by western

blots from cell lysates of MCF-10A cells and MDA-MB 435 human breast cancer cells. At 0 hours of exposure, all RhoA protein was located in the detergent-enriched phase following treatment with 0.5 mM perillyl alcohol in MCF-10A cells. However, after 18 hours of exposure RhoA protein accumulated in the aqueous phase in MCF-10A cells. For MDA-MB 435 cells even at 0 hours of treatment (treatment for 30 minutes) with 0.3 mM perillyl alcohol RhoA accumulated in the aqueous phase, which was again detected after 18 hours of exposure. The morphological effects of perillyl alcohol were investigated in MCF-10A cells and MDA-MB 435 cells following an 18-hour exposure to 0.5 mM or 0.3 mM, respectively. Perillyl alcohol treated MCF-10A and MDA-MB 435 cells showed a complete disassembly of stress fiber and focal adhesion complex formation, consistent with a loss of RhoA protein function.

Introduction

Small G-proteins have been reported to play an important role in the metastatic behavior of tumor cells (1). Protein isoprenylation, a form of post-translational modification, allows these proteins to associate with the plasma membrane where they activate various targets for a wide range of biological activity—contractility, adhesion, cytokinesis, proliferation, neoplastic transformation and apoptosis (2). Therefore, blocking the activation of low molecular weight GTPases by inhibiting their isoprenylation could reduce metastasis (3). The monoterpene perillyl alcohol (POH) has been shown to block isoprenylation of RhoA, a small GTPase involved in a migration-signaling

pathway, by competitively inhibiting the enzyme type I geranylgeranyl-protein transferase (4-5). However, the specifics of this inhibition vary among cell types and cellular environments (6). Perillyl alcohol's mode of action in normal and malignant human breast cells is unknown. Thus, in determining the anticancer mechanism of perillyl alcohol, it is important to evaluate this biochemical effect in different cell types.

To understand how cell migration may be affected by perillyl alcohol treatment, it is necessary to identify proteins that are able to coordinate both cell shape and signaling pathways. A good candidate is the Rho family of proteins. These small GTPases are able to relay extracellular stimuli such as mitogen-activated-integrin-derived signals, as well as organize the actin cytoskeleton (7). Microinjection with activated and dominant negative Rho family members leads to a defined hierarchy of GTPase activation: activated Cdc42 activates Rac1, which then activates RhoA (8-9). Rac appears to drive the development of lamellipodia, whereas Cdc42 stimulates the polymerization of actin at the front to the cell to form long, thin extensions called filopodia (8-9). RhoA activation leads to the production of large, highly organized focal adhesion complexes and actin stress fibers (8-9).

Activated RhoA binds to and activates the serine/threonine kinase Rho-kinase (ROKα) (8). This kinase is suggested to regulate cell contractility by indirectly increasing phosphorylation of myosin light chain through the inhibition of myosin phophatase activity (10) and by directly phosphorylating myosin light chain independently of myosin light chain kinase (11). Overall, through this

pathway, RhoA activation results in bundling of actin filament into stress fibers and clustering of integrins into focal adhesions (Fig. 4.1). This membrane-cytoskeleton interaction has been reported to increase invasiveness in tumor cells from liver and breast tissue (12-13). Additionally, the level of RhoA expression has been reported to be several times higher in tumors than in surrounding normal tissue (12).

The objective of this study was to determine whether the isoprenylation of RhoA protein in perillyl alcohol treated breast cancer (MDA-MB 435) and nontumorigenic breast (MCF-10A) cells was altered. We hypothesized that the decreased migration observed in POH-treated MCF-10A and MDA-MB 435 cells would be the result of the translocation of RhoA to the cytosol, thus impairing its migration signaling pathway. By western blot, we found that the RhoA protein of exposed cells accumulated in the aqueous phase following lysing with a detergent buffer. This result suggests that RhoA isoprenylation in MCF-10A and MDA-MB 435 cells is affected by perillyl alcohol and that this may account for the observed decreased in MCF-10A migration previously reported. Furthermore, the morphological loss of actin stress fiber and focal adhesion formation following exposure of MCF-10A to perillyl alcohol indicates loss of RhoA function.

Materials and Methods

Materials

MCF-10A and MDA-MB 435 cells were purchased from American Type Culture Collection. Epidermal growth factor, cholera toxin, insulin were obtained

from Calbiochem (La Jolla, CA). Hydrocortisone was purchased from Sigma-Aldrich Chemicals (St. Louis, MO). Horse serum was purchased from Irvine Scientific (Santa Ana, CA). Perillyl alcohol was purchased from Aldrich Chemical Co. (Milwaukee, WI). The MicroBCA kit was from Pierce (Rockford, IL). Immobilon-P transfer membrane is from Millipore (Bedford, MA). Mouse anti-human RhoA IgG, mouse anti-human vinculin IgG and secondary goat anti-mouse IgG-AP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). CDP-star chemiluminescence reagent was purchased from Dupont-New England Nuclear (Boston, MA). Cell culture plates were purchased from VWR (Plainfield, NJ). Triton X-100 and X-114 were purchased from Sigma-Aldrich Chemicals (St. Louis, MO). Goat anti-mouse rhodamine-conjugated IgG , FITC-phalloidin and ELF-97 Immunohistochemical Mounting Medium were purchased from Molecular Probes (Eugene, OR).

Cell Culture

Cells were grown in 75 cm^2 Falcon tissue-culture flasks and maintained at 37° C and 5% CO_2 in humidified chambers. MCF-10A cells were maintained in AM media—1:1 mixture of Ham's F-12 medium and Dulbecco's Modified Eagle's Medium High Glucose with 2 mM L-glutamine from Irvine Scientific (Santa Ana, CA) supplemented with the following: epidermal growth factor (20 ng/ml), cholera toxin (100 ng/ml), insulin (0.01 mg/ml), hydrocortisone (500 ng/ml) and 5% horse serum. MDA-MB 435 cells were maintained in RPMI Medium 1640 supplemented with 10% fetal bovine serum and 1% L-glutamine (29.2 mg/ml), penicillin G (10,000 units/ml) and streptomycin sulfate (10,000 μg/ml) from Irvine

Scientific (Santa Ana, CA). Cells were routinely passaged using trypsin/EDTA from Irvine Scientific (Santa Ana, CA).

Western Analysis

Subconfluent plates of MCF-10A and MDA-MB 435 cells treated with perillyl alcohol for 18 hours were washed in ice-cold PBS and lysed in ice-cold RIPA buffer and protein concentrations were determined by MicroBCA assay. Proteins were separated by 12% SDS-PAGE and transferred to Immobilon-P Transfer Membranes and processed for immunoblotting as previously described (15). Processed membranes incubated with primary antibody were then incubated with secondary goat anti-mouse IgG-AP and exposed to chemiluminescence reagent and developed on Kodak X-0MAT LS scientific imaging film.

Separating Proteins into Aqueous and Detergent-Enriched Phases

The isoprenylated and unprocessed forms of RhoA proteins were separated into the detergent-enriched phase and the aqueous phase of 1% Triton X-114 TBS (20 mM Tris and 150 mM NaCl, pH 7.5) buffer as described by Guiterrez et al. (14). Perillyl alcohol (18 hr)-treated and untreated control cells were washed in ice-cold PBS and lysed with ice-cold 1% Triton X-114 TBS buffer. The cell debris was pelleted by centrifugation and the supernatant was incubated at 37° C for 5 minutes. The turbid solution was centrifuged at 16,000 g for 2 minutes. and the upper (aqueous) and the lower (detergent-enriched) phases were separated. By volume, equal percentages of RhoA proteins in the

aqueous phase and the detergent-enriched phase were analyzed by SDS-PAGE and western blot as described above.

Immunohistochemistry

Subconfluent MCF-10A and MDA-MB 435 cells plated on coverslips in serum-containing media for 24 hr were washed in PBS and treated with POH for 18 hours. Cells were then washed in PBS, fixed in 4% paraformaldehyde, permeabilized with 0.3% Triton X-100 PBS, labeled with primary mouse anti-human vinculin antibody and double stained with secondary rhodamine-conjugated goat anti-mouse IgG antibody and FITC phalloidin after 30 minutes of incubation. Stained cells were analyzed with a Zeiss confocal microscope.

Results

To investigate whether perillyl alcohol decreases the isoprenylated RhoA levels by down-regulating protein production rather than through inhibiting type 1 GGPTase, we measured RhoA levels in MCF-10A and MDA-MB 435 cells treated with perillyl alcohol by western blot. After 18 hours of treatment there was no difference in protein levels between treated and untreated cells (Fig. 4.2). There was a difference in RhoA protein expression between cell lines. The non-tumorigenic MCF-10A cells express more RhoA protein compared to the MDA-MB 435 cancer cells as determined by equal protein loading.

Since perillyl alcohol inhibits type 1 GGPTase, we hypothesized that it would change the ratio of prenylated and unprenylated forms of RhoA protein.

After separating proteins into aqueous (unprenylated) and detergent-enriched (prenylated) phases, proteins were detected by western blot. At 0 hours (treatment for 30 minutes) of exposure, all treatments with MCF-10A cells showed only the RhoA protein the detergent-enriched phase (Fig. 4.3). While at 18 hours of exposure, there was an accumulation of RhoA protein in the aqueous phase in the treated MCF-10A cells as compared to the untreated (Fig. 4.4). Reciprocally, a decrease of RhoA protein in the detergent-enriched phase was seen in the treated versus untreated MCF-10A cells (Fig. 4.4). For MDA-MB 435 cells at 0 hours of exposure, the aqueous phase contained RhoA protein when treated with perillyl alcohol, whereas in the same phase the untreated sample was void of the protein. At 18 hours of treatment, MDA-MB 435 cells continued to show an accumulation of RhoA protein in the aqueous phase for the perillyl alcohol treated cells as compared to the untreated cells (Fig. 4.5).

RhoA activated by serum growth factors can induce formation of stress fibers and focal adhesion complexes(16). As shown in figures 4.6 and 4.7, MCF-10A and MDA-MB 435 cells in the presence of serum-containing media had numerous stress fibers and focal adhesion complexes. When exposed to migration media alone for 18 hours, MCF-10A and MDA-MB 435 cells showed a slight decrease in the number of stress fibers and focal adhesion complexes (Fig. 4.6 and 4.7). However, after 18 hours of treatment with perillyl alcohol in migration media, MCF-10A and MDA-MB 435 cells lost their stress fibers and the majority of their focal adhesion complexes (Fig. 4.6 and 4.7), resulting in the

condensation of actin filaments and focal adhesion complex proteins within the center of the cells that is consistent with a loss of RhoA function.

Discussion

Our earlier work showed a decrease in cell migration for the nontumorigenic human breast cell line MCF-10A after exposure to 0.5 mM perillyl alcohol (POH). Previous studies suggest that perillyl alcohol inhibits protein isoprenylation in several malignant and non-malignant cell lines (5,17). Perillyl alcohol has been shown to inhibit the enzymes FPTase and types I and II GGPTase that catalyze the protein isoprenylation of small G-proteins (4). Inhibition of these three enzymes, however, has not been uniform; inconsistencies have been seen in the reduction of FPTase activity in different cell lines (4,18). Therefore, we looked for a possible link between the observed decrease in migration of MCF-10A cells and a change in activity of a small G-Protein. RhoA is a small GTPase that requires the addition of a 20-carbon isoprene group geranylgeranylpyrophosphate by the enzyme GGPTase for it to associate with the plasma membrane, where it takes place in migration signaling pathways (9).

Initially, to investigate the possibility that POH decreases the isoprenylated RhoA levels by down-regulating the protein rather than inhibiting type I GGPTase, we measured RhoA levels in POH-treated cells by western blot. Our results corroborate those of others that POH exposure does not change the

levels of RhoA in comparison to those in control cells (4). We also found that the human breast cancer cell line MDA-MB 435 expresses less RhoA protein in comparison to MCF-10A cells. This was not what we anticipated because MDA-MB 435 is a highly invasive breast cancer cell line, and it has been shown that other types of tumor cells express several times the amount of RhoA protein compared to non-malignant cells (19). Additionally, cells overexpressing RhoA protein exhibit increased motility (12).

To confirm an effect of perillyl alcohol on RhoA protein isoprenylation in our two cell lines, we addressed the translocation of RhoA protein from the plasma membrane to the cytosol. The unprenylated form of RhoA protein cannot associate with the plasma membrane (20); therefore, upon exposure to perillyl alcohol RhoA accumulates in the cytosol. After an 18-hour treatment and with 0.5 mM POH, RhoA accumulated in the aqueous phase and, reciprocally, the detergent-enriched phase showed a decrease in RhoA protein in MCF-10A cells. At the 0-hour timepoint (treatment for 30 minutes), in the MCF-10A cell line no RhoA protein is detected in the aqueous phase for POH-treated or untreated cells. This may be due to the cells recruiting RhoA to the plasma membrane as they reorganize their cytoskeletons to form focal adhesion complexes and stress fibers for adhering to the fibronectin-coated plate.

Unlike the aforementioned observation in MCF-10A cells, a deposition of RhoA protein in the cytosolic phase was measured for the 0-hour timepoint (treatment for 30 minutes) following treatment with POH in the MDA-MB 435 cells. After 18 hours of treatment with 0.3 mM POH, a small loss of RhoA protein

from the detergent-phase was seen in the breast cancer cells. With MDA-MB 435 cells expressing less RhoA protein, it is possible that the cells are more susceptible to the consequences of exposure to perillyl alcohol, even at subcytotoxic concentrations. Another scenario is that alternative pathways are being activated/inactivated by perillyl alcohol that lead to the increased ease by which RhoA is removed from the plasma membrane. It is likely that an apoptotic mechanism is at a low level of activation at this level of treatment; it has been reported that POH induces the expression of the proapoptotic protein Bak in pancreatic tumor cells, but does not in non-malignant pancreatic cells (21). It could also be that perillyl alcohol treatment is activating a different domain of an effector protein such as ROKα that leads to apoptosis in the MDA-MB 435 cells (22).

One effector protein of RhoA that is involved in a migration signaling pathway is ROKα. The kinase activity of ROKα is essential for it to promote the formation of stress fibers and focal adhesion complexes (22). In both MCF-10A and MDA-MB 435 cell lines morphological changes occurred after treatment with perillyl alcohol. We observed a loss of stress fibers and focal adhesion complexes following an 18-hour exposure in both cell lines. This may be due to a loss of activated ROKα: a downstream target in the Rho-dependent pathway of stress fiber and focal adhesion complex formation (22).

The assembly and disassembly of focal adhesion complexes and concomitant cell spreading have been implicated in migration activities of tumor cells (23). Treatment of MCF-10A cells with the monoterpene perillyl alcohol

blocked their migration. One mechanism of action of POH is the inhibition of isoprenylation of such protein as small G-proteins. RhoA is a small GTPase involved in migration signaling pathway. Our investigation into an alteration of RhoA activity following POH treatment yielded positive results for both translocation of RhoA to the cytosol and loss of its effect on downstream cytoskeletal components: focal adhesion complex and stress fiber formation. Further examination into the mechanism of the diminished migration in POH-treated MCF-10A cells would be to measure the ability of ROKα to phosphorylate its substrates such as myosin light chain. However, the difference observed in the MDA-MB 435 cells following POH-treatment warrants investigation into the possibility of induced expression of pro-cell death proteins such as Bak. This would coincide with previous findings and advance the understanding of how perillyl alcohol affects normal tissues.

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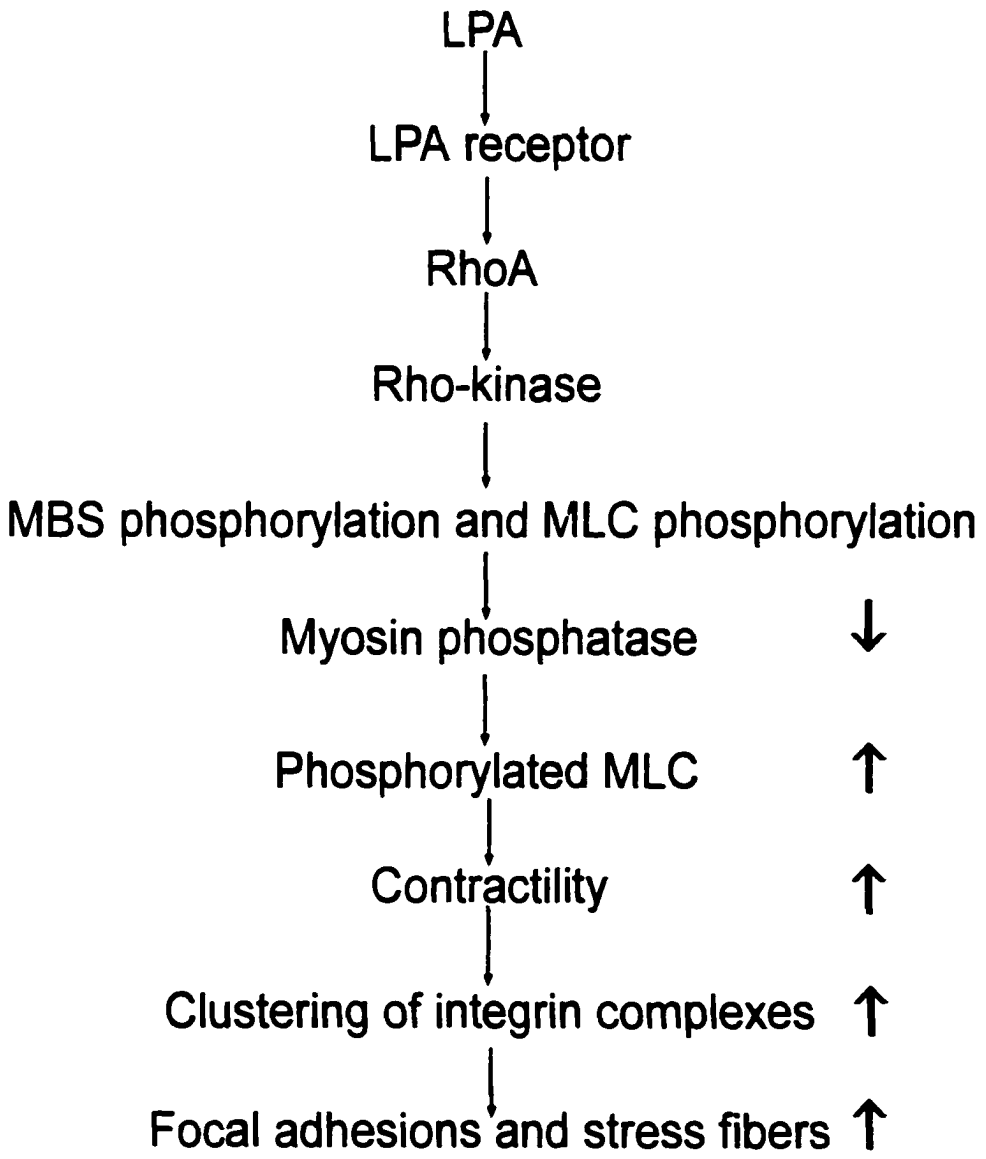

Figure 4.1. An established pathway for RhoA activation coupled with stress fiber and focal adhesion formation that induces cell migration. LPA; lysophosphatidic acid. MBS; myosin-binding subunit of myosin phosphatase. MCL; myosin light chain.

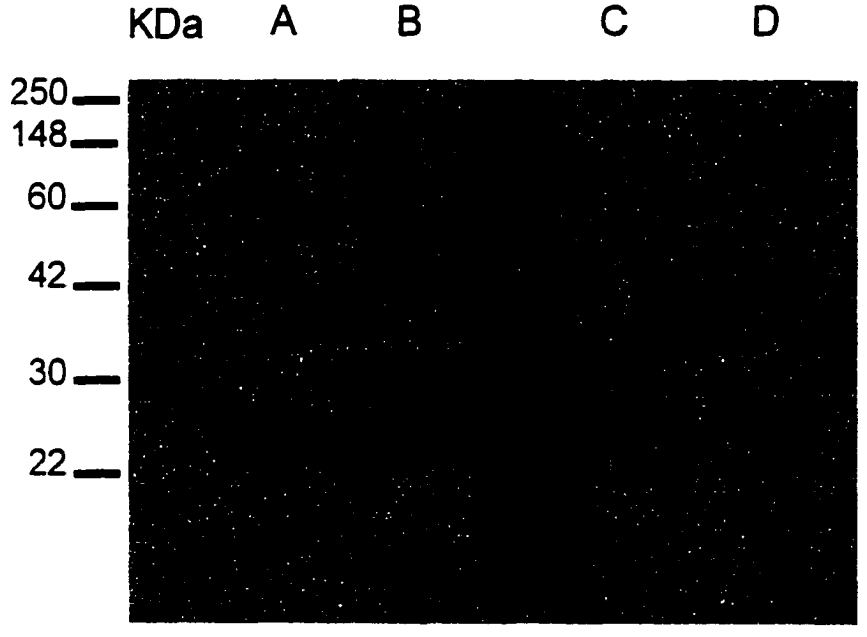

Figure 4.2. Following 18 hours of exposure to perillyl alcohol, RhoA protein levels in MCF-10A and MDA-MB 435 cells did not decrease, as measured by western blot. An equal amount of protein was loaded in each lane, as determined by a MicroBCA protein assay. A. MCF-10A with migration media. B. MCF-10A with migration media and 0.5 mM perillyl alcohol. C. MDA-MB 435 with migration media. D. MDA-MB 435 with migration media and 0.3 mM perillyl alcohol.

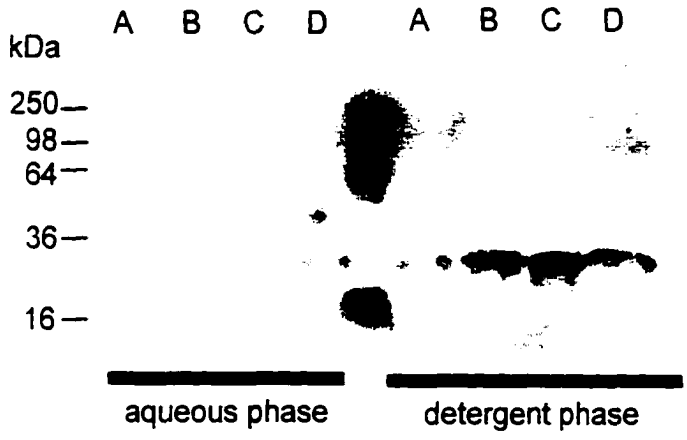

Figure 4.3. At 0 hours (treatment for 30 minutes) of exposure to 0.5 mM perillyl alcohol in MCF-10A cells, RhoA protein was found only in the detergent-enriched phase for all treatment conditions as measured by western blot. An equal percentage of overall volume for each sample was loaded. A. AM media and 0.5 mM perillyl alcohol. B. AM media. C. Migration media. D. Migration media and 0.5 mM perillyl alcohol.

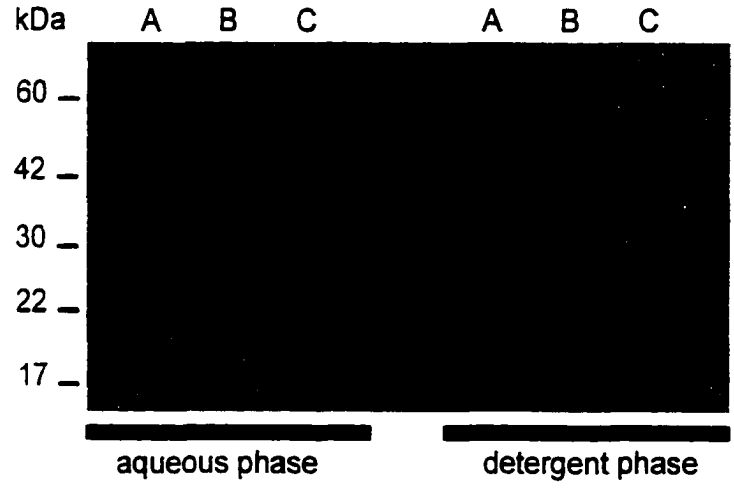

Figure 4.4. After an 18-hour incubation with 0.5 mM perillyl alcohol, RhoA protein in MCF-10A cells accumulated in the aqueous phase as determined by western blot. An equal percentage of overall volume was loaded for each sample. A. AM media. B. Migration media. C. Migration media and 0.5 mM perillyl alcohol.

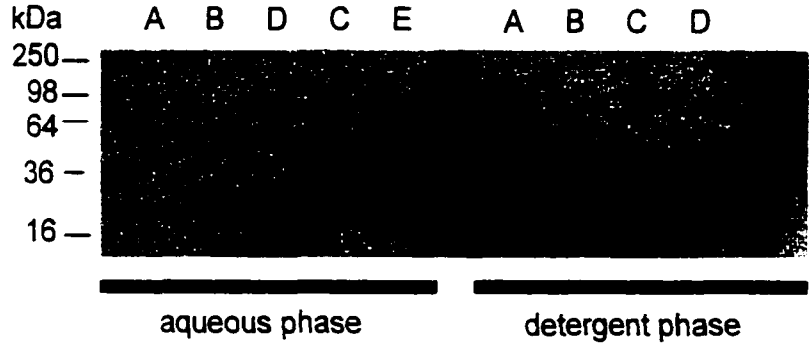

Figure 4.5. MDA-MB 435 cells showed an accumulation of RhoA protein in the aqueous phase after 0 (treatment for 30-minutes) and 18 hours of exposure to 0.3 mM perillyl alcohol as measured by western blot. An equal percentage of overall volume was loaded for each sample. A. Migration media, 0 hours. B. Migration media and 0.3 mM perillyl alcohol, 0 hours. C. Migration media, 18 hours. D. Migration media and 0.3 mM perillyl alcohol, 18 hours. E. Whole cell lysate in migration media, 18 hours.

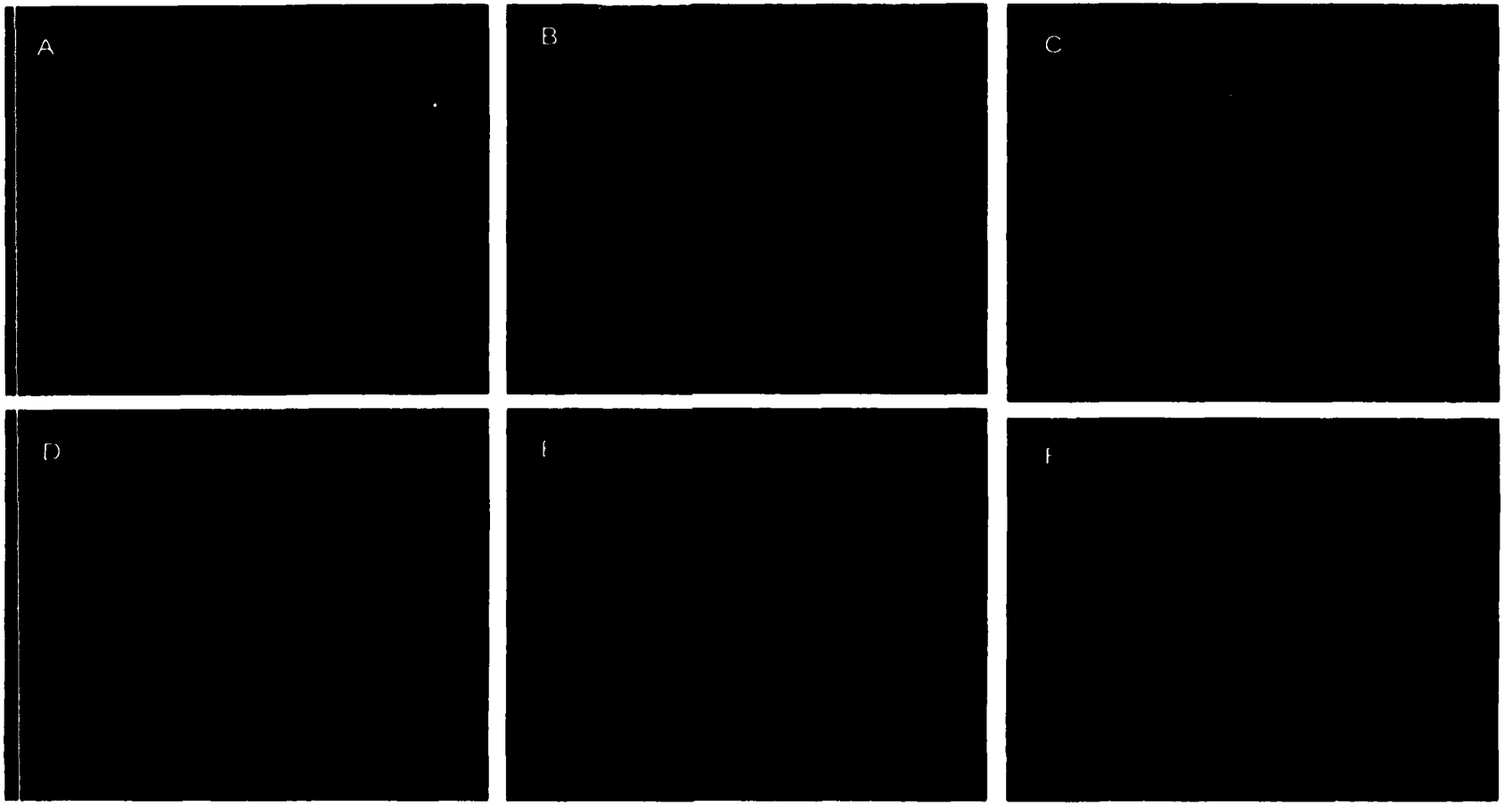

Figure 4.6. After 18 hours, MCF-10A cells grown in AM media (A and D) show extensive actin stress fibers and numerous focal adhesion complexes throughout the cell body and at the cell periphery. Migration media (B and E) reduced the number of both focal adhesions and stress fibers while 0.5 mM perillyl alcohol (C and F) eliminated most. Cells were stained with phalloidin (A-C) and anti-vinculin antibody (D-F) for actin stress fibers and focal adhesion complexes, respectively.

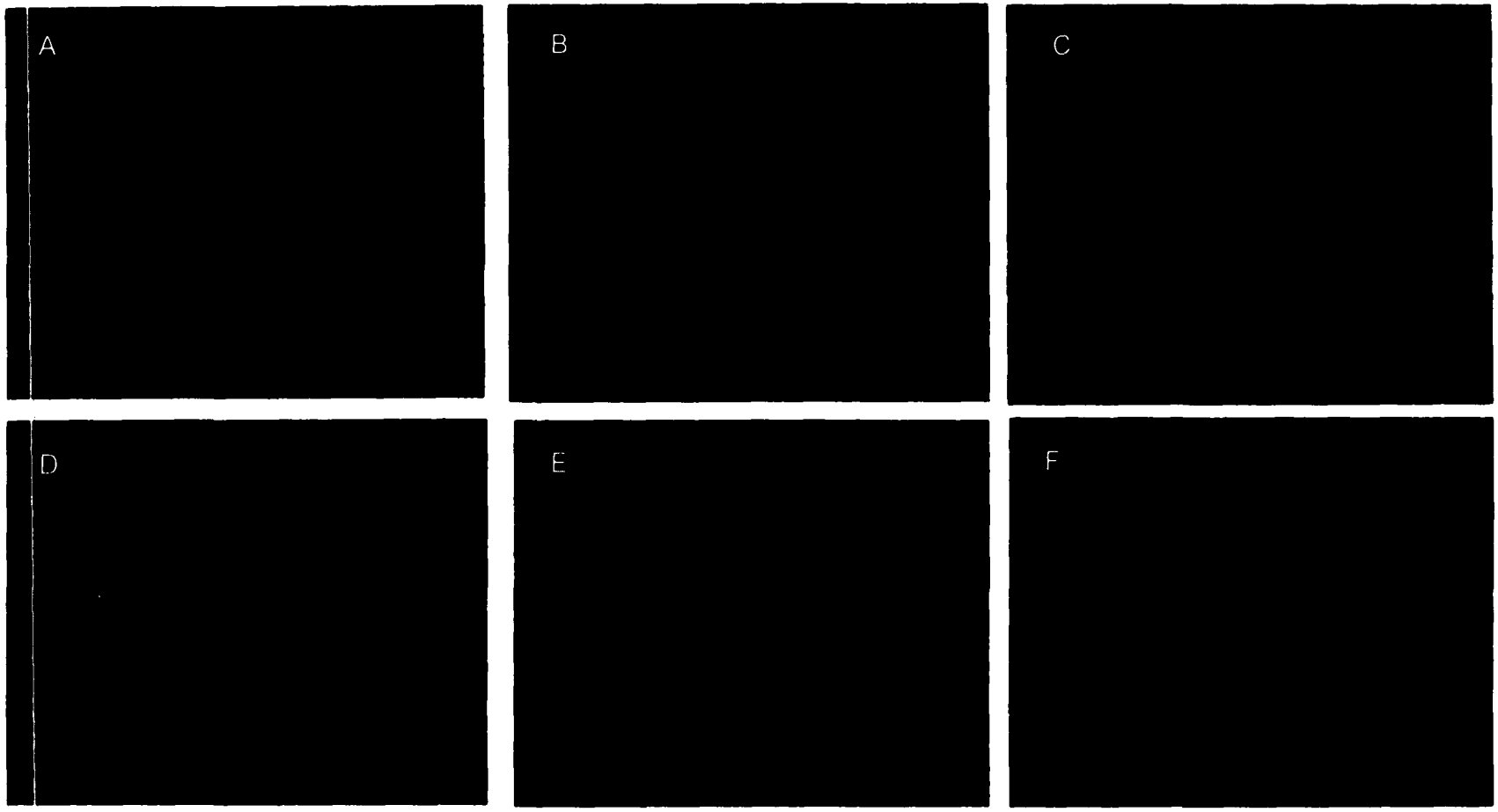

Figure 4.7. After 18 hours, MDA-MB 435 cells grown in media with serum (A and D) show extensive actin stress fibers and numerous focal adhesion complexes throughout the cell body and at the cell periphery. Migration media (B and E) reduced stress fibers and focal adhesions while 0.3 mM perillyl alcohol (C and F) eliminated most. Cells were stained with phalloidin (A-C) and anti-vinculin antibody (D-F) for actin stress fibers and focal adhesion complexes, respectively.

CHAPTER 5

GENERAL DISCUSSION

The process of metastasis is both multi-faceted and progressive. Multiple aspects of cell physiology must be appreciated in order to gain a sense both of the opportunities for therapeutic intervention and the challenges of actually making clinical use of any new anti-metastatic therapies. Cell migration and invasion into the surrounding tissue are major characteristics of malignant tumors. Their migratory potential is a major obstacle to effective surgical therapy. The mechanism by which cancer cells migrate and consequently invade and extravasate, are not fully understood. Alterations in the expression of growth factor receptors (1), changes in the extracellular matrix-cell interactions (2) and signal transduction pathways (3) are likely to be of importance.

Drugs that interact with the locomotive apparatus of malignant cells are of great interest in cancer therapy. The discovery of drugs with anti-migratory properties has been hampered by the lack of adequate technology that can quickly and accurately measure cell migration. Our lab's new 96-well migration plate, a tool for measuring cell migration and cell death, along with our migration assay can be used to screen for compounds that inhibit tumor cell migration (4). In this study, we define the conditions necessary for optimizing our high-

throughput assay to screen anti-cancer compounds for their ability to stop tumor cell migration of established cell lines of the National Cancer Institute *in vitro*.

We determined that the concentration of dimethyl sulfoxide (DMSO) that induces cell death is greater than the concentration that interferes with cell migration of the four cell lines. Our interest in delineating these effects of DMSO is due to the likelihood that DMSO will be a solvent used in drug packaging by such institutions as the National Cancer Institute: a major repository for anti-cancer compounds. Additionally, our results confirm that this assay protocol is capable of identifying non-cytotoxic, anti-migratory compounds (4). Importantly, we demonstrate that it is possible to separate the effect of DMSO from that of an anti-cancer drug for inhibiting cell migration.

Another parameter for optimization was selecting the preferred extracellular matrix (ECM) protein for migration in our four cell lines. Matrix proteins contribute to metastasis by stimulating tumor cell motility (5-9). Of these proteins we tested vitronectin, fibronectin, laminin and type IV collagen, finding that our four cell lines all maximally migrate on fibronectin; thus, simplifying the migration assay for a drug screen. These proteins stimulate chemotaxis (motility toward a chemical gradient) and haptotaxis (motility toward a bound substrate). The ability of ECM proteins to activate haptotaxis in tumor cells indicates that the intact ECM proteins may provide a path for the migration of tumor cells.

The relative number of cells needed to evenly cover the migration filter was determined. Ensuring that the seeded cells form a monolayer on the filter for the migration assay is necessary to mimic *in vivo* conditions. Of the four cell

lines. the small cell lung carcinoma (NCI-H460) cells required approximately a third more cells than the other cell lines to form a monolayer.

Compilation of these parameters for optimizing our migration assay for a large-scale drug screen signifies that we are ready to proceed. It can reasonably be concluded that we are able to discern between the cytotoxicity of DMSO and its effect on cell migration in our assay. With the common use of DMSO as drug solvent, we should be able to apply our plate and assay to screen repositories of anti-cancer drugs such as that held by the National Cancer Institute.

To apply our optimized conditions for screening anti-cancer agents for anti-migration properties, we chose to investigate the monoterpenes limonene, perillyl alcohol and perillaldehyde. We found that these monoterpenes varied in cytotoxicity. The induced cell death of MCF-10A non-tumorigenic breast cells by perillaldehyde may be attributable to its increased affinity for the enzymes farnesyl protein transferase (FPTase) and geranylgeranyl protein transferase (GGPTase) as compared to that of perillyl alcohol (POH) and limonene. Prior research has shown that the metabolite perillic acid of limonene and POH is much more effective in blocking FPTase and GGPTase (10). These enzymes are involved in post-translational modification of small G-proteins, which impact a plethora of cell activities such as growth, migration and apoptosis. The anti-tumor effects of limonene and POH are suspected to be based on their inhibition of FPTase and GGPTase (10).

However, there is a disparity between equal concentrations of perillyl alcohol and limonene and their effects on cell proliferation (10-11). We found a

decrease in POH-treated MCF-10A cell migration at non-cytotoxic levels. This was not attained for limonene. The most likely reason is that it is limonene metabolites, perillic acid and perillic acid methyl ester, that inhibit small G-protein isoprenylation effectively and not limonene (11-12).

A probable mediator of this inhibited migration is the small G-protein RhoA, a member of the Rho family of small GTPases that is involved in a cell migration signaling pathway (13-14). Interfering with the isoprenylation of RhoA would effectively block activation of Rho-kinase (ROKα), one of its downstream effector proteins (15). Overall this would deplete the cell of stress fibers and focal adhesion complexes—necessary components for contraction in cell migration (16).

Unlike migration, adhesion was much less affected by POH treatment in both MCF-10A and MDA-MB 435 cell lines. The requirements for adhering are less demanding on the cell than migrating. Turnover of focal adhesion complexes and remodeling of the plasma membrane are not required for adhesion. Thus, it is possible that after POH-treatment, the cells retain the capacity to bind to a fibronectin-coated surface, but not are not able to migrate through it.

To further assess POH as a possible inhibitor of cancer cell migration, we tested it in the human breast cancer cell line MDA-MB 435. Finding that POH could not inhibit migration without also being cytotoxic in this cell line was surprising. Previous research has shown that other tumor cell lines express more RhoA compared to non-tumorigenic cells (17). Additionally, an increased

level of expression of RhoA has been associated with an invasive phenotype (17-18), and MDA-MB 435 is a highly motile cell line. However, our finding that POH is more cytotoxic in this cancer cell line compared to the non-tumorigenic one is supported by earlier research. Stayrook, et al. (19) found that POH significantly increased apoptosis in pancreatic cancer cells relative to non-malignant pancreas cells. Therefore, it is conceivable that POH is affecting a different mechanism in MDA-MB cells than in MCF-10A cells, which may be attributable to changes in RhoA function.

We initially investigated the effect of POH on RhoA by measuring the RhoA protein level in POH-treated cells to verify that its expression was not down-regulated. Our results support prior findings that RhoA protein expression is not altered by POH exposure (13). However, contrary to our assumptions, MDA-MB 435 cells express less RhoA than MCF-10A cells. Although, it has also been published that excessive RhoA activity in rat fibroblasts inhibits migration probably through the formation of strong focal adhesions (14).

To confirm an effect of perillyl alcohol on RhoA protein isoprenylation in our two cell lines, we addressed the translocation of RhoA protein from the plasma membrane to the cytosol. It is the isoprenylated form of RhoA that is capable of activating downstream targets such as Rho-kinase. Our finding with the MCF-10A cell line supports our hypothesis. After 18-hours of exposure, RhoA protein accumulates in the aqueous phase with a reciprocal depletion from the detergent-enriched phase. A difference was observed at the 0-hour (treatment for 30 minutes) timepoint; treated cells did not have a detectable

amount of RhoA protein in the aqueous phase. After one hour of adhering to the plate surface, the cells were still not spread out, and therefore, RhoA may be recruited to the plasma membrane in an effort to reestablish stress fiber and focal adhesion complex formation.

In the MDA-MB 435 cell line translocation of RhoA to the aqueous phase was measured at the 0-hour timepoint although their appearance was similar to the MCF-10A cells. It may be that the lower expression of RhoA in this breast cancer cell line makes them more susceptible to the effects of perillyl alcohol treatment, even at a subcytotoxic dose. The increased sensitivity of the MDA-MB 435 cell line to POH suggests that it may experience more adverse effects with POH-treatment due to activation a pro-apoptosis pathway (20-21). Another study showed that expression of the pro-apoptotic protein Bak was induced by perillyl alcohol in pancreatic tumor cells, but was innocuous in non-malignant pancreas cells (19).

One target protein of activated RhoA is Rho-kinase. Phosphorylation of this serine/threonine kinase leads to the generation of stress fibers and focal adhesion complexes that provide the cell with contractile forces needed for migration (15). Following treatment with POH, we observed considerable morphological changes in both cell lines. Cells were round in appearance owing to their loss of stress fibers and focal adhesions; a possible manifestation due to the loss of Rho-kinase activity (15). From this point it is valid to advocate continued examination of the activity of Rho-kinase through more direct methods.

Ideally, following perillyl alcohol treatment the activity of Rho-kinase should be measured through substrate phosphorylation.

It is known that the assembly and disassembly of focal adhesion complexes is integral to migration activities of tumor cells (22). Perillyl alcohol treatment in MCF-10A cells effectively inhibited their migration. Blocking the isoprenylation of small G-proteins is an established effect of perillyl alcohol. The small GTPase RhoA is involved in a migration signaling pathway. We were able to show that following POH-treatment RhoA accumulated in the aqueous phase and that morphological changes occurred. These cells lost stress fibers and focal adhesion complexes indicative of a loss of RhoA function. However, what remains to be clarified is why this loss of isoprenylated proteins impacts different cell types in diverse ways as seen in our study with non-tumorigenic breast cells and malignant breast cells. Perhaps the intracellular level of the geranylgeranyl diphosphate, the substrate for GGPTase, is much lower in contrast to the much higher intracellular farneslyl diphosphate concentration (23) and with further variation depending on the cell type (13). If this is true, then perillyl alcohol could effectively compete with the endogenous substrate for GGPTase I but not FPTase. Oncogenic Ras proteins are present in 30% of all human cancers, and normal and oncogenic Ras are substrates for isoprenylation by FPTase (24). This may partially explain the observed difference between our non-malignant breast cells and breast cancer cells following perillyl alcohol treatment.

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