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TITLE: Delphinidin: A Novel Agent for Inhibition of Breast Tumor Kinase Signaling by Targeting EGFR

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<b>14. ABSTRACT</b> Epidermal growth factor receptor (EGFR) is expressed at high levels in at least 25% of breast cancers and is associated with poor prognosis. Upon epidermal growth factor (EGF)-stimulation, breast tumor kinase (Brk) is recruited to the EGFR, and this event activates the catalytic activity of Brk, which in turn phosphorylates paxillin a binding partner and substrate for Brk. The phosphorylation of paxillin promotes the activation of Rac1, thereby stimulating cell migration and invasion in response to EGF. Many synthetic inhibitors of EGFR are known, but their use is limited because of their unacceptable cytotoxic effects on normal cells. Therefore, identification of a natural, nontoxic agent(s) as an inhibitor of EGFR is of utmost importance. Delphinidin, a major anthocyanin known to be present in pigmented fruits and vegetables, inhibits constitutive and EGF-induced phosphorylation of EGFR, activation of PI3K, phosphorylation of AKT, and MAPK. We also found that delphinidin treatment inhibits constitutive and EGF-induced activation of Brk signaling mediated through EGFR. Furthermore, treatment of breast cancer cells with delphinidin inhibited cell growth and invasion and induced apoptosis. Taken together, the composite result suggest that delphinidin is an effective inhibitor of EGFR signaling at least in breast cancer cells that act through novel Brk signaling pathway and holds great promise for its treatment.						
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## INTRODUCTION:

The epidermal growth factor receptor (EGFR/HER1, also ErbB1) belongs to a family of four transmembrane receptor tyrosine kinases (EGFR/HER1, HER2, HER3, and HER4) that is activated in many epithelial tumors (1). Overexpression of EGFR occurs in about 25 % of human breast cancers and is associated with a poor clinical prognosis and therapeutic response (2). EGFR was the first tyrosine kinase receptor to be linked directly to human tumors (3). EGFR becomes biologically functional by forming a homodimer with itself or a heterodimer with other members of the receptor tyrosine kinases family. The mechanisms, which promote the formation of receptor dimers, include ligand binding and high receptor density due to overexpression. Several studies have shown that in many tumors epidermal growth factor (EGF)-related growth factors are produced either by the tumor cells themselves or are available from surrounding stromal cells, leading to constitutive activation of EGFR. The EGFR signaling pathway is involved in several cellular processes associated with malignant behavior, including cell proliferation, differentiation, apoptosis, migration, and cell adhesion. The signaling pathways induced by activated EGFR include the phosphatidylinositol 3-kinase (PI3K)/AKT and mitogen activated protein kinases (MAPK), both of which play a significant role in the mitogenic and cell survival responses mediated by EGFR. Abnormalities in the expression and signaling pathways downstream of the EGFR contribute to progression, invasion, and maintenance of the malignant phenotype in breast cancer (4).

Upon EGF-stimulation, breast tumor kinase (Brk/PTK6) is recruited to the EGFR, and this event activates the catalytic activity of Brk, which in turn phosphorylates paxillin a binding partner and substrate for Brk. The phosphorylation of paxillin promotes the activation of Rac1, thereby stimulating cell migration and invasion in response to EGF (5). Brk is a nonreceptor protein tyrosine kinase expressed in breast carcinoma tissue samples and breast tumor cell lines, and its expression is low or undetectable in normal mammary tissue or benign lesions (6). Brk expression sensitizes the mammary epithelial cells to the mitogenic response of EGF and potentiates their anchorage-independent growth (7). Accordingly, Brk association with EGFR enhances EGF-dependent phosphorylation of erbB3, which subsequently leads to an increased recruitment of PI3K and activation of Akt (8). EGFR is an upstream regulator of Brk signaling pathway through which Brk mediates EGF-induced phosphorylation of paxillin and activation of Akt, and involved in cell migration and invasion. Migration is a key process in tumor cell invasion and is frequently increased in cancer biogenesis. It occurs in part via the assembly of focal adhesion complexes along the leading edge of the cell membrane linking the tumor cell to the actin cytoskeleton allowing for coordinated movement (9,10). The EGFR is in the crossroads of numerous pathways controlling cell function, including cell growth, survival, adhesion, and migration.

Many synthetic inhibitors of EGFR are known, but their use is limited because of their unacceptable cytotoxic effects on normal cells. Therefore, identification of a natural, nontoxic agent(s) as an inhibitor of EGFR is of utmost importance. Delphinidin, a major anthocyanin known to be present in pigmented fruits and vegetables (such as pomegranate, berries, dark grapes, egg plant, tomato, carrot and red onion) possesses potent antioxidant and antiproliferative properties. Therefore, modulating Brk's signaling with delphinidin by targeting EGFR may provide a promising cancer chemopreventive and cancer therapeutic target for breast cancer. In

this study, employing human breast cancer cells AU-565 and non-tumorigenic human mammary epithelial cells MCF-10A that overexpress EGFR, we evaluated the effect of delphinidin on EGFR and its downstream signaling pathways. Through this study we envision identification of a natural, nontoxic dietary agent that can be exploited for the treatment of breast cancers that act through novel Brk signaling pathway by targeting EGFR.

## **BODY:**

### **MATERIALS AND METHODS:**

**Materials:** Delphinidin (>98% pure) was purchased from Extrasynthase (Lyon, France). The monoclonal and polyclonal antibodies for ERK1/2 (phospho-p44/42, Thr202/Tyr204), JNK (phospho-p54/46, Thr183/Tyr185), p38 (phospho-p38, Thr180/Tyr204), EGFR and phospho-EGFR at specific tyrosine sites 1068, 1045, and 845 were obtained from Cell Signaling Technology (Beverly, MA, USA). The antibodies for PI3K, phospho AKT, Rac1, Paxillin-phospho (Tyr118), Bcl-2, Bax and caspase-3 were procured from Upstate Biotechnology (Lake Placid, NY). Monoclonal antibody for PARP was purchased from Promega (Madison, WI, USA). Monoclonal anti-EGFR C225 antibody was obtained from Sigma (St. Louis, MO, USA). The antibody for Brk was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The antibody for Paxillin-phospho (Tyr31) was procured from Chemicon International Inc. (Teleucula, CA, USA). Anti-mouse secondary horseradish peroxidase conjugate was obtained from Amersham Biosciences Limited (Buckinghamshire, England). Annexin-V-FLUOS staining kit was procured from Roche Diagnostic Corporation (Indianapolis, IN, USA). Antibiotic (Penicillin and Streptomycin) used were obtained from Cellgro Mediatech, Inc. (Herndon, VA, USA). Protein was estimated using BCA protein assay kit obtained from Pierce (Rockford, IL, USA).

**Cell Culture:** The AU-565, MCF-10A and 184A1 cells were obtained from ATCC (Manassas, VA, USA). The human breast cancer AU-565 cells were cultured in RPMI 1640 media (ATCC) supplemented with 10% FBS and 1% penicillin-streptomycin. The human immortalized MCF-10A cells were cultured in mammary epithelial growth medium (MEGM), from Cambrex (Walkersville, MD) supplemented with 100 ng/ml cholera toxin from EMD Biosciences, Inc. (San Diego, CA). The normal mammary epithelial cell line (184A1) was cultured in MEGM from Cambrex supplemented with 0.005 mg/ml transferrin and 1 ng/ml cholera toxin. The cells were maintained under standard cell culture conditions at 37 °C and 5% CO<sub>2</sub> in a humid environment.

**Treatment of Cells:** Delphinidin (dissolved in DMSO) was used for the treatment of AU-565, MCF-10A and 184A1 cells. The final concentration of DMSO used was 0.1% (v/v) for each treatment. For dose-dependent studies cells were treated with delphinidin (5-40 μM) for 3 and 48 hours in complete cell medium. Control cells were treated with vehicle alone. In additional experiments, serum starved AU-565 cells were treated with delphinidin (5-40 μM) or with anti-EGFR monoclonal antibody C225 (1-4 μg/ml) for 3 hours and then incubated without or with EGF (50 ng/ml) for 15 min. In pilot experiments, it was established that DMSO (0.1% v/v) had no effects when measured at 48 h.

**Cell Viability:** The effect of delphinidin on the viability of cells was determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide) assay. The cells were plated at  $1 \times 10^4$  cells per well in 200  $\mu\text{l}$  of complete culture medium containing 5, 10, 20 and 40  $\mu\text{M}$  concentrations of delphinidin in 96-well microtiter plates for 48 h at  $37^\circ\text{C}$  in a humidified chamber. Each concentration of delphinidin was repeated in 10 wells. After incubation for specified times at  $37^\circ\text{C}$  in a humidified incubator, MTT reagent (4  $\mu\text{L}$ , 5 mg/mL in PBS) was added to each well and incubated for 2 hours. The microtiter plate containing the cells was centrifuged at 1,800 rpm for 5 minutes at  $4^\circ\text{C}$ . The MTT solution was removed from the wells by aspiration and the formazan crystals were dissolved in DMSO (150  $\mu\text{L}$ ). Absorbance was recorded on a microplate reader at 540 nm wavelength. The effect of delphinidin on growth inhibition was assessed as percentage inhibition in cell growth where vehicle-treated cells were taken as 100% viable.

**Preparation of Cell Lysates and Western Blot Analysis:** Following treatment of cells with delphinidin, the medium was aspirated and the cells were washed with PBS (10 mmol/L, pH 7.45). The cells were then incubated in ice cold lysis buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1mmol/L EGTA, 1mmol/L EDTA, 20 mmol/L NaF, 100 mmol/L  $\text{Na}_3\text{VO}_4$ , 0.5% NP-40, 1% Triton X-100, 1mmol/L phenyl methyl sulfonyl fluoride (PMSF) (pH 7.4), with freshly added protease inhibitor cocktail (Protease Inhibitor Cocktail Set III, Calbiochem, La Jolla, CA) over ice for 20 minutes. The cells were scraped and the lysate was collected in a microfuge tube and passed through a 21.5 G needle to break up the cell aggregates. The lysate was cleared by centrifugation at  $14000 \times g$  for 15 minutes at  $4^\circ\text{C}$ , and the supernatant (total cell lysate) collected, aliquoted and was used on the day of preparation or immediately stored at  $-80^\circ\text{C}$  for use at a later time. For western blotting, 25-50  $\mu\text{g}$  protein was resolved over 12 % polyacrylamide gels and transferred onto a nitrocellulose membrane. The non-specific sites on blots were blocked by incubating in blocking buffer (5% non fat dry milk/ 1% Tween 20 in 20 mmol/L TBS, pH 7.6) for 1 hour at room temperature, incubated with appropriate monoclonal primary antibody in blocking buffer for 90 minutes to overnight at  $4^\circ\text{C}$ , followed by incubation with anti-mouse or anti-rabbit secondary antibody horse-radish peroxidase conjugate and detected by chemiluminescence and autoradiography using Hyperfilm obtained from Amersham Biosciences (UK Ltd.).

**Detection of Apoptosis by Confocal Microscopy:** The Annexin-V-FLUOS staining kit was used for the detection of apoptotic cells according to vendor's protocol. This kit uses a staining protocol in which the apoptotic are stained with annexin V (green fluorescence). MCF-10A and AU-565 cells were grown to about 60% confluence and then treated with delphinidin (5, 10, 20 and 40  $\mu\text{M}$ ) for 48 hours. The fluorescence was measured by a Zeiss 410 confocal microscope (Thornwood, NY). Confocal images of green annexin-FITC fluorescence were collected using 488 nm excitation light from an argon/krypton laser, a 560 nm dichroic mirror, and a 514–540 nm bandpass barrier filter.

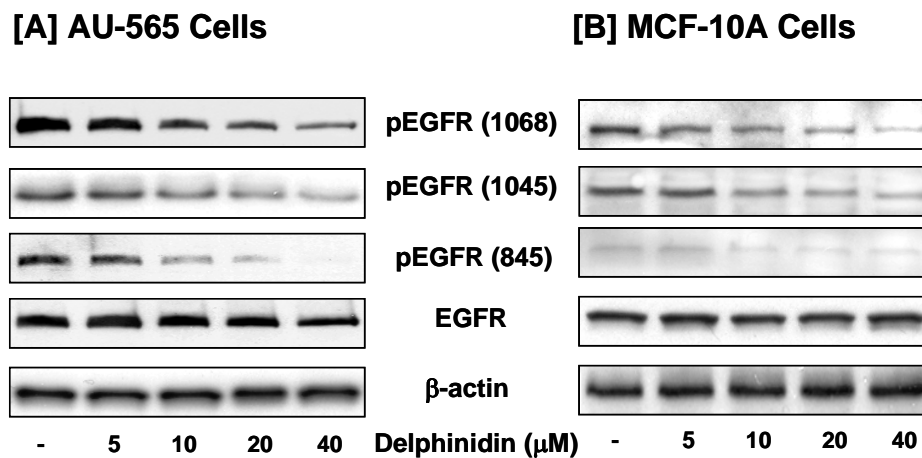
**Cell invasion assay:** Cell invasion was performed using CHEMICON<sup>®</sup> QCM<sup>™</sup> 24-well invasion assay kit. Cells treated with delphinidin (5-40 $\mu\text{M}$ ) or C225 antibody (1, 2, and 4  $\mu\text{g}/\text{ml}$ ) were loaded into the chamber. EGF (50 ng/ml) was added to each of the wells and cells were allowed to invade through the membrane by incubating at  $37^\circ\text{C}$  for 18 h after which the invaded

cells on the bottom of the insert membrane were detached, lysed and subsequently detected by CyQUANT<sup>®</sup> GR dye using a fluorescence plate reader at 480/520 nm filter set.

## RESULTS AND DISCUSSION:

### Delphinidin treatment inhibits phosphorylation of EGFR in AU-565 and MCF-10A cells:

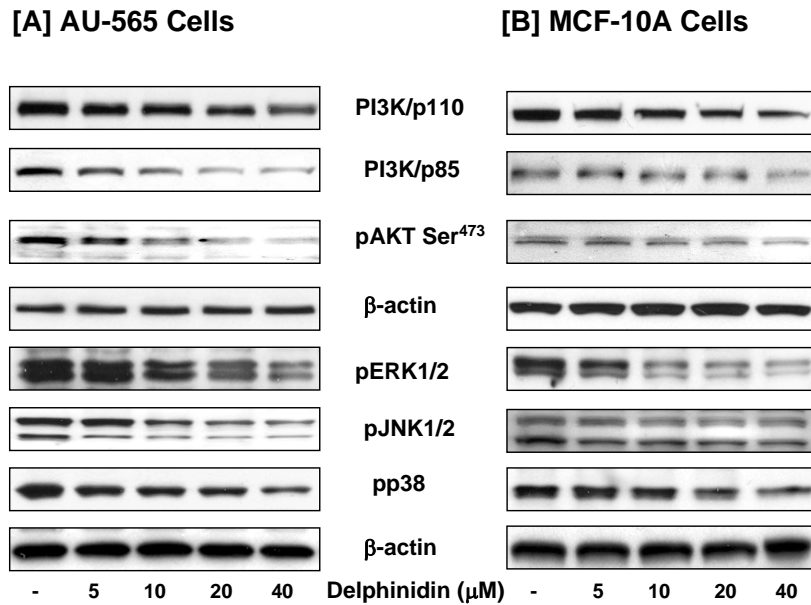
Aberrant expression and/or activation of EGFR has been reported in a wide range of human malignancies, including non-small cell lung carcinoma, breast, prostate, colorectal and head and neck cancers, and provides a strong rationale for targeting this growth factor receptor for EGFR positive human cancers (11,12). We used western blot analysis to assess the effect of delphinidin in human breast cancer cells AU-565 and non-tumorigenic human mammary epithelial cells MCF-10A that overexpress EGFR. Using phosphospecific antibodies that detect specific tyrosine residues of EGFR at the 1068, 1045 and 845 sites, we found that the receptor was phosphorylated at all this residues in both the cell lines, although the level of phosphorylation differed in both the cell lines. Delphinidin (5-40  $\mu$ M; 3 hours) treatment to EGFR positive cells AU-565 and MCF-10A resulted in a dose-dependent decrease in the phosphorylation of specific tyrosine residues of EGFR at 1068, 1045 and 845 sites (Figure 1A,B).



**Figure 1: Effect of delphinidin on the phosphorylation of EGFR in AU-565 and MCF-10A cells.** AU-565 and MCF-10A cells were treated with various concentrations of delphinidin (5–40  $\mu$ M) for 3 hours after which cells were harvested and total cell lysates were prepared for western blot analysis as detailed in 'Materials and methods'. Equal protein loading was confirmed by  $\beta$ -actin. The data shown here are from representative experiment repeated two times with similar results.

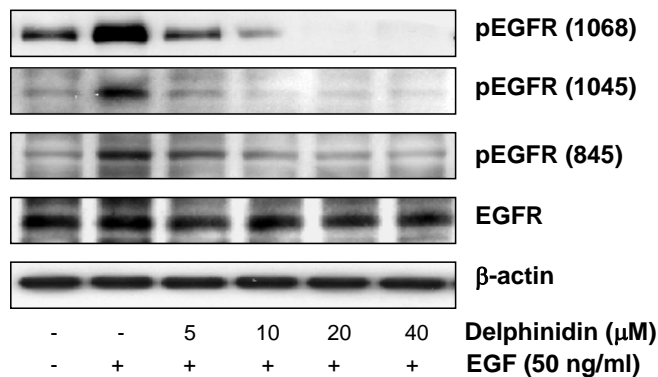
**Delphinidin treatment inhibits protein expression of PI3K and phosphorylation of AKT and MAPK in AU-565 and MCF-10A cells:** The signaling pathways induced by activated EGFR include the PI3K/AKT and MAPK, both of which play a significant role in the mitogenic and cell survival responses mediated by EGFR. We next evaluated the effect of delphinidin (5-40  $\mu$ M; 3hours) treatment to EGFR positive cells AU-565 and MCF-10A on protein expression of PI3K and phosphorylation of AKT and MAPK. We found that delphinidin treatment inhibited

protein expression of PI3K, phosphorylation of Akt at Ser<sup>473</sup> and phosphorylation of MAPK (such as pERK1/2, pJNK1/2 and pp38) in a dose-dependent manner (Figure 2A,B).



**Figure 2: Effect of delphinidin on protein expression of PI3K and phosphorylation of AKT and MAPK in AU-565 and MCF-10 A cells.** AU-565 and MCF-10A cells were treated with various concentrations of delphinidin (5–40 μM) for 3 hours after which cells were harvested and total cell lysates were prepared for western blot analysis as detailed in 'Materials and methods'. Equal protein loading was confirmed by β-actin. The data shown here are from representative experiment repeated two times with similar results.

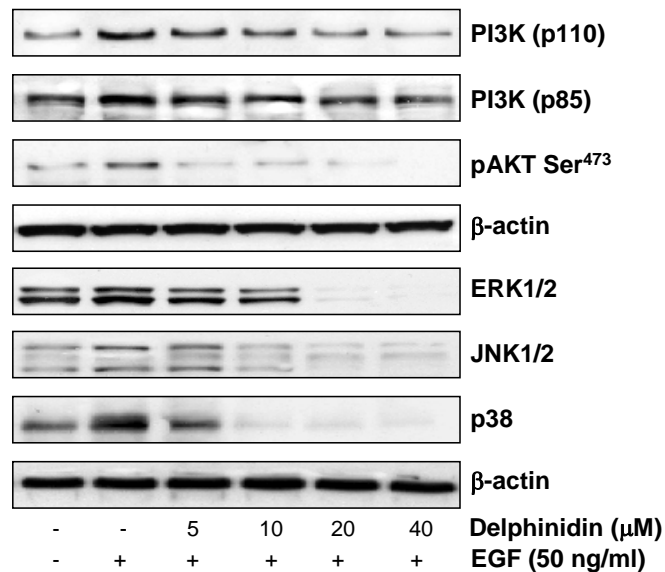
**Delphinidin treatment inhibits EGF-induced autophosphorylation of EGFR in AU-565 cells:** In the next experiments we used western blot analysis to assess the effect of delphinidin on EGFR autophosphorylation by its ligand, EGF. AU565 cells, which express high levels of phospho-EGFR, were serum starved and then incubated with delphinidin (5-40 μM) for 3 hours followed by a 15-minute incubation with EGF (50 ng/ml). Delphinidin treatment inhibited EGF-induced phosphorylation of EGFR at specific tyrosine residues 1068, 1045 and 845 (Figure 3). The total levels of EGFR remained unchanged. These results verified the ability of delphinidin to inhibit the activation of the EGFR.





**Figure 3: Effect of delphinidin on EGF-induced autophosphorylation of EGFR in AU-565 cells.** Serum-starved AU565 cells were treated with various concentrations of delphinidin (5–40  $\mu$ M) for 3 hours and then incubated without or with 50 ng/ml EGF for 15 minutes after which cells were harvested and total cell lysates were prepared for western blot analysis as detailed in 'Materials and methods'. Equal protein loading was confirmed by  $\beta$ -actin. The data shown here are from representative experiment repeated three times with similar results.

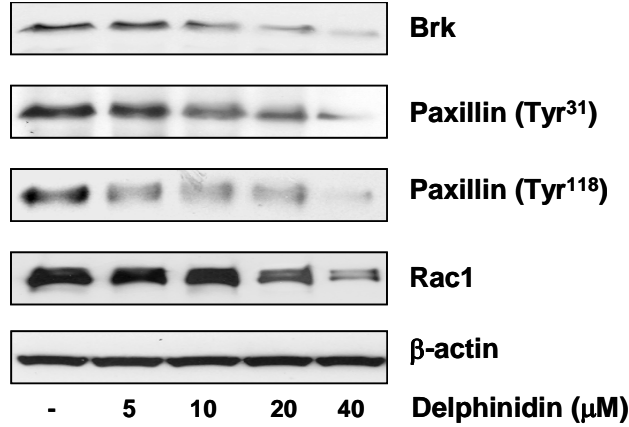
**Delphinidin treatment inhibits EGF-induced activation of PI3K and phosphorylation of AKT and MAPK in AU-565 cells:** Based on its ability to inhibit EGF-induced activation of the EGFR, we evaluated the effect of delphinidin treatment on the signaling cascades triggered by this receptor as well. The signaling pathways induced by activated EGFR include the MAPK and the PI3K/AKT pathways, both of which play a significant role in the mitogenic and cell survival responses mediated by this receptor. We found that delphinidin treatment inhibits EGF-induced activation of PI3K in a dose-dependent manner (Figure 4). The phosphorylation of Akt at Ser<sup>473</sup> was almost completely inhibited at 20  $\mu$ M dose of delphinidin and the phosphorylation of MAPK were successfully inhibited at 10-20  $\mu$ M doses of delphinidin (Figure 4).



**Figure 4: Effect of delphinidin on EGF-induced activation of PI3K and phosphorylation of AKT and MAPK in AU-565 cells.** Serum-starved AU565 cells were treated with various concentrations of delphinidin (5–40  $\mu$ M) for 3 hours and then incubated without or with 50 ng/ml EGF for 15 minutes after which cells were harvested and total cell lysates were prepared for western blot analysis as detailed in 'Materials and methods'. Equal protein loading was confirmed by  $\beta$ -actin. The data shown here are from representative experiment repeated three times with similar results.

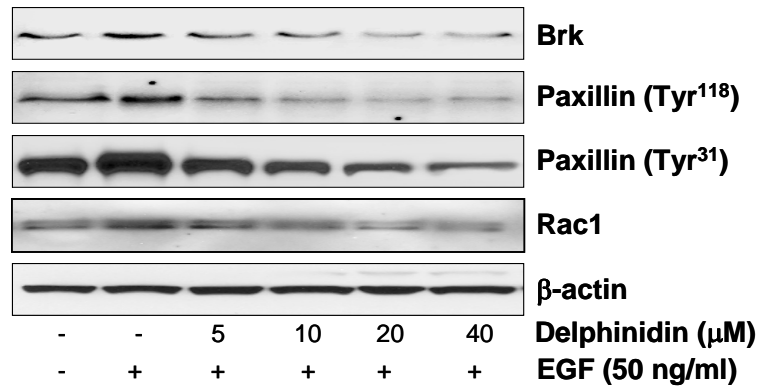
**Delphinidin treatment inhibits Brk protein expression and phosphorylation of paxillin and activation of Rac1 in AU-565 cells:** We used western blot analysis to assess the effect of

delphinidin in human breast cancer cells AU-565 that overexpress EGFR. Delphinidin treatment inhibited constitutive levels of Brk protein expression as well as phosphorylation of paxillin at Tyr<sup>31</sup> and Tyr<sup>118</sup> and activation of Rac 1 protein expression (Figure 5).



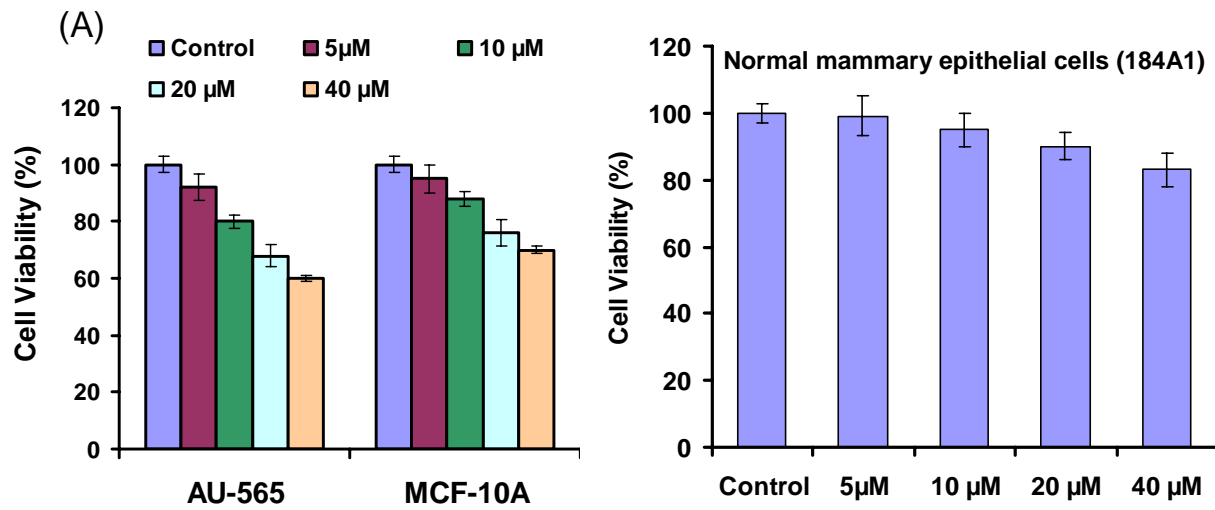
**Figure 5: Effect of delphinidin on the levels of Brk protein expression and phosphorylation of paxillin and activation of Rac1 in AU-565 cells.** AU-565 cells were treated with various concentrations of delphinidin (5–40 μM) for 3 hours after which cells were harvested and total cell lysates were prepared for western blot analysis as detailed in 'Materials and methods'. Equal protein loading was confirmed by β-actin. The data shown here are from representative experiment repeated two times with similar results.

**Delphinidin treatment inhibits EGF-induced Brk protein expression and phosphorylation of paxillin and activation of Rac1 in AU-565 cells:** Since EGF stimulation activates the catalytic activity of Brk, which in turn phosphorylates paxillin at Tyr<sup>31</sup> and Tyr<sup>118</sup>. These phosphorylation events promote the activation of small GTPase Rac1 via the function of CrkII. Through this pathway, Brk is capable of promoting cell motility and invasion and functions as a mediator of EGF-induced migration and invasion. Our findings suggest that treatment of AU-565 cells with delphinidin inhibits EGF-induced expression of Brk and also inhibits phosphorylation of paxillin at Tyr<sup>31</sup> and Tyr<sup>118</sup> and activation of Rac 1 protein (Figure 6).



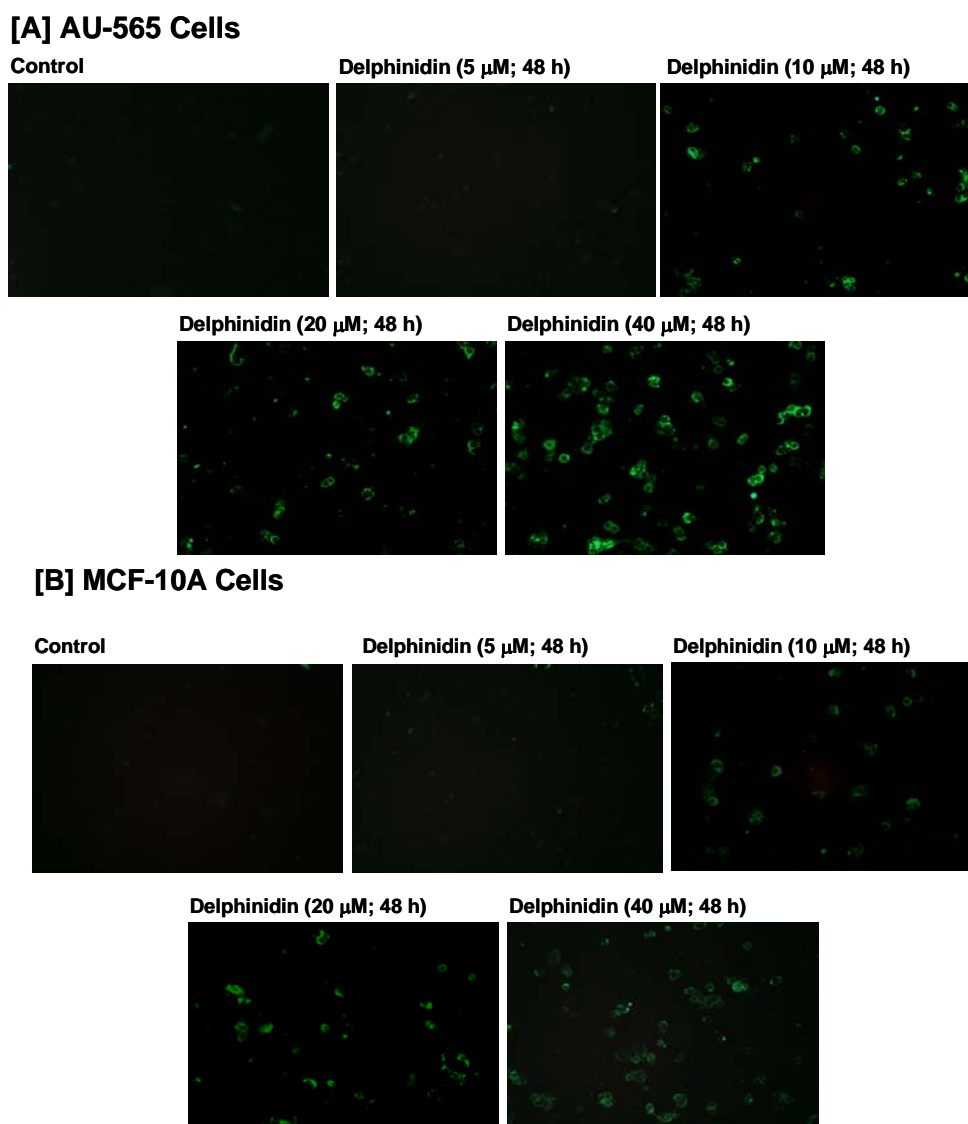
**Figure 6: Effect of delphinidin on EGF-induced Brk protein expression and phosphorylation of paxillin and activation of Rac1 in AU-565 cells.** Serum-starved AU565 cells were treated with various concentrations of delphinidin (5–40  $\mu\text{M}$ ) for 3 hours and then incubated without or with 50 ng/ml EGF for 15 minutes after which cells were harvested and total cell lysates were prepared for western blot analysis as detailed in 'Materials and methods'. Equal protein loading was confirmed by  $\beta$ -actin. The data shown here are from representative experiment repeated three times with similar results.

**Delphinidin treatment of AU-565 and MCF-10A cells results in growth inhibition:** We next investigated whether delphinidin treatment imparts antiproliferative effects against AU-565 and MCF-10A cells. As shown in Figure 7A, delphinidin treatment (5-40  $\mu\text{M}$  for 48 hours) of AU-565 and MCF-10A cells resulted in a dose-dependent inhibition of cell growth as assessed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide assay. We found that delphinidin treatment (5-40  $\mu\text{M}$  for 48 hours) of normal mammary epithelial cell line (184A1) had only marginal effect on cell growth at these doses (Figure 7B).



**Figure 7: Effect of delphinidin treatment on cell growth.** The cells were treated with delphinidin (5-40  $\mu\text{M}$ ) for 48 h, and the viability of cells was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide assay.

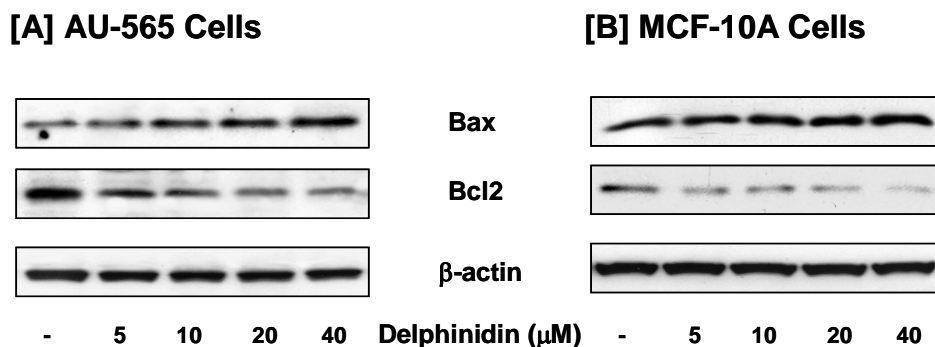
**Delphinidin treatment of AU-565 and MCF-10 A cells results in apoptosis:** To assess whether delphinidin-induced growth inhibition of the cells is mediated through induction of apoptosis. The extent of apoptosis was quantified by fluorescence microscopy of delphinidin (5-40  $\mu\text{M}$ ; 48 hours) treated and untreated cells. As shown by the data delphinidin treatment resulted in induction of apoptosis in both AU-565 and MCF-10A cells as determined by fluorescence microscopy after staining the cells with Annexin V (Figure 8A,B). The effect of delphinidin was more pronounced in AU-565 cells.



**Figure 8: Delphinidin treatment of AU565 and MCF-10A cells results in apoptosis:** The cells were treated with delphinidin (5-40  $\mu$ M) for 48 h, and the apoptosis was determined by fluorescence microscopy. The annexin-V-FLUOS staining kit was used for detection of apoptotic cells. The kit uses a staining protocol in which apoptotic cells are stained with annexin V (green fluorescence). A representative picture from two independent experiments with similar result is shown.

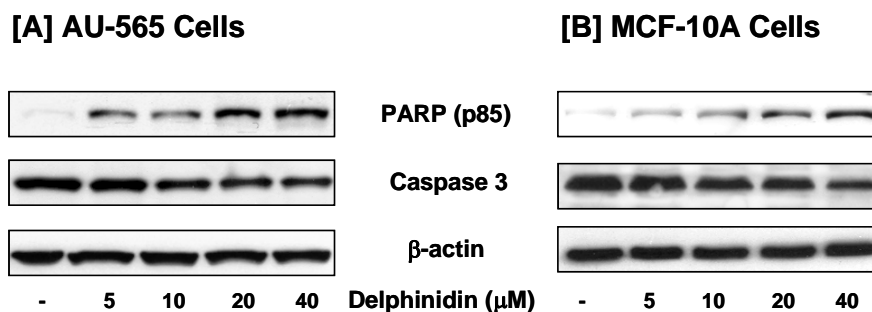
**Delphinidin treatment of AU-565 and MCF-10A cells results in upregulation of Bax and downregulation of Bcl2:** Because Bax and Bcl-2 play crucial roles in apoptosis, we next studied the effects of delphinidin on the protein levels of Bax and Bcl-2 in AU565 and MCF-10A cells. The immunoblot analysis exhibited an increase in the protein expression of Bax (Figure 9). In

contrast, the protein expression of Bcl-2 was significantly decreased by delphinidin treatment in a dose-dependent fashion (Figure 9), thus further confirming the induction of apoptotic process.



**Figure 9: Effect of delphinidin treatment of AU-565 and MCF-10A cells on protein expression of Bax and Bcl2.** The cells were treated with delphinidin (5-40  $\mu$ M) for 48 hours and cells were harvested, and cell lysates were prepared. The data are representative of three independent experiments with similar results. Equal protein loading was confirmed by  $\beta$ -actin. The data shown here are from representative experiment repeated three times with similar results.

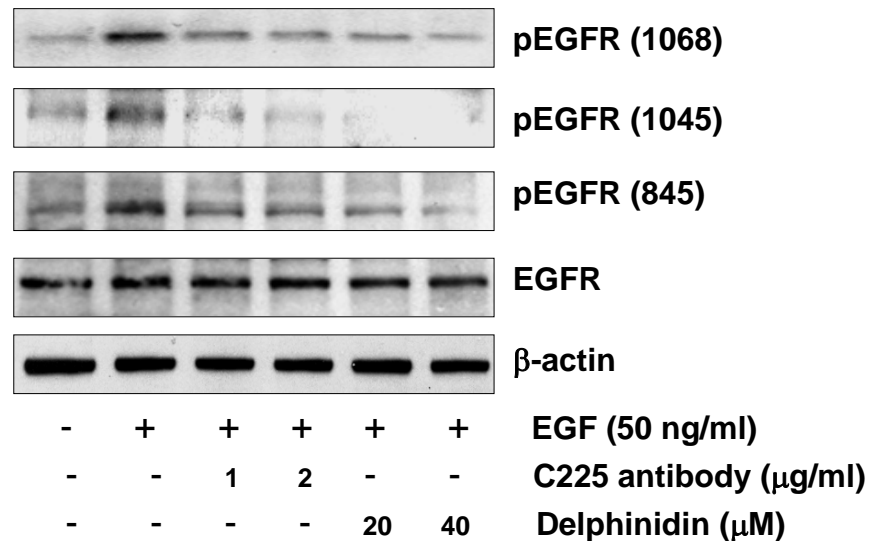
**Delphinidin treatment of AU-565 and MCF-10A cells results in cleavage of PARP protein and activation of caspase 3.** Next, we examined whether delphinidin treatment could induce poly(ADP-ribose) polymerase (PARP) cleavage. PARP is a 116 kDa protein that is cleaved into 85 kDa fragment during apoptotic cell death. As shown in Figure 10, employing immunoblot analysis, we found that the full-size PARP protein (116 kDa) was cleaved to yield an 85-kDa fragment after treatment of cells with delphinidin (5-40  $\mu$ M) for 48 hours. We next examined whether delphinidin treatment of AU-565 and MCF-10A cells resulted in activation of caspase 3. Our results suggest that treatment of cells with delphinidin (5-40  $\mu$ M) resulted in a decrease in the protein expression of procaspase-3 in both the cell lines (Figure 10).



**Figure 10: Effect of delphinidin treatment of AU-565 and MCF-10A cells on cleavage of PARP protein and activation of caspase 3.** The cells were treated with delphinidin (5-40  $\mu$ M) for 48 hours and cells were harvested, and cell lysates were prepared. The data are representative of three independent experiments with similar results. Equal protein loading was confirmed by  $\beta$ -

actin. The data shown here are from representative experiment repeated three times with similar results.

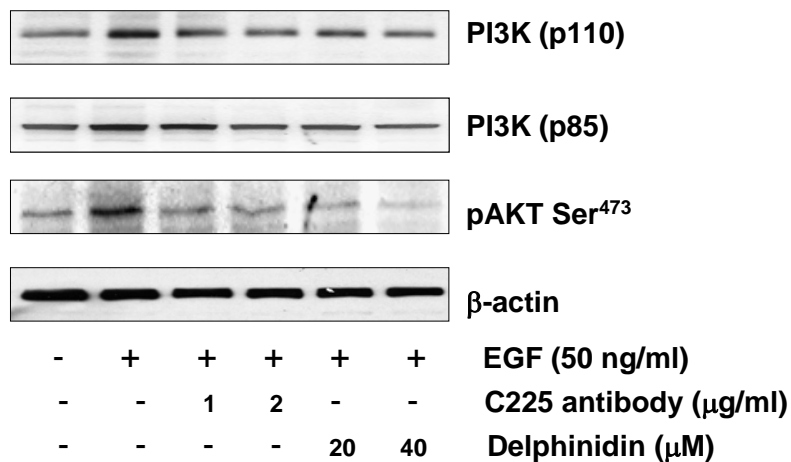
**Both delphinidin and C225 antibody treatment inhibit EGF-induced autophosphorylation of EGFR in AU-565 cells:** In the next experiments we used western blot analysis to assess the effect of delphinidin and anti-EGFR antibody C225 on EGFR autophosphorylation by its ligand, EGF. The anti-EGFR monoclonal antibody C225 specifically binds to EGFR with high affinity, blocking growth factor binding, receptor activation and subsequent signal-transduction events leading to cell proliferation. AU565 cells, which express high levels of phospho-EGFR, were serum starved and then incubated with delphinidin (20 and 40  $\mu$ M) or C225 antibody (1 and 2  $\mu$ g/ml) for 3 hours followed by a 15-minute incubation with EGF (50 ng/ml). Both delphinidin and C225 antibody treatment inhibited EGF-induced phosphorylation of EGFR at specific tyrosine residues 1068, 1045 and 845 (Figure 11). These results verified the ability of delphinidin and C225 antibody to inhibit the activation of the EGFR.



**Figure 11: Effect of delphinidin and C225 antibody on EGF-induced autophosphorylation of EGFR in AU-565 cells.** Serum-starved AU565 cells were treated with delphinidin (20 and 40  $\mu$ M) or C225 antibody (1 and 2  $\mu$ g/ml) for 3 hours and then incubated without or with 50 ng/ml EGF for 15 minutes after which cells were harvested and total cell lysates were prepared for western blot analysis as detailed in 'Materials and methods'. Equal protein loading was confirmed by  $\beta$ -actin. The data shown here are from representative experiment repeated two times with similar results.

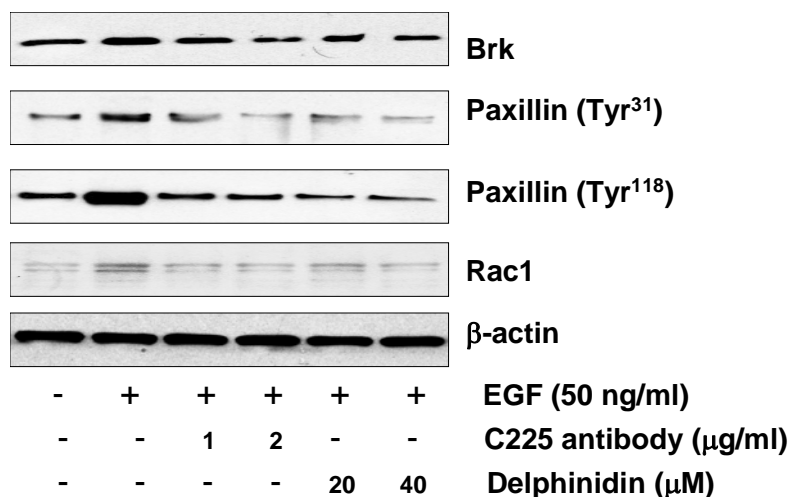
**Both delphinidin and C225 antibody treatment inhibit EGF-induced activation of PI3K and phosphorylation of AKT and MAPK in AU-565 cells:** We next evaluated the effect of delphinidin and C225 antibody treatment on the signaling cascades triggered by EGFR. The signaling pathways induced by activated EGFR include the PI3K/AKT pathways, which play a significant role in the mitogenic and cell survival responses mediated by this receptor. We found that delphinidin and C225 antibody treatment inhibited EGF-induced activation of PI3K (Figure

12). The phosphorylation of Akt at Ser<sup>473</sup> was also inhibited by both delphinidin and C225 antibody (Figure 12).



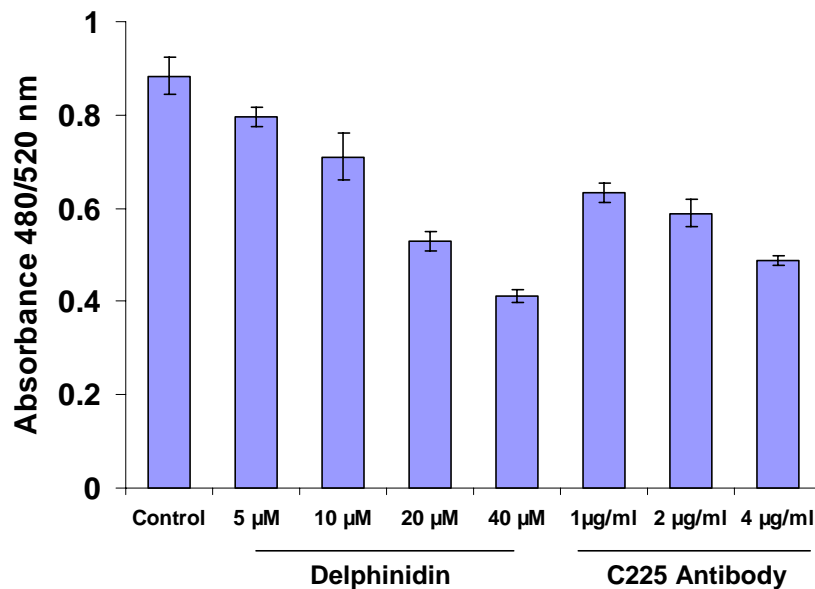
**Figure 12: Effect of delphinidin and C225 antibody on EGF-induced activation of PI3K and phosphorylation of AKT in AU-565 cells.** Serum-starved AU565 cells were treated with delphinidin (20 and 40 µM) or C225 antibody (1 and 2 µg/ml) for 3 hours and then incubated without or with 50 ng/ml EGF for 15 minutes after which cells were harvested and total cell lysates were prepared for western blot analysis as detailed in 'Materials and methods'. Equal protein loading was confirmed by β-actin. The data shown here are from representative experiment repeated two times with similar results.

**Both delphinidin and C225 antibody treatment inhibit EGF-induced Brk protein expression and phosphorylation of paxillin and activation of Rac1 in AU-565 cells:** Since EGF stimulation activates the catalytic activity of Brk, which in turn phosphorylates paxillin at Tyr<sup>31</sup> and Tyr<sup>118</sup>. These phosphorylation events promote the activation of small GTPase Rac1 via the function of CrkII. Through this pathway, Brk is capable of promoting cell motility and invasion and functions as a mediator of EGF-induced migration and invasion. Our findings suggest that treatment of AU-565 cells with delphinidin and C225 antibody inhibit EGF-induced expression of Brk and also inhibit phosphorylation of paxillin at Tyr<sup>31</sup> and Tyr<sup>118</sup> and activation of Rac 1 protein (Figure 13).



**Figure 13: Effect of delphinidin and C225 antibody on EGF-induced Brk protein expression and phosphorylation of paxillin and activation of Rac1 in AU-565 cells.** Serum-starved AU565 cells were treated with delphinidin (20 and 40  $\mu\text{M}$ ) or C225 antibody (1 and 2  $\mu\text{g/ml}$ ) for 3 hours and then incubated without or with 50 ng/ml EGF for 15 minutes after which cells were harvested and total cell lysates were prepared for western blot analysis as detailed in 'Materials and methods'. Equal protein loading was confirmed by  $\beta$ -actin. The data shown here are from representative experiment repeated three times with similar results.

**Both delphinidin and C225 antibody inhibit invasive potential of AU-565 cells:** One of the hallmark characteristic of cancer cells is enhanced motility. Penetration of the extracellular matrix and the basement membrane is a key step in tumor dissemination and invasion. The EGFR is in the crossroads of numerous pathways controlling cell function, including cell growth, survival, adhesion, and migration. It is therefore critical that EGFR expression is well coordinated. EGFR is an upstream regulator of Brk signaling pathway through which Brk mediates EGF-induced phosphorylation of paxillin and activation of Akt, and involved in cell migration and invasion. Since delphinidin is an inhibitor of EGFR, we determined the effect of delphinidin and C225 antibody on the invasive capacity of AU-565 cells grown with exogenous EGF by determining the number of cells that penetrated the Matrigel. We found that both delphinidin and C225 antibody treatment inhibited EGF-induced invasion of AU-565 cells in a dose-dependent manner (Figure 14).



**Figure 14: Effect of delphinidin and C225 antibody treatment on EGF-induced invasion of AU-565 cells.** AU-565 cells were treated with delphinidin (5–40  $\mu\text{M}$ ) or C225 antibody (1–4  $\mu\text{g/ml}$ ) and were loaded into the chamber. EGF (50 ng/ml) was added to each of the wells and cells were allowed to invade through the membrane by incubating at 37°C for 18 h after which the invaded cells on the bottom of the insert membrane were detached, lysed and subsequently



detected by CyQUANT<sup>®</sup> GR dye using a fluorescence plate reader at 480/520 nm filter set. The data represent the means  $\pm$  SE of three experiments in which each treatment was performed in multiple wells.

#### **KEY RESEARCH ACCOMPLISHMENTS:**

- Delphinidin, a major anthocyanin known to be present in pigmented fruits and vegetables, inhibits constitutive and EGF-induced phosphorylation of EGFR, activation of PI3K, phosphorylation of AKT and MAPK.
- Delphinidin treatment inhibits constitutive and EGF-induced activation of Brk signaling mediated through EGFR.
- Delphinidin treatment inhibits EGF-induced cell invasion.
- The composite result suggest that delphinidin is an effective inhibitor of EGFR signaling at least in breast cancer cells that act through novel Brk signaling pathway and holds great promise for its treatment.

#### **SIGNIFICANCE:**

Abnormalities in the expression and signaling pathways downstream of the EGFR contribute to progression, invasion, and maintenance of the malignant phenotype in breast cancer. EGFR is expressed at high levels in at least 25 % of breast cancers and is associated with poor prognosis. Many synthetic inhibitors of EGFR are known, but their use is limited because of their unacceptable cytotoxic effects on normal cells. Therefore, identification of a natural, nontoxic agent(s) as an inhibitor of EGFR is of utmost importance. Upon EGF-stimulation, Brk is recruited to the EGFR, and this event activates the catalytic activity of Brk, which in turn phosphorylates paxillin a binding partner and substrate for Brk. The phosphorylation of paxillin promotes the activation of Rac1, thereby stimulating cell migration and invasion in response to EGF. Delphinidin, a major anthocyanin known to be present in pigmented fruits and vegetables, inhibits constitutive and EGF-induced phosphorylation of EGFR, activation of PI3K, phosphorylation of AKT, and MAPK. We also found that delphinidin treatment inhibits constitutive and EGF-induced activation of Brk signaling mediated through EGFR. Furthermore, we found delphinidin inhibits Brk-induced paxillin phosphorylation and activation of Rac 1 signaling. We then compared the growth inhibitory effects of delphinidin and found that its treatment resulted in a decrease in cell growth of cancer cells but had only minimal effects on normal mammary epithelial (184A1) cells. Treatment of breast cancer cells with delphindin inhibited cell growth and invasion and induced apoptosis. Taken together, the composite result suggest that delphinidin is an effective inhibitor of EGFR signaling at least in breast cancer cells that act through novel Brk signaling pathway and holds great promise for its treatment.

## **REPORTABLE OUTCOMES:**

### **Abstract**

Zaman N, **Afaq F**, Khan N, Syed DN, and Mukhtar H. Delphinidin, a major anthocyanin in pigmented fruits and vegetables is a potent inhibitor of epidermal growth factor receptor and its downstream signaling pathway. The 97<sup>th</sup> Annual Meeting of the American Association for Cancer Research, Washington, DC, USA (April 1-5, 2006).

## **CONCLUSIONS:**

This study identifies an abundant fruits and vegetables based anthocyanin delphinidin as an effective inhibitor of EGFR signaling at least in breast cancer cells that act through novel Brk signaling pathway and holds great promise for its treatment.

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<b>Abstract Number:</b>	2266
<b>Presentation Title:</b>	<b>Delphinidin, a major anthocyanin in pigmented fruits and vegetables is a potent inhibitor of epidermal growth factor receptor and its downstream signaling pathway</b>
<b>Presentation Start/End Time:</b>	Monday, Apr 03, 2006, 8:00 AM -12:00 PM
<b>Location:</b>	Exhibit Hall, Washington Convention Center
<b>Poster Section:</b>	22
<b>Poster Board Number:</b>	17
<b>Author Block:</b>	<i>Najia Zaman, Farrukh Afaq, Naghma Khan, Deeba N. Syed, Hasan Mukhtar.</i> Univ. of Wisconsin, Madison, WI

Abnormalities in the expression and signaling pathways downstream of the epidermal growth factor receptor (EGFR) contribute to progression, invasion, and maintenance of the malignant phenotype in breast cancer. EGFR is expressed at high levels in at least 30% of breast cancers and is associated with poor prognosis. Many synthetic inhibitors of EGFR are known, but their use is limited because of their unacceptable cytotoxic effects on normal cells. Therefore, identification of a natural, nontoxic agent(s) as an inhibitor of EGFR is of utmost importance. Delphinidin, a major anthocyanin known to be present in pigmented fruits and vegetables (such as pomegranate, berries, dark grapes, egg plant, tomato, carrot and red onion) possesses potent antioxidant and antiproliferative properties. In this study, employing EGFR positive breast cancer cell lines, we evaluated the effect of delphinidin on EGFR and its downstream signaling pathways. Delphinidin (5-40  $\mu$  M; 3h) treatment to EGFR positive breast cancer cells AU-565 and MCF-10A was found to result in a dose-dependent decrease in the phosphorylation of specific tyrosine residues of EGFR at 1068, 1045 and 845 sites. Delphinidin was also found to inhibit phosphorylation of EGFR in other EGFR positive cancer cells such as A431 and A549. The signaling pathways induced by activated EGFR include the PI3K/AKT and MAPK, both of which play a significant role in the mitogenic and cell survival responses mediated by EGFR. Therefore, we analyzed the expression of these proteins and found that delphinidin treatment of AU-565 and MCF-10A cells inhibited the (i) activation of PI3K, (ii) phosphorylation of AKT, and (iii) phosphorylation of MAPK in a dose-dependent manner. In additional experiments, serum starved AU-565 cells were treated with delphinidin (5-40  $\mu$  M; 3 h) and then incubated without or with EGF (50 ng/ml) for 15 minutes. We found that delphinidin treatment of AU-565 cells inhibited EGF-induced phosphorylation of EGFR, AKT and MAPK, and activation of PI3K in a dose-dependent manner. We next investigated whether blockage of EGFR signaling pathways by delphinidin would inhibit cell growth and induce apoptosis. Treatment of AU-565 and MCF-10A cells with delphinidin (5-40  $\mu$  M; 48 h) inhibited cell growth and induced apoptosis in both these cell lines as determined by MTT assay, confocal microscopy and TUNEL assay. Delphinidin treatment of AU-565 and MCF-10A cells also resulted in cleavage of PARP protein, activation of caspase-3, downregulation of Bcl-2 and increased expression of Bax protein. In summary this study identifies an abundant fruits and vegetables based anthocyanin delphinidin as an effective

blocker of EGFR signaling at least in breast cancer cells. Delphinidin could be developed as an agent for the management of EGFR positive human cancers.

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