Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2006, Article ID 87246, Pages 1–10
DOI 10.1155/JBB/2006/87246

*Research Article*

# Apocynin Derivatives Interrupt Intracellular Signaling Resulting in Decreased Migration in Breast Cancer Cells

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Received 8 September 2005; Accepted 13 October 2005

Cancer cells are defined by their ability to divide uncontrollably and metastasize to secondary sites in the body. Consequently, tumor cell migration represents a promising target for anticancer drug development. Using our high-throughput cell migration assay, we have screened several classes of compounds for noncytotoxic tumor cell migration inhibiting activity. One such compound, apocynin (4-acetovanillone), is oxidized by peroxidases to yield a variety of oligophenolic and quinone-type compounds that are recognized inhibitors of NADPH oxidase and may be inhibitors of the small G protein Rac1 that controls cell migration. We report here that while apocynin itself is not effective, apocynin derivatives inhibit migration of the breast cancer cell line MDA-MB-435 at subtoxic concentrations; the migration of nonmalignant MCF10A breast cells is unaffected. These compounds also cause a significant rearrangement of the actin cytoskeleton, cell rounding, and decreased levels of active Rac1 and its related G protein Cdc42. These results may suggest a promising new route to the development of novel anticancer therapeutics.



## INTRODUCTION

Cancer remains the second highest cause of death in the US [1, 2]. Unlike primary tumors that can be surgically removed and treated with adjuvant chemotherapy and/or radiotherapy, secondary tumors (metastases) are difficult to treat because metastatic tumor cells disseminate throughout the body, making them almost impossible to target. While the majority of anticancer drugs target the hyperproliferation of metastatic cells and are efficacious in treating the beginning stages of cancer, none are curative for metastatic disease [3, 4]. Many of these drugs are ineffective if the cancer is not treated immediately and may prove toxic to healthy tissue. Cytotoxic anticancer drugs also generate a variety of adverse side effects, including nausea, vomiting, suppressed immune system, and hair loss [5].

Nontoxic inhibitors of cancer cell migration are therefore an attractive new class of potential anticancer drugs, offering the promise that potentially malignant tumors could be confined to their tissue of origin through multiple rounds of traditional adjuvant therapy. However, identifying such compounds is complicated by the highly complex and tightly controlled cell migration process [6–8]. Migrating cells use proteolytic enzymes to digest "holes" in the surrounding ECM, and then extend cytoplasmic projections (pseudopodia) from the cell body in the direction of migration, forming a "leading edge," behind which the remainder of the cell follows [9]. Extension and contraction of pseudopodia occur in a cyclic pattern, giving rise to the typical "crawling" behavior of moving cells. Pseudopodia are enriched in proteins thought to control the direction and rate of cell migration [10]. These include extracellular proteases, extracellular matrix receptors (eg, integrins) and adapter proteins that link these receptors to the actomyosin cytoskeleton, as well as numerous signaling molecules, including GTPases (Rac1, Cdc42, RhoA) that control the assembly and activation of this cytoskeleton [11]. However, how these molecules work to choreograph the sequential rearrangement of cytoskeletal elements during cell migration is not well understood.

The number of known compounds that specifically inhibit this cyclical process is likewise very low. To address this issue, we have developed an automated high-throughput screening assay for identifying nontoxic inhibitors of cancer cell migration. We have previously used

this assay to characterize the antimigratory behavior of carboxyaminoimidazole, perillyl alcohol, and tamoxifen on breast cancer cells [12, 13]. Having illustrated the nontoxic effects of these well-known compounds on cancer cell migration, we have now turned our attention to identifying new, previously unidentified inhibitors of tumor cell migration.

Natural products present a potentially rich source for novel anticancer drugs. Plants, in particular, are repositories of biodiversity, and therefore, represent a source of many medicines. Several therapeutic cancer treatments have been derived from compounds found in plants (eg, taxol, paclitaxel, perillyl alcohol) [14, 15]. Apocynin, obtained from the roots of *Picrorhiza kurroa*, is another potential anticancer compound. Its rhizomes have been used in oriental traditional medicine for thousands of years, treating a variety of diseases of the liver and lungs [16]. Apocynin disrupts the assembly of the NADPH oxidase complex, which includes the same Rac1 protein that regulates the actin cytoskeleton during cell migration [17, 18]. The possible link between apocynin and Rac1 inhibition suggests that apocynin may be a source for inhibitors of Rac1-mediated tumor cell migration. In this study, we report the application of an in vitro screening assay to identify apocynin-derived inhibitors of Rac1-based tumor cell migration.

## MATERIALS AND METHODS

Tissue culture media (DMEM, RPMI) and penicillin G-streptomycin sulfate (GPS) were purchased from Mediatech (Cellgro, Va). Fetal bovine serum (FBS) was purchased from Gemini Bio-Products (Woodland, Calif). Trypsin-EDTA was obtained from Sigma Chemical Co (St Louis, Mo). F-actin/G-actin in vivo assay kit (BK037) was obtained from Cytoskeleton Inc (Denver, Colo). PAK-1 PBD agarose beads were obtained from Upstate Cell Signaling (Lake Placid, NY). Mouse monoclonal IgG2b antibody against Rac1 (Cat# 610650) and mouse monoclonal IgG1 antibody against Cdc42 (Cat# 610928) were purchased from BD Transduction Laboratories (San Diego, Calif). Horseradish peroxidase (HRP)-conjugated goat antimouse IgG and HRP-conjugated goat antirabbit IgG secondary antibodies were obtained from Jackson Immuno Research (West Grove, Pa). The protein assay kit was purchased from Pierce (Rockford, Ill). MTT and apocynin were purchased from Sigma. Phalloidin and calcein AM were purchased from Molecular Probes (Eugene, Ore). MIC plates were generously donated by Millipore (Danvers, Mass). Unless otherwise specified, the other standard reagents were obtained from Fisher Scientific (Fair Lawn, NJ).

### *Synthesis of compounds*

Apocynin (1 g) was dissolved in 5 ml dimethylformamide and transferred to 490 ml phosphate buffer (20 mM, pH 7) to obtain a concentration of 12 mM apocynin. To this solution, 5 ml of a 1 mg/ml soybean peroxidase (SBP) solution in aqueous buffer were added. The reaction vessel was wrapped in aluminum foil and the solution was magnetically stirred at room temperature. The reaction was initiated by the addition of a concentrated $H_2O_2$ solution (30% w/v), added continuously via syringe pump at 0.1 ml/h for 12 hours) resulting in a total of 20 mM $H_2O_2$ fed to the reaction. The resulting precipitate was collected by centrifugation in 50 ml conical centrifugation tubes at 3000 rpm for 15 minutes, and washed three times with DI water. The individual pellets were pooled, transferred to 1.5 ml microcentrifuge tubes, and dried under vacuum. All compounds except A5 were synthesized at pH 7-8 and collected after the first wash. Compound A5 was synthesized at pH 5 and collected similarly.

### *Cell culture*

Cryopreserved MDA-MB 435 breast cancer cells were obtained from the ATCC (Manassas, Va) and were grown according to the manufacturers' instructions. Briefly, cells were plated at $5 \times 10^3$ cells/cm$^2$ in a T75 flask (75 cm$^2$) for continuous passaging in RPMI medium supplemented with 10% FBS, 1% L-glutamine [29.2 mg/mL], penicillin G [10,000 U/mL], and streptomycin sulfate [10,000 $\mu$g/mL]. Medium was changed twice weekly and cells were detached by trypsin-EDTA and passaged into fresh culture flasks at a ratio of 1 : 10 upon reaching confluence. Cultures were incubated at 37°C in a humidified atmosphere containing 95% air and 5% $CO_2$.

### *Adhesion assays*

Cell adhesion assays were performed as previously described using Sarstedt 96-well suspension cell culture plates [19]. Tissue culture plates were coated with purified fibronectin at a concentration of 20 $\mu$g/mL for 1 hour at room temperature. Wells were washed twice with PBS and incubated with nd-blotto (5% nondairy creamer in PBS + 0.2% Tween 20) for 30 minutes prior to the assay. Cells were allowed to attach for 30 minutes at 37°C in the presence of either 203 ng/$\mu$l, 36 ng/$\mu$l, or 3.6 ng/$\mu$l per well of each of the four compounds, or their respective vehicle controls. Cells were subsequently fixed with 3% paraformaldehyde, washed twice in PBS, and incubated in crystal violet dye for 15 minutes. Wells were washed thoroughly with water and the violet dye was extracted with 10% SDS solution. Absorbance was measured using a TECAN SPECTAFluor spectrophotometer at 595 nm and relative adhesion was compared to cells attached to nd-blotto.

### *Migration assays*

Cell migration assays were performed using 8 $\mu$m MIC plates. Control filters were coated with purified fibronectin at a

concentration of 20 $\mu$g/ml or nd-blotto for 1 hour at room temperature prior to assay. Basal chambers for the nd-blotto wells were filled with migration medium (DMEM + 1% sodium pyruvate + 1X GPS) while the basal chambers for the remaining wells were filled with control medium. Cell suspensions in migration medium were seeded at a density of $5 \times 10^3$ cells per well. One lane of cells was left untreated while the rest were given either one of the four compounds at final concentrations of either 203 ng/$\mu$l, 36 ng/$\mu$l, or 3.6 ng/$\mu$l per well, or their respective vehicle control. Migrations were allowed to run for 18 hours at 37°C. Filters were then incubated for 30 minutes with 5 $\mu$M calcein AM and washed thoroughly with PBS. Residual cells were swabbed from the top of the wells to avoid false readings. To quantitate migration, plates were read at 485Ex/535Em with a TECAN SPECTAFluor spectrophotometer. Relative fluorescence values for each experimental condition were expressed relative to FN and nd-blotto controls.

### *Viability*

Cell viability assays were performed using Sarstedt 96-well suspension cell culture plates. Cells were plated at a density of $5 \times 10^3$ cells per well in a half and half mixture of migration medium and control medium. One lane was left untreated as a positive control while the remaining lanes were treated as per the migration plates with the compounds and vehicles. After aspirating the medium, cells were supplied with fresh medium containing 5 mg/ml of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and incubated for 4 hours at 37°C. Medium was again aspirated and 100 $\mu$l of 10% SDS was added to each well. Plates were placed back in the incubator for 2 hours and absorbance was measured using a TECAN SPECTAFluor spectrophotometer at 570 nm. Viability was expressed relative to the untreated control.

### *Immunohistochemistry*

MDA-MB 435 cells were grown on glass cover slips coated with 20 $\mu$g/ml of fibronectin for 4 hours in the presence of one of the four compounds at a final concentration of 15.2 ng/$\mu$l or the respective ethanol vehicle control. Cells were then fixed with 3% paraformaldehyde for 30 minutes and washed thoroughly with PBS. Cover slips were blocked with PBS + 1% BSA for 30 minutes and again washed thoroughly with PBS. TRITC conjugated phalloidin was incubated with the cover slips at a 1 : 200 concentration in blocking solution for 1 hour at room temperature. Cover slips were mounted using Prolong antifade medium (Molecular Probes). Cells were visualized with a Nikon TE2000-S inverted fluorescence/phase contrast microscope equipped with a digital SPOT camera.

### *Actin isolation*

Actin was isolated using an F-actin/G-actin in vivo assay kit. Briefly, cells were plated on four separate 25 cm tissue culture plates which were coated with fibronectin for 4 hours at a concentration of $30 \times 10^6$ cells per plate. Compounds 5 and 9 were each added to one of the plates at a final concentration of 36 ng/$\mu$l. The other plates were used as an untreated condition and vehicle control. Cells were grown for 4 hours and harvested in approximately 1 ml of warm LAS2 buffer (LAS1 stabilization buffer with 100 mM ATP and protease inhibitors). Cell lysates were homogenized using a 25 G needle and incubated at 37°C for 10 minutes, followed by centrifugation at 100,000 x*g* for 60 minutes at 37°C. Supernatants containing G-actin were immediately removed and the pellets containing the F-actin were dissociated using cytochalasin-D in ice cold $dH_2O$.

### *Rac1/Cdc42 isolation*

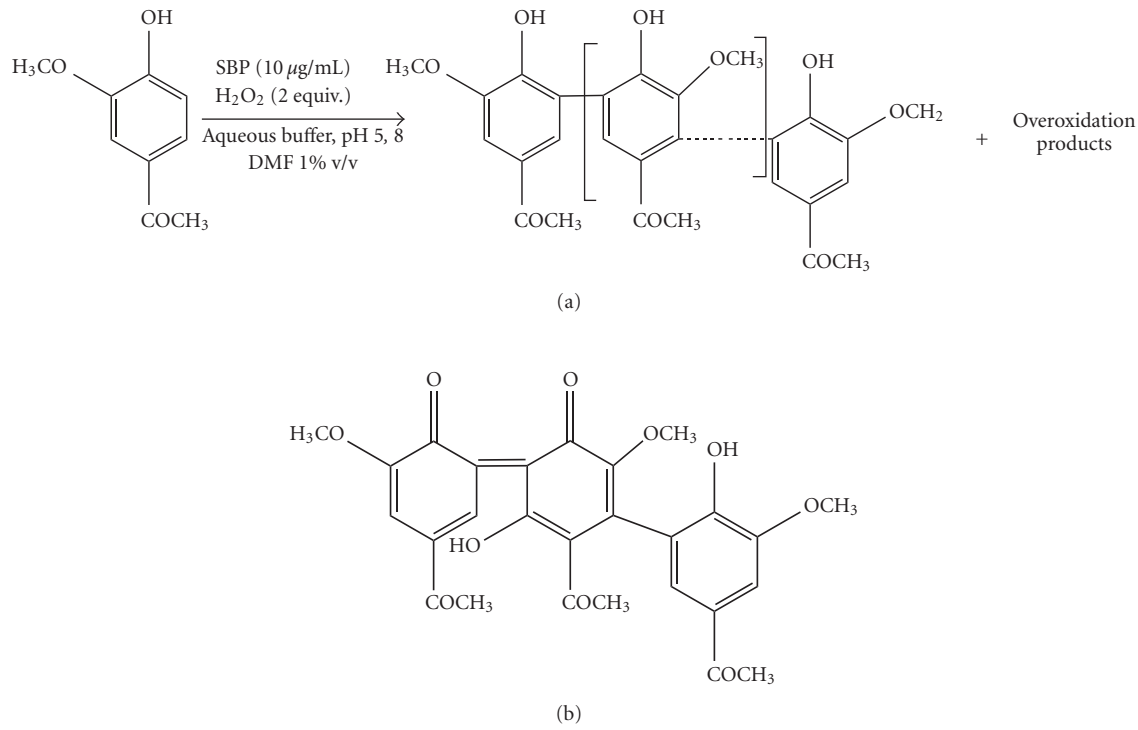
Rac1 and Cdc42 isolation was done using PAK-1 PBD agarose beads. Briefly, treated and untreated cells were lysed with 1 ml of MLB (magnesium-containing lysis buffer) (25 mM HEPES pH 7.5, 150 mM NaCl, 1% NP-40, 10% glycerol, 25 mM NaF, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM sodium orthovanadate, protease inhibitors). PAK-1 agarose (30 $\mu$l) was added to each lysate and agitated for 1 hour at 4°C. Beads were collected by centrifugation and the supernatant discarded. Beads were then washed three times with MLB.

### *Western blotting*

Western blots were performed on actin lysates and Rac1/Cdc42 lysates. Once protein concentrations were determined using a Pierce micro-BCA kit, lysates were suspended in Laemmli sample buffer and resolved on a 10% SDS-PAGE gel. The gels were then electrophoretically transblotted to Trans-Blot nitrocellulose membranes (0.2 $\mu$m) (Bio-Rad, Hercules, Calif). The membranes were incubated with blocking solution (5% nonfat dried milk in 1X PBS + 0.2% Tween-20 (PBST)) for 1 hour, then probed with either a G-actin antibody (1 : 1000), Rac1 antibody (1 : 250), or a Cdc42 antibody (1 : 250) for 2 hours at room temperature. After three washes with PBST, membranes were incubated with HRP-conjugated secondary IgG (1 : 25, 000) for 1 hour, followed by another three washes with PBST. Immunoreactive bands were detected using the SuperSignal chemiluminescent reagent (Pierce) and quantitatively analyzed by normalizing band intensities to the controls on scanned films by IMAGEJ software.

### *Statistical analysis*

All experiments were repeated a minimum of two times and the representative data were presented as mean ± SE.




Scheme 1: (a) Typical products obtained by the SBP catalyzed oxidation of apocynin. (b) Probable structure of the major product obtained from the SBP catalyzed oxidation of apocynin at pH 8 that had a significant inhibition on cancer cell migration.

Statistical analyses were preformed using Student's unpaired $t$ test, and a $p$ value less than 0.05 was considered significant.

## RESULTS

### *A subset of apocynin derivatives inhibit cancer cell migration at nonlethal doses*

We employed our high-throughput assay to determine if apocynin and its derivatives had a physiological effect on cancer cell migration. Apocynin itself had no substantial effect on migration, nor did other similarly structured compounds and their dimers, such as vanillin (data not shown). Of the nearly 100 compounds screened, only a handful yielded any significant inhibition of migration. Peroxidase catalyzed oxidation of apocynin leads to a mixture of products (Scheme 1(a)); however, at pH 8 the major oxidation product is a trimeric hydroxylated quinone of m/z 508[19a] (Scheme 1(b)). Results from a representative ineffective apocynin derivative, A5, are shown in Figure 1(a). At sublethal doses, the effective compounds blocked migration by at least 40%, and as high as 80% (Figures 1(b)–1(d)); at higher, toxic doses, migration was completely inhibited as expected (not shown). The inhibition of cell migration seen with the mixture of apocynin oxidation products at pH 8 could be predominantly due to the trimer hydroxylated quinone.

### *Apocynin derivatives inhibit migration at the level of the actin cytoskeleton*

To address the possible mechanisms underlying this nontoxic migration inhibition activity, we further characterized the cells treated with these compounds. In 30-minutes-adhesion assays, the active compounds had no appreciable inhibitory effect on cell adhesion to fibronectin as compared to the controls (Figure 2). The slight decreases seen in adhesion caused by compounds A5, A8, and 9 correlated to the minor levels of cell death observed in Figure 1. Staining with fluorescently tagged phalloidin revealed that, unlike control MDA-MB 435 cancer cells, which spread and formed well-defined actin stress fibers when plated on fibronectin (Figure 3, panels (a) and (b)), cells treated with the migration inhibiting drugs (compound A8, Figure 3, panel (c); compound 9, panel (e)) failed to organize their filamentous actin (F-actin) cytoskeleton and remained round. Cells treated with the ineffective compound A5 (Figure 3, panel (d)) spread well and organized distinct stress fibers as seen in controls. These changes in actin organization were likewise reflected in western blots for monomeric actin (G-actin) and F-actin in treated and untreated cell lysates (Figure 4): cells treated with active compounds 5 and 9 contained approximately 50% less F-actin compared to control cells, while the amount of G-actin was comparable in all conditions.

Small G-proteins are well-known moderators of the actin cytoskeleton. To test their possible sensitivity to these

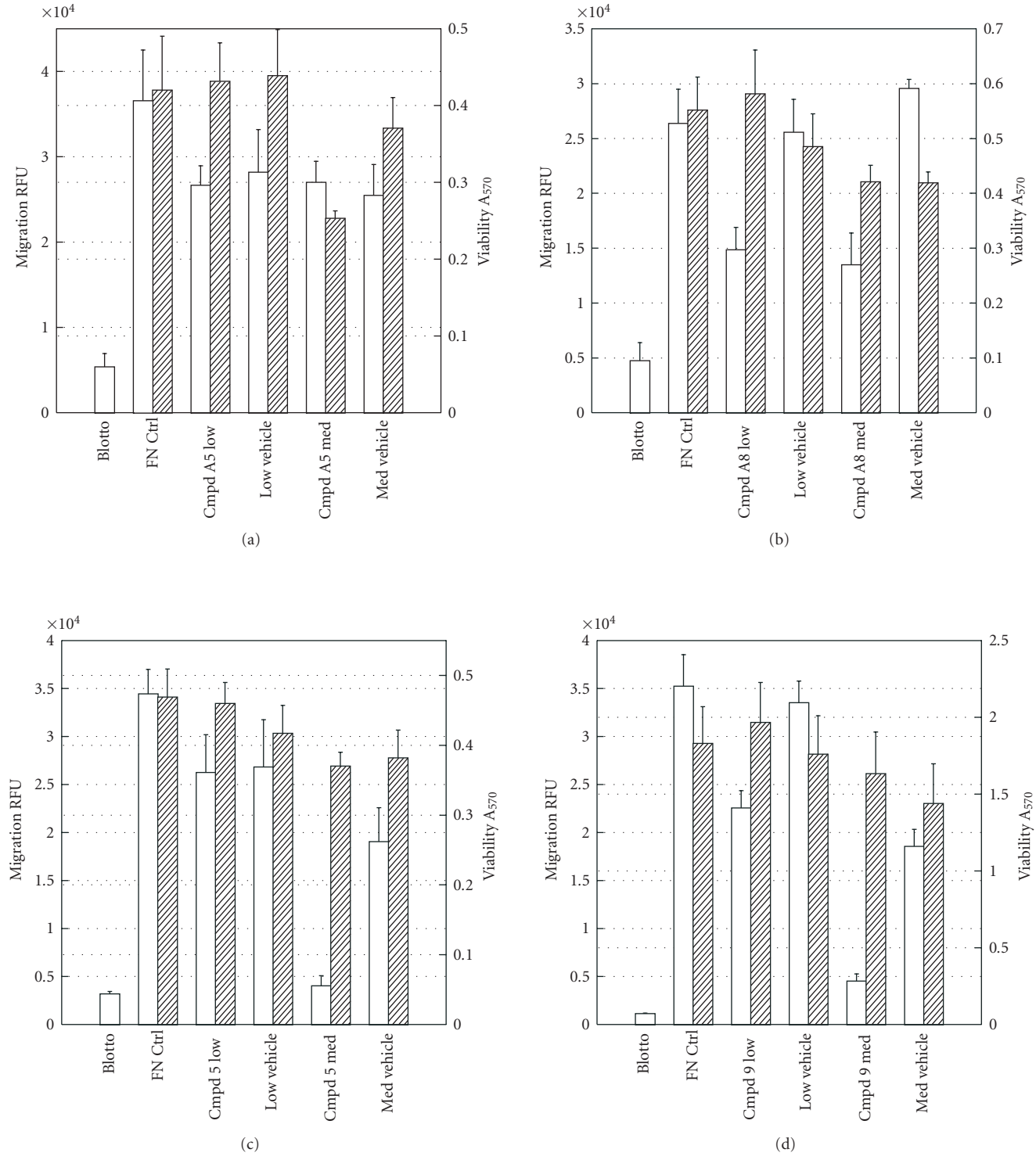



Figure 1: Migration (solid) and viability (shaded) assays of MDA-MB 435 breast cancer cells in the presence or absence of compound A5 (panel (a)), compound A8 (panel (b)), compound 5 (panel (c)), and compound 9 (panel (d)). Migration studies were performed for 18 hours using Millipore MIC plates, and cells were stained with calcein AM. Viability was assessed by utilizing an MTT assay and measuring absorbance at 570 nm. Three concentrations of each compound were used; [low] = 3.6 ng/$\mu$l, [med] = 36 ng/$\mu$l, and [high] = 203 ng/$\mu$ l. Data not shown for high concentration.

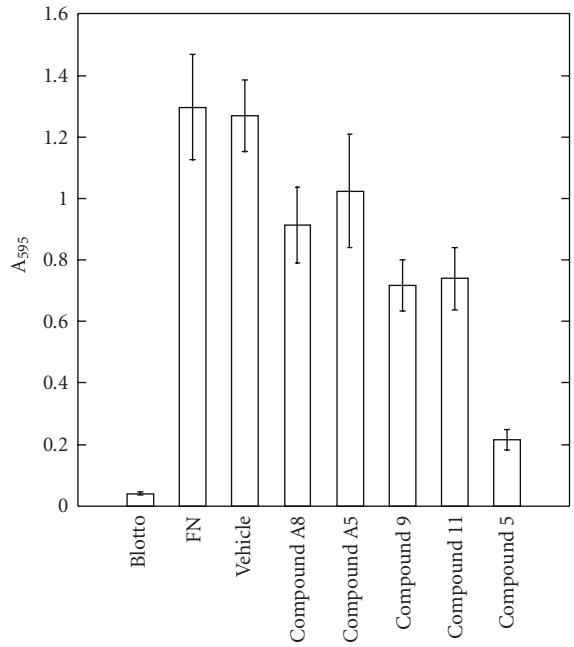



Figure 2: Static 5 hours adhesion of MDA-MB 435 breast cancer cells to purified fibronectin in the presence and absence of test compounds. Concentrations used were 36 ng/$\mu$l. Adherent cells were stained with crystal violet, solubilized in 1% SDS, and absorbance determined at 570 nm. Values represent mean ± standard deviation.

compounds, we used PAK-PDB pull down assays to quantitate active amounts of both Rac1 and Cdc42 (Figures 5 and 6, resp). As shown in Figure 5, levels of active Rac1 changed very little between untreated cells, vehicle treated cells, and cells treated with the inactive compound A5. In contrast, cells treated with active compounds experienced a 40%–70% decrease in the amount of active Rac1. Similar results were seen with active Cdc42 in Figure 6. Relative to the untreated control there was a small decrease in cells treated with A5, presumably due to the presence of vehicle which yielded the same slight decrease. However, we observed an even greater decrease in cells treated with compounds A8, 5, and 9.

## DISCUSSION

Our screening assay has previously identified three noncytotoxic compounds that inhibit tumor cell migration. All the three are already in clinical trials or have been approved for clinical use [20–22]. Here we expanded our search to compounds that inhibit cell migration but whose effects on cancer are unknown, starting with compounds that inhibit intracellular signaling events that may be linked to cancer cell migration. The target of this study, apocynin, upon peroxidase-catalyzed metabolic activation, interferes with NADPH oxidase and inhibits lymphocyte migration through a G-protein regulated pathway without affecting adhesion. Reactive oxygen species generated by NADPH oxidase also control actin structure [23]. Apocynin or its metabolites have also been shown to affect the migration of polymorphonuclear granulocytes, suggesting its mechanism of action is conserved throughout cell types [24].

Our data collectively suggests that the compounds we identified induce sufficient rearrangement of the actin cytoskeleton to inhibit migration but not cause cell death over an 18-hour period. Major disruptors of the actin cytoskeleton such as cytochalasin-D trigger apoptosis [25], but our compounds do not elicit such a severe response. This disruption of the actin cytoskeleton leads to cell rounding without an appreciable decrease in cell adhesion. The highest effective doses used in our study reduced adhesion by 20%–40% (Figure 2), but lower doses which also inhibited cell migration resulted in no significant decrease in adhesion (data not shown). That these cells continued to be adherent may explain why they remained viable even after treatment. MDA-MB 435 breast cancer cells contain the characteristic integrin receptors to bind fibronectin; $\alpha 3\beta 1$, $\alpha 5\beta 1$, and $\alpha V\beta 3$ [26]. It is through these integrins, particularly the $\alpha 5\beta 1$ pairing, that cells are able to receive survival signaling which includes activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway [27]. Akt signaling pathways have significant roles in response to extracellular stimuli, serving to regulate a number of cellular functions including nutrient metabolism, cell growth, apoptosis, and survival [28].

The Rho family of proteins control remodeling of the actin cytoskeleton [29, 30]. These small G-proteins, which include Rac1 and Cdc42, are well known for their ability to modulate and rearrange the actin cytoskeleton. They regulate signal transduction pathways that mediate distinct cytoskeletal rearrangements required for the production

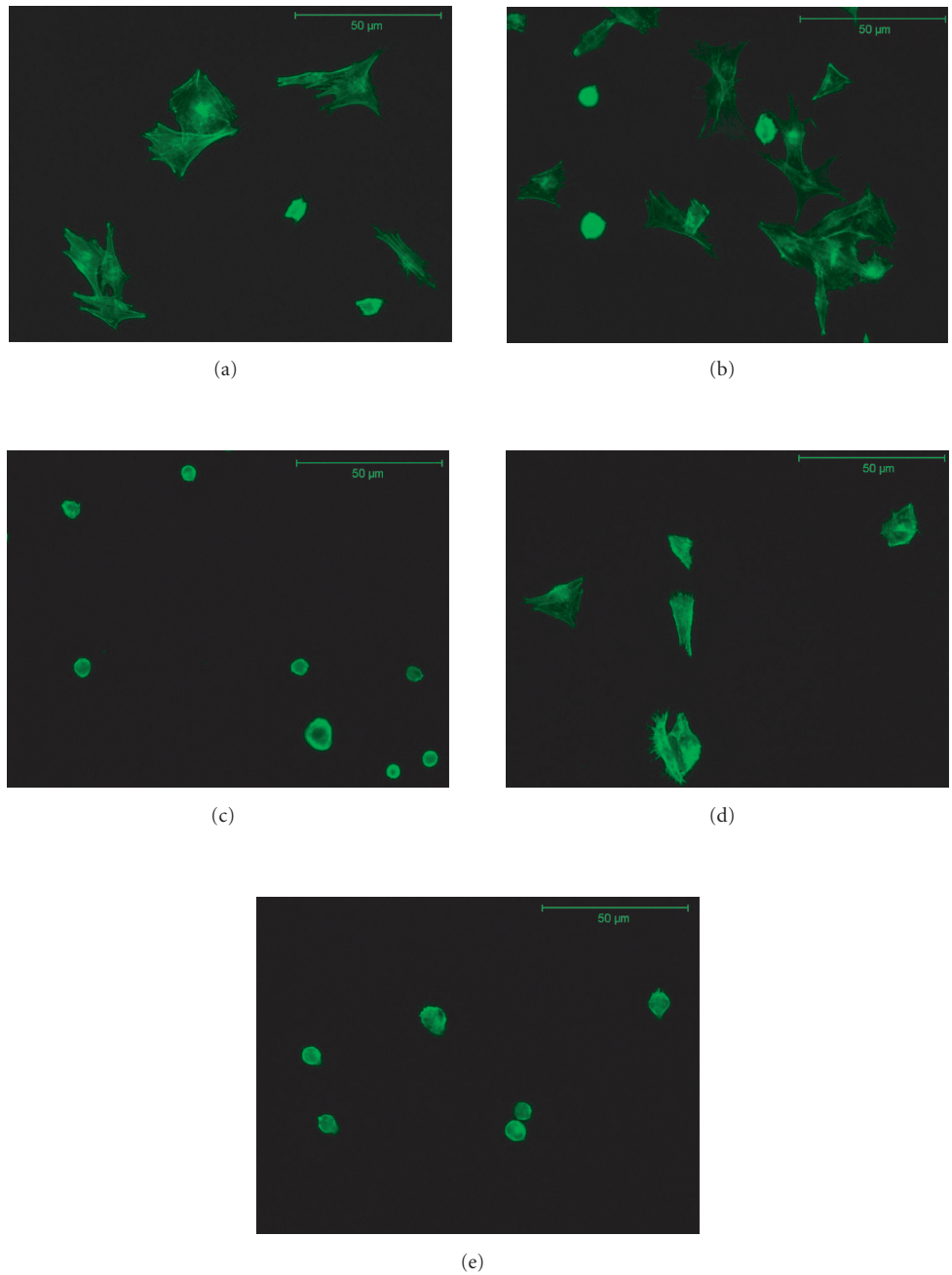



Figure 3: Rearrangement of actin cytoskeleton in treated and untreated MDA-MB 435 breast cancer cells. Cells were plated on fibronectin for 4 hours in the presence of either compound 9 (panel (c)), compound A5 (panel (d)), or compound A8 (panel (e)) at a final concentration of 15.2 ng/$\mu$l and were compared to the positive control (panel (a)) and the ethanol vehicle (panel (b)).Cells were fixed and stained for F-actin using TRITC-phalloidin. Bar = 50 $\mu$m.

of actin-rich protrusions called lamellipodia and fillipodia and then subsequent cell migration [31]. Our data suggest that the active derivatives of apocynin might be inhibiting migration by altering the activity of these proteins.

Apocynin or its oxidation products inhibit translocation of the cytosolic p47-phox and p67-phox proteins to their membrane fraction counterparts, causing inactivation of NADPH oxidase [32]. Active Rac1 is necessary for the translocation of p47-phox and p67-phox, though it does not mediate it directly. Rac1's role in NADPH oxidase activation is not well understood, but it is able to bind p67-phox, and this binding may be what causes the final formation of the active NADPH oxidase complex. When Rac1 is in its inactive form, there is a decreased level of $O_2^-$, signifying inactive NADPH oxidase [33]. NADPH oxidase has also been shown

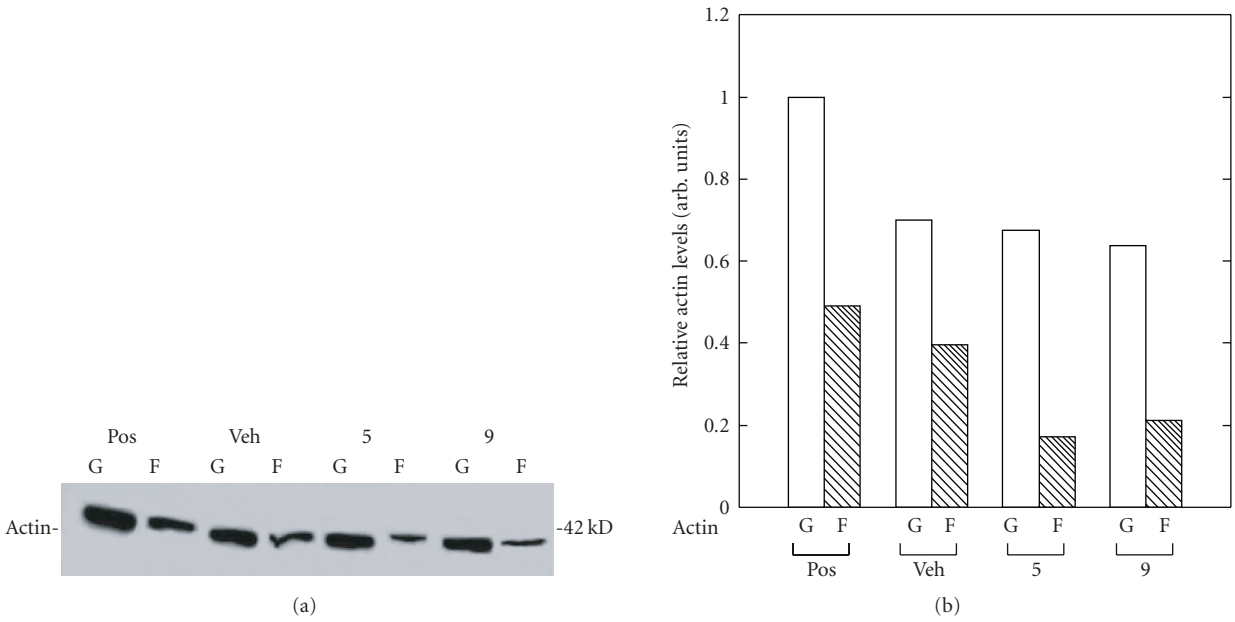



Figure 4: (a) Western blot for G- and F-actin levels in MDA-MB 435 breast cancer cells cultured on fibronectin for 4 hours. Positive control (lanes 1 and 2) and ethanol control (lanes 3 and 4) were compared to cells in the presence of 36 ng/$\mu$l of compound 5 (lanes 5 and 6) and 36 ng/$\mu$l of compound 9 (lanes 7 and 8) by probing with an antibody for G-actin. Odd numbered lanes represent G-actin while even numbered lanes represent F-actin totals. (b) Densitometry of the intensity of G- and F-actin bands.


Figure 5: (a) Western blot of MDA-MB 435 breast cancer cells cultured on fibronectin for 1 hour. Active Rac1 was isolated using a PAK-PBD pull down assay. Positive control (lane 1) and ethanol control (lane 2) were compared to cells treated with 36 ng/$\mu$l of either compound A5, A8, 5, or 9 (lanes 3, 4, 5, and 6, resp) by probing with a monoclonal antibody for Rac1. (b) Densitometric analysis of Rac1 band intensities.

to associate with the actin cytoskeleton, implicating another mode by which Rac1 may manage cytoskeletal structure [34]. Other inhibitors of NADPH oxidase also result in decreased cell migration [35]. This could be caused by the decreased activation of Rac1 from low levels of reactive oxygen species, attributed to diminished activity of NADPH oxidase [36]. Our data suggests that the compounds may act through Rac1 also.

The oxidation products of peroxidase catalysis on apocynin may mimic the active metabolite synthesis of myeloperoxidase found in blood. Thus, the inhibitory effect may be due to the in vivo activation of a biologically inert compound

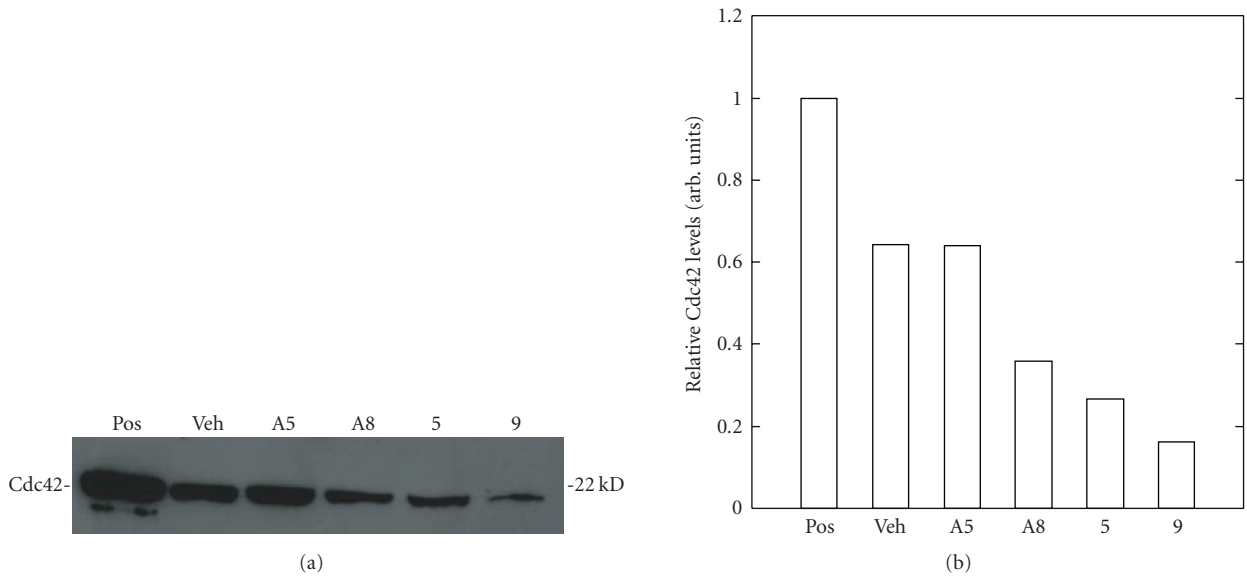



Figure 6: (a) Western blot of MDA-MB 435 breast cancer cells cultured on fibronectin for 1 hour. Active Cdc42 was isolated using a PAK-PBD pull down assay. Positive control (lane 1) and ethanol control (lane 2) were compared to cells treated with 36 ng/$\mu$l of either compound A5, A8, 5, or 9 (lanes 3, 4, 5, and 6, resp) by probing with a monoclonal antibody for Cdc42. (b) Densitometric analysis of Cdc42 band intensities.

to give a highly potent cell proliferation inhibitor. Peroxidase catalysis does not result in single products [37]. Rather, the enzyme generates a mixture of oxidation products. We have purified several of these compounds in this work and tested their abilities to inhibit cell proliferation. In therapy, however, the action of myeloperoxidase would most likely generate a mixture of metabolites. The influence of this mixture on biological activity may serve to increase the potency of the inhibitory effect. Such a study is left for future investigations.

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