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Efficacy and mechanism of action of Deguelin in suppressing metastasis of 4T1 cells

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Abstract

Cancer related deaths in breast cancer patients are due to metastasis of the disease. Murine 4T1 cells (Murine mammary cancer cell line developed from 6-thioguanine resistant tumor) provide an excellent research tool for metastasis related studies because these cells are highly aggressive and readily metastasize to the lungs. In this study we determined the effect of Deguelin on in vivo/ vitro growth and metastasis of 4T1 cells. Deguelin inhibited the in vitro growth of 4T1 cells in a time and dose dependent manner accompanied with reduced nuclear PCNA immunostaining. In cells treated with Deguelin, reduced expression of nuclear c-Met, and its downstream targets such p-ERK and p-AKT was observed. Deguelin reduced the cell migration in 4T1 cells as determined by scratch wound assay. Combined treatment with Deguelin + ERK or PI3K/AKT inhibitor had no additional effect on cell migration. These results indicated that the action of Deguelin on cell migration may be mediated by AKT and ERK mediated signaling pathways. In vivo, Deguelin treatment significantly inhibited growth of 4T1 cells. Deguelin also reduced the occurrence of metastatic lung lesions by 33% when cells were injected intravenously into Balb/c female mice. There was no difference in the body weight as well as liver and spleen weights between vehicle treated control and Deguelin treated animals indicating that Deguelin was nontoxic at the dose used in the present study. These results provide rationale for developing Deguelin as a chemotherapeutic agent for triple negative breast cancer patients.

Keywords

4T1 cells; metastasis; biomarkers; Deguelin; TNBC

Introduction

Over the years the approach or the treatment of breast cancer patients has been streamlined. The classification of breast cancer has now been expanded to include not only steroid receptors and HER2/neu positive or negative breast cancers but to include Luminal A for high ER score higher than 200, Luminal B for ER score between 11 to 199 but HER2/neu negative, ERBB2 positive when ER score is less than 10 but HER2 positive, Luminal and HER2 hybrids when both these are present and finally triple negative breast cancers (TNBC) where all three prognostic markers are absent (1). There are well defined and successfully implemented treatment regimens for patients with steroid receptor and HER2 positive tumor

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types. However, 15–20% of the patients have characteristic of TNBC, they receive aggressive chemotherapy, albeit without significant success rates (2). A large proportion of these patients expressing the TNBC phenotype are metastatic (3). Therefore there is an urgent need to identify new drugs that can be employed to treat this sub-class of breast cancer patients. Over the years these studies have been conducted by employing human TNBC cell lines, specifically MDA-MB-231 cells. However the major problem is the fact that human breast cancer cell lines rarely metastasize when inoculated into athymic mice. Therefore 4T1 murine mammary cancer cells have been extensively used for evaluating efficacy of chemotherapeutic agents on metastasis. These cells when transplanted into syngeneic mice develop tumors with 100% incidence and induce lung metastasis.

In this study we evaluated Deguelin as a potential therapeutic agent using 4T1 cells. Deguelin (7aS, BaS)-13, 13a-dihdro-9-10-dimethoxy-3,3-dimethyl-3H-bis[1]benzo-pyrano[3,4-b:6',5'-e]pyran-7(7aH), a rotenoid, was first isolated from an African plant *Mundulea sericea* (Leguminosae) and was originally identified as a chemopreventive agent (4,5). In addition to anticarcinogenic action Deguelin also showed growth inhibitory action in various cancer cell types such as prostate, lung and others (5, 6,7, 8 9,10 11, 12, 13, 14, 15, 16, 17,18). Previous reports have indicated several modes of action for Deguelin including altered β catenin – Wnt pathway, down regulation of PI3K-AKT signaling, XIAP family members and Survivin levels (9,11,13) as well as inhibition of HSP90 binding to its chaperon proteins(6,12,13,17,19,20). Recent study in pancreatic cells showed that Deguelin not only has growth inhibitory action but also has a potent antimetastatic property (21). In vivo rotenone was found to be toxic, however Deguelin, which is an analog of rotenone was tolerated at a very high dose (22). To the best of our knowledge antimetastatic and therapeutic properties of Deguelin in breast cancer have not been reported. In this study we explored the effect of Deguelin on in vitro and in vivo growth and metastasis of murine mammary cancer cells.

Material and Methods

Cell Culture

The 4T1 mouse mammary carcinoma cells (The cell line was originally developed by Dr. Fred Miller from Michigan Cancer Foundation, Michigan, MI) was obtained from were obtained from American Type Culture Collection (Rockville, MD) and cultured in monolayers in MEM-E supplemented with 10% heat-inactivated fetal bovine serum. 100 εg/mL penicillin and 100 εg/mL streptomycin (Invitrogen) FBS Invitrogen™ by Life Technologies Grand Island, NY) and maintained at 37°C in the atmosphere of 5% CO2 and 95% air. According to ATCC "4T1 is a 6-thioguanine resistant cell line selected from the 410.4 tumor without mutagen treatment. When injected into BALB/c mice, 4T1 spontaneously produces highly metastatic tumors that can metastasize to the lung, liver, lymph nodes and brain while the primary tumor is growing in situ. The primary tumor does not have to be removed to induce metastatic growth." Thus 4T1 is the complete name for the cell line.

Deguelin synthesis

Deguelin was synthesized via a four-step process using commercially available rotenone as starting material (23). Newly synthesized Deguelin was compared with that of commercially available using HPLC and characterized by NMR (Supplemental Results)

Preparation of solutions used in the study

For in vitro studies Deguelin was dissolved in absolute alcohol at concentration of 10mM as stock solution and working solutions were prepared by appropriate dilutions. For in vivo

studies, Deguelin was administered to BALB/c mice in the form of a suspension in 1% Gum Arabic (Sigma-Aldrich® St. Louis, MO) in PBS. In brief, 1mg of Deguelin and 2mg gum Arabic powder were mixed uniformly using a mortar and pestle and then resuspended in 1ml PBS. Animals received 0.05ml suspension which delivered 2mg/kg body weight Deguelin if animal weighed 25g. The inhibitors UO126 and LY294002 were obtained from Santa Cruz Biotechnology, Santa Cruz, CA and were initially dissolved in DMSO. For in vitro assays inhibitors in DMSO were further diluted (1:1000 in ethanol) as working solution (1εl/2 ml media gave final concentration of inhibitors as indicated).

Effect of Deguelin on in vitro cell proliferation

For determining the effects of Deguelin on cell proliferation 4T1 cells (30,000 cells/well) were incubated with increasing concentration of Deguelin ranging from 0 to 500nM for 24, 48 and 72h. At the termination the cells were trypsinized and cell proliferation was evaluated by counting cells using the Z-series Coulter counter (Beckman Inc. Brea, CA) according to the procedure described earlier (12). Data are presented as Mean±SE of percent of vehicle treated control.

Immunohistochemical staining

For immunohistochemical staining of various proteins, cells cultured on glass coverslips and treated with vehicle (ethanol) or Deguelin, at the end of treatment cells were rinsed with PBS and fixed in 4% formalin (10min), permeabilized in ice cold methanol (4min) and then rinsed again with PBS. Cells were incubated with 5% BSA and then incubated with primary antibodies (c-Met, PCNA, p-ERK, ERK, AKT, p-AKT) for 2h. For staining of tumors and lung metastasis 4εm-thick sections of formalin-fixed paraffinembedded tissues were mounted on frosted micro slides, and sections were deparaffinized in xylene and rehydrated by processing through a graded series of alcohol. (100-0%). The sections were rinsed in phosphate-buffered saline (PBS) and then microwaved two times for 5 min in citrate buffer (Vector Laboratories, Burlingame, CA) for antigen retrieval. This method is described in detail previously (24). After PBS wash nonspecific staining was blocked by incubating tissue sections in 5% BSA. Tissues sections were incubated with primary antibodies (c-Met, PCNA, p-ERK, ERK, AKT, p-AKT) in a humidified chamber. All antibodies were diluted according to manufacturer's instructions in PBS containing 1.0% BSA. All primary antibodies (c-Met, p-ERK, PCNA, ERK) were obtained from Santa Cruz Biotechnology, Santa Cruz, Burlingame, CA. After incubation with primary antibody, cells/tumor sections were rinsed extensively with PBS and then treated with appropriate biotinylated antibody followed by biotinylated horseradish peroxidase macromolecular complex using ABC Vectastain kit (Vector Laboratories, Burlingame, CA). Immunoreactivity to specific protein was visualized using DAB (3,3’ Diaminobenzidine, Vector Laboratories, Burlingame, CA) as a chromogen. For c-Met and p-ERK in cells immunostaining was enhanced using $NiCl_2$ with DAB. The cells/tumors were counterstained with hematoxylin, (except c-Met and p-ERK) dehydrated by passing through increasing series of ethanol (0–100%) and cleared in xylene and then mounted using permount. Images were taken at 40x objective (400x magnification) using Olympus BX-51 microscope.

In Vivo studies

Female BALB/c mice, 6 to 7 weeks old, were purchased from Charles River Laboratories (Wilmington, MA) and housed at IIT Research Institute’s Animal facility. Animals were kept in groups of five per cage and fed with AIN76A control diet and water ad libitum and were maintained at 24 ± 2°C temperature, 50 ± 10% relative humidity, and 12-hour light/12-hour dark cycle throughout the study. In a second experiment we used n=10 animals per group, Deguelin dose was increased to 6mg/kg body weight, other experimental details were similar to those mentioned above for 2mg/kg body weight Deguelin dose. All the in vivo

experiments in mice were performed according to approved protocol by IIT Research Institute Animal care and use committee. Every effort was made to prevent any unnecessary pain to the animals.

For in vivo tumor growth, 4T1 cells (500,000 cells/animal) were inoculated subcutaneously with viable cells in dorsal flank of 6–7 weeks old female Balb/C mice. Number of cells was determined using Coulter Counter (12). The next day following cell inoculation, animals were divided in to two groups, one receiving vehicle only and the other with Deguelin (2mg/kg body weight in gum Arabic /PBS solution). Each group consisted of n=4 animals. Deguelin/vehicle was injected daily by intraperitoneal route. Animals were monitored for any signs of drug associated toxicity including body weight changes or regular mobility. Once the palpable tumor developed at the site of injection (on day 12), the tumor size (cm) was measured in three different planes (length), width and depth, these will be 2x radiuses in each plane) twice weekly using Verneer calipers (24). Tumor volume (cm^3) was calculated using the standard mathematical formula (Volume = 4/3xπ/6x(1/2) l (1/2)w (1/2)d for volume determination of an ellipsoid object. At the termination (on day 20 post cell inoculation) of the experiment, animals were sacrificed; tumors were excised and fixed in formalin. Experiments were repeated three times. Data presented are from single independent experiment. Tumor volume is shown as Mean±SE in each group.

The effects of Deguelin on metastasis was investigated by injecting 10,000 4T1 cells in 50εL PBS through tail vein in 5–6 week old Balb/c female mice using 27 gauge needle. Animals were randomized into two groups. Deguelin was prepared as a suspension (PBS: gum Arabic) as described earlier. One group received vehicle whereas the second group received 2mg Deguelin/kg mouse body weight daily by intraperitoneal injections for 20 days. Each group consisted of n= 5 animals. Animals were sacrificed by CO2 asphyxiation, lungs were exposed and trachea was detached. Lungs were perfused with 7.5% of India ink solution (diluted in distilled water) through the trachea, lungs were excised and then placed in destaining solution (85% of 70% Ethanol, 10% of 37% formaldehyde and 5% acetic acid). After 24h metastatic lesions in each lung were counted using dissecting microscope. Data represent mean number of lesions in each group. After counting lesions lungs were processed for immunohistochemical staining. In brief, lungs were dehydrated by passing through series of alcohol, cleared in xylene and then embedded in paraffin. Paraffin (4 εm) sections of lungs were mounted on glass slide and processed for immunohistochemical staining as described earlier. In order to access toxicity, body weight, liver and spleen weights were recorded at termination. In a separate experiment we delivered Deguelin at 6mg/kg body weight dose for 20 days using above protocol. Each, vehicle and Deguelin treatment group consisted of n=10 animals.

Effect of Deguelin on cell migration

The scratch assay was performed to detect the effect of Deguelin on cell migration on 4T1 cells. Briefly, 4T1 cells were plated in six well plates and allowed to grow to 90% confluence. Cell monolayers were wounded (151.6 ± 2.4εm) with a sterile 1ml pipette tip; cultures were washed with fresh media to remove detached cells from the plates. Cells were treated with either media containing vehicle (ethanol) or 250nM Deguelin and incubated for 16h at 37°C. In order to determine whether the effect of Deguelin is mediated through either the ERK or AKT pathways, cells were pretreated with inhibitors (10εM UO126 or 10εg/ml LY 294002) and incubated at 37°C for 8h and then wounded as described above. Inhibitor-pretreated cells were incubated with Deguelin, appropriate inhibitor, Deguelin and inhibitor (as indicated) or vehicle containing media for additional 16h. At termination of experiment cultures were fixed in 4% formalin, and then stained with Hematoxylin. Distance of wounded area in each well was measured using DP-70 imaging software equipped with scale bar on an Olympus BX-51 microscope attached to a digital camera. Data represent

mean ± SE distance of wounded area. Data represent mean of triplicates and minimum of 3 readings from each well. Representative photographs of culture wells treated with vehicle and Deguelin are shown.

Western blot analysis

Western blot analysis was performed according to the procedure described previously (12). In brief, Deguelin and vehicle treated cells were lysed in freshly prepared protein extraction buffer (20mM HEPES, pH 7.9, 400nM NaCl, 0,1%NP-40, 10% glycerol, 1mM Sodium vanadate, 1mM Sodium fluoride, 1mM Dithiothreitol, 1mM Phenylmethylsulfonyl fluoride, 10(εg/ml aprotinin, 10εg/ml leupeptin) for 45 min on ice. Lysates were centrifuged and the supernatant was collected. Total protein concentration in the lysate was determined using modified Lowry's method (Bio Rad, Hercules, and CA). Protein samples were separated on 10% polyacrylamide gels and then transferred to nitrocellulose membrane. The membranes were blocked by incubating in 5% BSA in PBS and then incubated overnight with primary antibody (diluted according to manufacturer's instruction in 5% BSA) followed by appropriate secondary antibody. Primary antibodies against p-ERK, p-AKT, AKT, ERK, c-Met and PCNA are similar to those used for immunostaining assay. G-6-PDH, tubulin or actin (Santa Cruz Biotechnology, Santa Cruz, CA) detection was used as a loading control. Chemiluminescence reaction was performed to visualize protein band using ECL kit from Amersham Pharmacia Biotechnology (Piscataway, NJ). Pixel intensities of each protein specific band and the ratio of specific protein-pixel intensity/actin-pixel intensity were calculated using UN-SCAN-IT imaging software (Silk Scientific, Inc. Orem, Utah 84059 USA).

Statistical Analyses

All experiments were performed in triplicates Data generated were subjected to appropriate statistical analysis using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA). Differences between means from two different groups were subjected to student's '*t*' test, whereas one-way analysis of variance (ANOVA) followed by Bonferroni adjustment was used to analyze significant differences between three or more groups. Results were considered to be significantly different when $P < 0.05$.

Results

Deguelin inhibits growth of 4T1 cells

In this study we evaluated effect of Deguelin on highly aggressive metastatic 4T1 cells. As shown in Fig. 1, Deguelin significantly ($P<0.001$) inhibited growth of 4T1 cells in a time and dose dependent manner. At 250nM Deguelin, following 48 h exposure approximately 80% growth inhibition was observed (Fig. 1a). Also number of cells showing nuclear PCNA (a biomarker of cell proliferation-shown by an arrow) immunostaining was less in Deguelin treated cells as compared to that in control (Fig. 1b). Effect of Deguelin on PCNA protein levels was further confirmed by western blots. PCNA (36 Kda) level was significantly reduced in cells treated with Deguelin as compared to those treated with vehicle only (Fig. 1b). We also examined effect of Deguelin on cell proliferation evident by Hematoxylin staining. As shown in Fig. 1b, Deguelin treated cells showed fewer cells undergoing mitosis than those cells treated with vehicle (Fig. 1b)

Deguelin affects c-Met, p-ERK and p-AKT expression

4T1 cells express c-Met receptor. (25). These cells are stimulated by HGF, a known ligand of c-Met (25) and cell migration is inhibited by Gefitinib, a small tyrosine kinase inhibitor. We therefore examined whether Deguelin affects c-Met expression in these cells. In control

vehicle treated cells intense c-Met immunostaining was present in the nucleus. In cells incubated in the presence of 250nM Deguelin for 48h, weak c-Met staining was seen. We examined whether effect of Deguelin is evident on the downstream targets of c-Met, which are ERK and AKT. In cells treated with Deguelin reduced nuclear expression of p-ERK, p-AKT was seen as compared to those cells treated with vehicle only (Fig. 1c). Results obtained by immunohistochemistry were further confirmed by western blot analysis. As shown in Fig. 1d relative levels of c-Met (143Kda), p-AKT (60Kda), p-ERK (42/44Kda) were decreased in cells treated with Deguelin for 48h than in vehicle treated controls (Fig. 1d). Although there was reduced expression of total ERK, the total expression of AKT was not altered following Deguelin treatment (Fig. 1d).

Deguelin inhibits cell migration

We pretreated confluent monolayer of 4T1 cells with UO126 or LY294002 or vehicle containing media and incubated at 37°C for 8h. At the end of incubation a wound was produced and cells in each group were further treated with vehicle (ethanol), Deguelin (250nM) alone, inhibitor alone or Deguelin plus inhibitor. Cells were incubated at 37°C for additional 16h. At that time cells were fixed, stained with hematoxylin and wounded area were examined and distance between the two edges of culture was measured under 20x objective of a light microscope. As shown in Fig. 2a vehicle treated cells migrated from the two edges of the wound and minimum (22εm) distance between two edges of wound was seen. On the other hand, Deguelin treated cells showed significantly ($P<0.001$) reduced wound closure, a gap between wounded edges was approximately 60εm. Cultures pretreated for 8h with UO126 and then incubated in regular vehicle containing media showed significantly ($P< 0.001$) reduced cell migration as compared to control. Presence of UO126 for entire time of the study showed significantly reduced cell migration as compared to that of vehicle, effect was similar to that of Deguelin treatment only. Combination treatment with Deguelin and UO126 (24h) was not significantly different from the single treatments with Deguelin or UO126 (24h). Pretreatment with PI-3k inhibitor LY294002 for 8h showed no significant effect on cell migration as compared to vehicle treated control. However similar to UO126, Deguelin treatment in cells pretreated for 8h with LY 294002 significantly reduced cell migration. However the effects of combination treatment of Deguelin and LY294002 (24h) on suppression of healing was similar to those observed following Deguelin or LY294002 treatments singly. These results suggest that Deguelin effect is mediated through ERK/AKT pathway (Fig. 2a). Fig. 2b shows mean + SE distance (εm) between the edges of wounded area following treatment with Deguelin or inhibitors as indicated.

Deguelin inhibits in vivo growth of 4T1 cells

To the best of our knowledge, effect of Deguelin has not been examined on in vivo growth of triple negative mammary cancer cells. We examined effect of Deguelin on in vivo growth of 4T1 cells transplanted s.c. Next day Deguelin was given at 2mg/kg body weight daily as a suspension by i.p. route. Control group received vehicle only. As shown in Fig. 3a, tumor size was significantly ($P<0.05$) smaller in Deguelin treated animals at days 16, 18 and 20 post cell inoculation of cells as compared to vehicle treated group. At termination (on day 20 after cell inoculation) tumor size in animals treated with Deguelin was 57 ± 7.50% of that in the vehicle treated animals (Fig. 3a). Deguelin treated animals showed normal appetite as evident from food consumption and normal mobility. In a subsequent study we followed identical protocol using 10 animals per group but increased dose of Deguelin (6mg/kg body weight). The tumor size in Deguelin group was 59± 10.22 % of control. These results suggested that even though 6mg dose of Deguelin used was non-toxic, increasing dose of Deguelin had no added benefit on reducing tumor growth. Results also indicate that Deguelin is effective in vivo at a non-toxic concentration.

Deguelin alters expression of various biomarkers in 4T1 tumors

The in vitro data (Fig. 1b) obtained in 4T1 cells on effect of Deguelin on PCNA, c-Met, p-ERK and p-AKT prompted us to further examine expression of the later proteins in 4T1 xenografts originated in vivo experiment in mice. As shown in Fig. 3b, relative nuclear expression of PCNA, c-Met, p-AKT, and P-ERK was reduced in tumors from Deguelin treated animals as compared to those treated with vehicle only. We also observed the presence of nuclear expression of HIF-1α in tumors from control animals, and reduced expression of this protein in those tumors from Deguelin treated animals. These results suggest that Deguelin inhibits both ERK and p-AKT pathway leading to reduced expression of HIF −1α.

Deguelin inhibits lung metastasis of 4T1 cells

We examined the effect of Deguelin on metastasis of 4T1 cells to the lungs. 4T1 cells (10,000 cells/animal) were injected intravenously into adult female mice via tail vein. After 24h animals received either Deguelin (2mg/kg body weight) or vehicle daily by i.p. route. After 20 days animals were sacrificed, lungs were exposed and perfused with India ink, fixed and examined under dissecting microscope. The lesions in lungs appear as white spots with black ink background. Number of metastatic lesions in the lungs in Deguelin treated animals was significantly ($P<0.006$) less as compared to those in vehicle treated animals (Fig. 4a). Metastatic lesions varied in size in both groups. In vehicle treated control group 300± 25 (lesions/lung) and in Deguelin group 199±11 metastatic lesions/lungs were found. These results were further confirmed. In a separate study (n=10 animals/group), we injected 7500cells/animal, animals received vehicle or Deguelin as mentioned above. Metastatic lesions in the lungs were significantly less in Deguelin treated animals as compared to those in controls (data not shown). Body weight, liver and spleen weights at termination were not significantly different in control and Deguelin treated groups (Table 1).

Deguelin reduces expression of PCNA, c-Met, p-ERK and other biomarkers in the lung lesions

We examined whether PCNA, c-Met, p-ERK p-AKT and HIF-1α expressions in metastatic lesions in the lungs of vehicle and Deguelin treated animals were different. Similar to that observed in in tumors nuclear PCNA expression in metastatic lung lesions was reduced in Deguelin treated animals. Similarly we observed reduced expression of c-Met, HIF-1α, p-ERK and p-AKT in lung lesions of Deguelin treated animals. These results clearly suggest that inhibitory effect of Deguelin on the in vivo and in vitro growth as well as metastasis of 4T1 cells is partially mediated through inhibition of c-Met expression, which eventually affects ERK and p-AKT pathway and their downstream targets such as HIF-1α (Fig. 4b).

Discussion

Deguelin was originally identified as a chemopreventive agent and has shown growth inhibitory action in various cancer cell types including breast, prostate, lung and others (9, 10, 11, 13, 16). Deguelin is reported to modulate β-catenin – Wnt pathway (12,13) down regulate PI3K-AKT signaling (10), XIAP family members of proteins, COX-2 and NF-κB (7,9). Although recently, the antiproliferative and therapeutic efficacy was studied in various cancer types, it is still not known whether Deguelin can inhibit growth of mammary cancer cells in vivo and suppress metastatic disease. The major reason for holding back on exploring potential use of Deguelin as therapeutic agent has been its reported neurologic toxicity (26). Previously it was reported that animals receiving Deguelin developed Parkinson's like disease (26) when it was delivered by continuous infusion. However, recent studies reported in the literature as well as data presented in this study clearly suggest that Deguelin is quite safe and effective as antiproliferative or antimetastatic agent at non-toxic

doses. It is quite possible that schedule, dose, route of administration and vehicle used to deliver the drug may have a major impact on inducing a toxic side effect. In an earlier study Deguelin was infused continuously using a peristaltic pump and as a result neurological toxicity was evident, we and others(,8,12,14,17) have administered Deguelin at 2–10 mg/kg body weight dose by gavage / i.p. route without any toxic symptoms. In our study Deguelin mixed as a suspension in PBS –gum Arabic solution and administered by intraperitoneal route daily up to 6mg/kg body weight 20days did not affect food/water intake, regular normal mobility, body weight or liver and spleen weights. No signs of Parkinson-like symptoms were observed in these animals. These data clearly suggest that Deguelin is quite safe even at 3 times the effective dose. An antiproliferative or antimetastatic activity of Deguelin was achieved at 2mg/kg body weight. Thus Deguelin treatment warrants further evaluation as therapeutic agent for breast cancer.

Metastasis is a complex pathological disease process, and it is a major cause of cancer related deaths. In this study we examined the effect of Deguelin both in vitro and in vivo tumor growth as well as metastasis of 4T1, mammary cancer cells. These cells are highly aggressive and their disease progression in syngeneic animals mimics stage IV disease progression in women. Moreover, 4T1 cells are ER-/PR- and Her-2 negative and thus represent a triple negative breast cancer phenotype.

We showed that Deguelin treatment resulted in significant inhibition of 4T1 cell proliferation both in vitro and in vivo. In vitro evaluation proliferation marker PCNA suggested that Deguelin treatment given daily in vivo by i.p. route reduced the growth of 4T1 cells transplanted s.c. in Balb/c animals. Interestingly, Deguelin treatment reduced the formation of metastatic lesions in the lungs of these mice following intravenous injection of 4T1 cells. This is the first report to indicate that Deguelin may be of significant value in treating highly aggressive metastatic breast cancer.

Many critical functions such as cell growth, differentiation, cell migration are regulated by various growth factor receptors. c-Met is a member of tyrosine kinase receptor kinase family and its known ligand is a Hepatocyte growth factor (HGF). c-Met is classified as an oncogene and is often overexpressed in various tumor types including 4T1 cells (27). The role of c-Met in breast cancer biology has been well documented (28). It plays a major role in tumor growth, cancer invasion and metastasis and is overexpressed in a variety of cancers such as lung, breast, ovary, kidney, colon, thyroid, liver, and gastric (29, 30, 31, 32, 33,34,35, 36,37, 38,39). In general, reports available in the literature clearly suggest that high c-Met expression is associated with invasive human breast cancer, shorter recurrence free survival and overall reduced overall survival. The intracellular domain of Met contains a kinase domain and a docking site for multiple molecules known to regulate specific downstream cell signaling pathways such as Ras-MAPK , ERK1/2, p38, PI3K – AKT, PKCα, IKKα -NFκB as well as p-FAK (40,41). c-Met also regulates beta catenin mediated activation of various genes by regulating LEF-1. Since Deguelin inhibited cell migration in our studies, we examined the effect of Deguelin on c-Met expression in 4T1 mammary cancer cells. While c-Met was present largely in the nucleus in 4T1 cells in vitro, it was expressed both in the cytoplasm and nucleus in the tumors and metastatic lesions. We observed reduced expression of nuclear c-Met in 4T1 cells, tumors and metastatic lesions following Deguelin treatment. Reduced c-Met expression following Deguelin treatment was accompanied by reduced cell growth, as well as PCNA expressions. We also observed reduced levels of p-AKT, and p-ERK (also known as MAPK42/44), which are downstream targets of c-Met.

In solid tumors, low oxygen tension or hypoxia can induce changes in homeostasis of tumor which lead to impaired cell growth and survival (42). Under hypoxia tumor cells undergo

adaptive changes in expression of various proteins and genes enabling them to successfully continue to grow and survive in hostile environment (42). The later molecular changes necessary for continued growth of tumor cells are regulated by HIF-1α, a transcription factor known to regulate >30 genes involved in angiogenesis, tumor growth, cell survival, proliferation and metastasis (42). HIF-1α is a downstream target of ERK1/2, ERK is also known to play a crucial role in the growth of solid tumors. Phosphorylation of ERK phosphorylates HIF-1α and activates the protein (43). On the other hand c-Met (upstream regulator of ERK) expression is shown to be regulated by HIF-1α(44). HIF-1α is activated (phosphorylated) under hypoxia, it activates c-Met transcription by binding to c-Met promoter, resulting in increased expression of c-Met (44) . In breast cancer, expression of HIF-1α is implicated as an independent prognostic biomarker for node negative as well as node positive breast cancer. Generally HIF-1 α expression in breast cancer is associated with poor overall and disease free survival (42). In our study we examined HIF-1 α expression in 4T1 cells in vitro under normoxic culture condition. We failed to observe detectable HIF-1 α in control and Deguelin treated cells. This was expected as under normal oxygen condition HIF-1 α protein has short half-life and is quite unstable and undergoes ubiquitination process. These results suggest that reduced c-Met expression observed in 4T1 cells in culture following Deguelin treatment is independent of HIF-1 α action. On the other hand we observed higher nuclear expression of both c-Met and HIF-1 α in both, tumors and metastatic lesions from control vehicle treated as compared to those of Deguelin treated animals. These results clearly suggest that Deguelin affects both c-Met and HIF-1 α levels in vivo. Nuclear expression of HIF-1 α is directly involved in metastatic spread of cancer, it increases at the transcriptional level production of VEGF, a critical growth factor for neovascular growth and thereby promote metastasis (45).

Both ERK and AKT are major players in regulating cell migration and therefore we used UO126, an inhibitor for ERK and LY294002, an inhibitor for PI3K/AKT to confirm the effects of ERK and AKT. Similar to Deguelin, incubation of cells with these inhibitors also reduced cell migration..

In conclusion, our data in the present study clearly indicate that Deguelin is a potent antiproliferative and antimetastatic agent. Deguelin reduced the in vitro/in vivo growth of highly metastatic murine mammary cancer cells. Deguelin action is mediated through down regulation of c-Met activation and thereby inhibiting downstream key molecules regulating multiple pathways such as PI3K-AKT, ERK1/2. Deguelin effect on ERK1/2 and p-AKT reduces the expression of various downstream gene (HIF-1α) involved in cell proliferation, cell survival, angiogenesis and metastasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

(4T1)	Mouse mammary tumor cell line 4T1
(MDA-MB231)	Human breast cancer cell ine MDA-MB-231
(ER)	estrogen receptor

(PR)	progesterone receptor
(HER-2)	human epidermal growth factor receptor-2
(PCNA)	Proliferating Cell Nuclear Antigen
(c-Met)	MET or MNNG HOS Transforming gene
(HGF)	hepatocyte growth factor
(ERK)	extracellular-signal-regulated kinases
(p-ERK)	phosphorylated ERK
(AKT)	Mouse strain Ak-thymoma (t)
(p-AKT)	phosphorylated AKT
(COX-2)	cyclooxyhenase-2
(ERB-B2)	avian erythroblastosis oncogene B
(TNBC)	triple negative breast cancer
(XIAP)	X-linked inhibitor of apoptosis protein
(hsp90)	heat shock protein 90
(RT)	reverse transcriptase
(PBS)	phosphate buffered saline
(DAPI)	4',6-diamidino-2-phenylindole
(PI)	propidium iodide
(BSA)	bovine serum albumin
(DAB)	diaminobenzedene
(AIN 76A)	American Institute of Nutrition 76A
(PI3K)	Phosphatidylinositol 3-kinases
(DMSO)	`dimethylsulfoxide
(FAK)	focal adhesion kinase-1
(HIF1α)	hypoxia inducing factor 1alpha
(i.p.)	intraperitoneally
(i.v.)	intravenous
(NFκB)	nuclear factor kappa-light-chain-enhancer of activated B cells
(Ras)	rat sarcoma
(MAPK)	mitogen activated protein kinase
(PKC)	protein kinase C
(IKK)	inhibitor of kappa B (IKB) kinase
(LEF1)	Lymphoid enhancer-binding factor-1
(TCF)	transcription factor
(Wnt)	combination of integration 1 (int) and drosophila wingless (wg)
(c-MYC)	myelocytomatosis oncogene

(VEGF)	vascular endothelial growth factor
(MMP-7)	matrix metalloproteinase 7
(uPA)	urokinase-type plasminogen activator;

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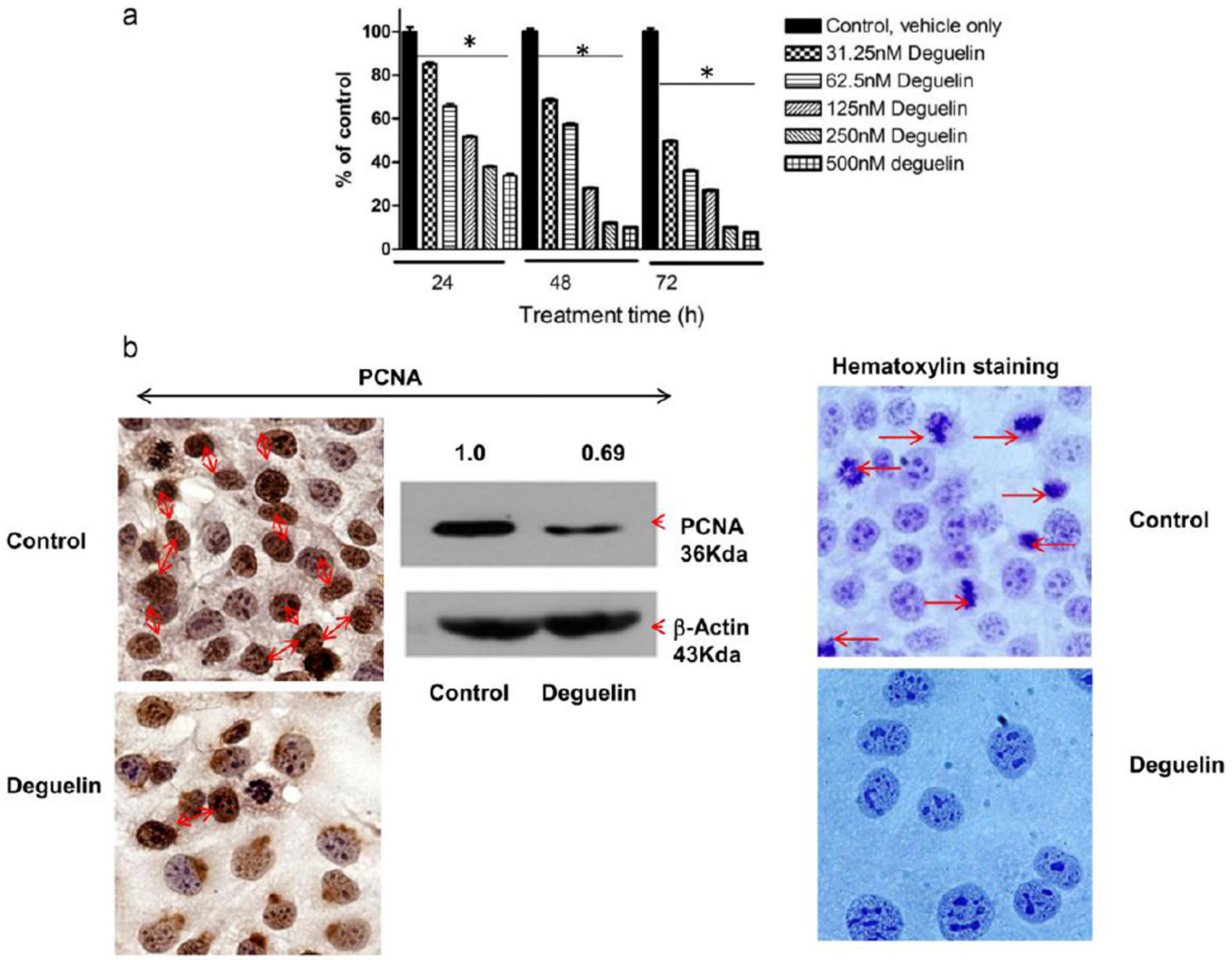


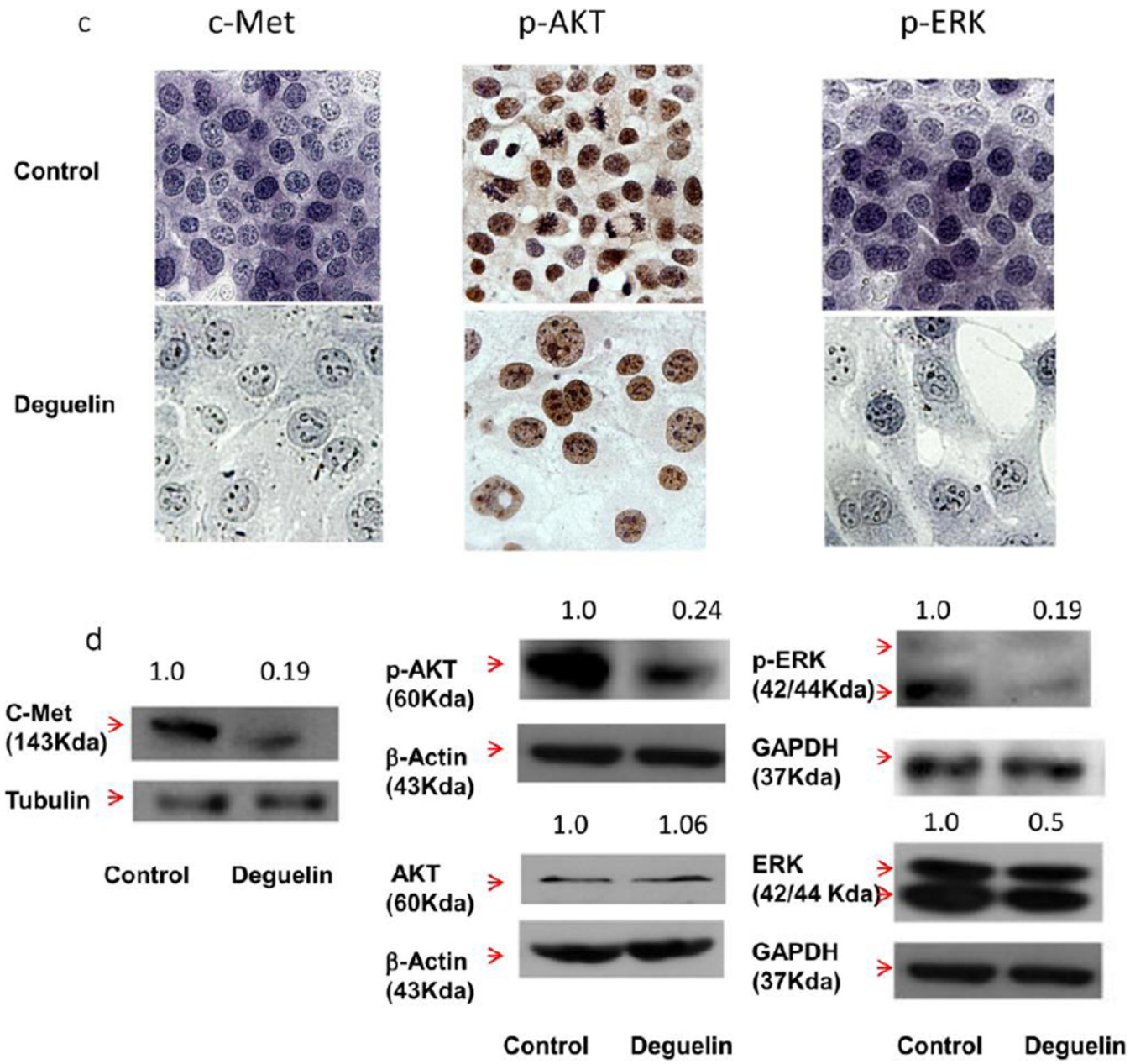


Fig. 1.

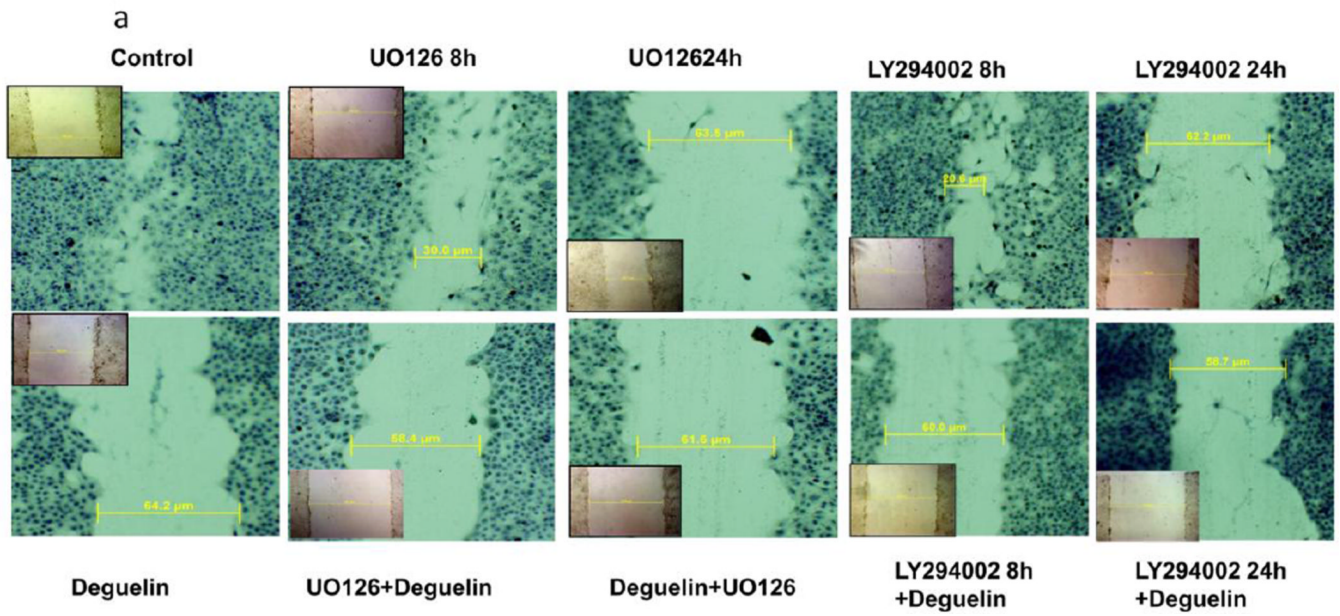
a Effect of Deguelin on proliferation of 4T1 cells. Cells were plated in six well culture plates at 30,000 cells/well density, incubated at 37°c under the culture condition described in methods section. After 24h, media was replaced with either vehicle (ethanol) or Deguelin (0–500nM) supplemented media. After 72h incubation cells were trypsinized and counted using coulter counter. Each bar represents Mean± SE of quadruplet's observation. Experiment was repeated three times. * indicates significant difference ($p<0.05$) between control and indicated treatment.

b Effect of Deguelin on PCNA expression and cell proliferation (mitosis, hematoxylin staining) in 4T1 cells. . Cells were cultured on glass coverslips an then treated with vehicle (ethanol) only or Deguelin (250nM) and incubated at 37°c for 48h. Immunohistochemical stating for PCNA was performed as described in detail under methods section. Brown (DAB

staining) indicates immunoreactivity to PCNA protein. Immunohistochemical results were further confirmed by western blots. Protein extracts from Deguelin and vehicle treated cells were electrophoresed, immunblotted and developed as described earlier. PCNA specific (36Kda) band was compared. β-Actin (43Kda) immunoblot is shown as a loading control. All images for PCNA immunostaining and Hematoxylin staining are taken using 40x objective lens. Arrow in hematoxylin stained cells indicate mitotic figures, in PCNA immunostaining show positive staining in the nucleus. Arrows in the western blots show band specific to indicated protein.

c. Effect of Deguelin on c-Met, p-AKT and p-ERK expression in Deguelin treated 4T1 cells. Cells cultured on coverslips were treated with vehicle (ethanol) only or Deguelin (250nM) and incubated at 37°C for 48h. Immunohistochemical stating for indicated proteins was performed as described in detail under methods section. For c-Met and p-ERK DAB containing NiCl2 (Bluish gray staining) as a chromogen. No nuclear counterstaining was performed. For p-AKT immunostaining only DAB (brown) was used as a chromogen. Nuclear counter staining was done with hematoxylin (blue). All images are taken at 40x objective lens.

d. Western blots of c-Met, p-AKT, AKT, p-ERK, ERK in protein lysates from 4T1 cells treated for 48h with Deguelin (250nM) or vehicle only. For c-Met 7.5% and ERKs and AKTs 10% SDS poly acrylamide gel was used. β actin/ Tubulin/ G-6-PDH was used as protein loading control. Arrows shows band specific to the indicated protein. Number in the parenthesis shows molecular weight (Kda) of specific protein. Numbers on the top of the blots indicate relative protein intensities in control and Deguelin treated cell. Protein band intensities were normalized in relation to loading control proteins (ratio of pixel intensity of protein/pixel intensity of control protein). Ratio in vehicle treated cells was considered as 1.0.



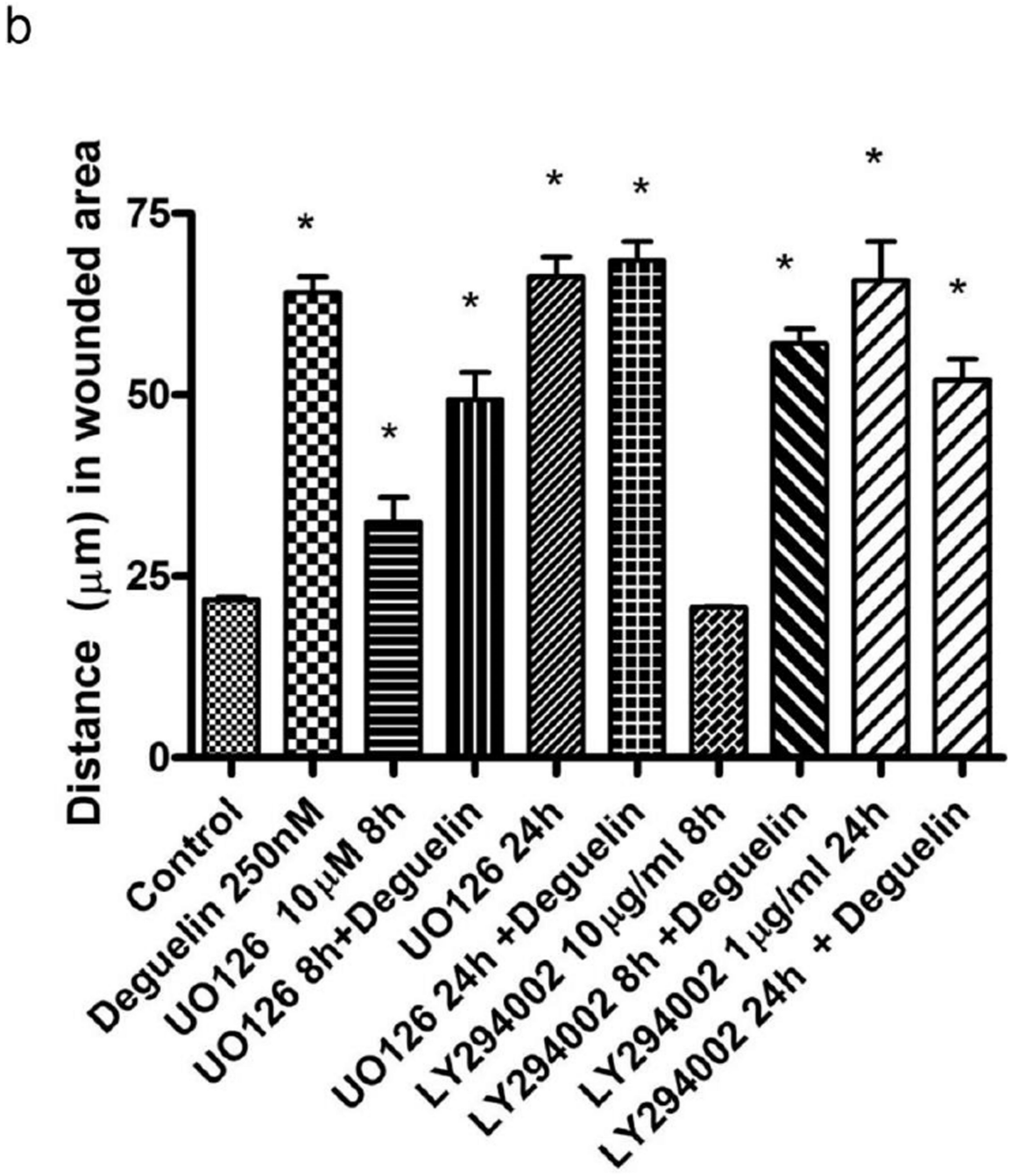


Fig. 2.

a Effect of Deguelin, UO126 (ERK inhibitor), LY294002 (PI3K/AKT inhibitor) either alone or in combination on cell migration: 4T1 cells were pretreated (8h) with indicated inhibitors or vehicle only as control. After 8h incubation at 37°C a scratch wound was inflicted and cultures were further incubated at 37°C for 16h with media containing vehicle only (ethanol), Deguelin (250nM) only, inhibitors only or combination of Deguelin (250nM) + inhibitors (UO126 at 10εM and LY294002 at 10εg/ml)) as indicated. Cultures were fixed, stained with hematoxylin and examined under light microscope. Distance between wounded

areas was determined for each treatment. Representative images are shown. Inset shows wounded area prior to the treatment.

b Quantitative measurement of cell migration following Deguelin (250nM), UO126 (10εM, LY294002 (10εg/ml) and combinations. Each bar represents mean value of minimum of 4 independent observations. Data represent Mean±SE (εm) distance between wounded areas. * indicates significant ($p<0.05$) difference between control and indicated treatment.

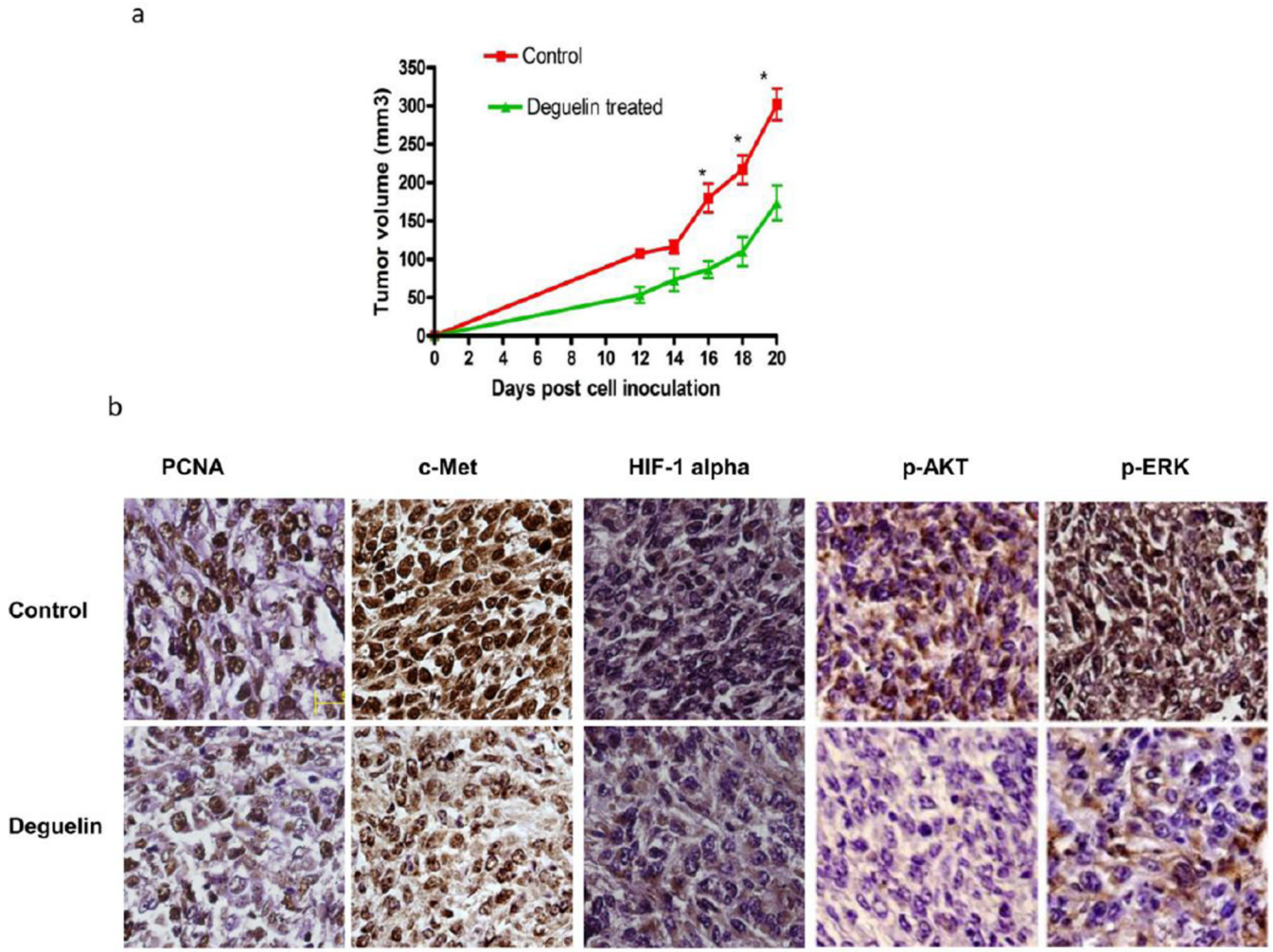


Fig. 3.

a Effect of Deguelin on in vivo growth of 4T1 cells transplanted s.c. in Balb/c mice. 4T1 cells (500,000 cells/0.1ml) were injected s.c. in the dorsal flank of female athymic mice. Animals received vehicle only or Deguelin (2mg/kg body weight) daily by i.p. injections. Tumor volume was measured twice /weekly) once it became palpable using calipers. Data represent mean± SE tumor volume in each group. * Indicates significant difference between control and treated group.

b Effect of Deguelin on various biomarkers. Tumors developed in animals treated with vehicle (ethanol) only or Deguelin (250nM) were fixed, embedded in paraffin and sectioned (4εm thickness), paraffin sections were processed for immunohistochemical staining of PCNA (proliferation marker), c-Met, HIF-1ε , p-AKT, p-ERK. Brown staining indicates immunoreactivity to indicated antigen, Tumor sections were counterstained with Hematoxylin. Images were taken using 40x objective lens (400x magnification).

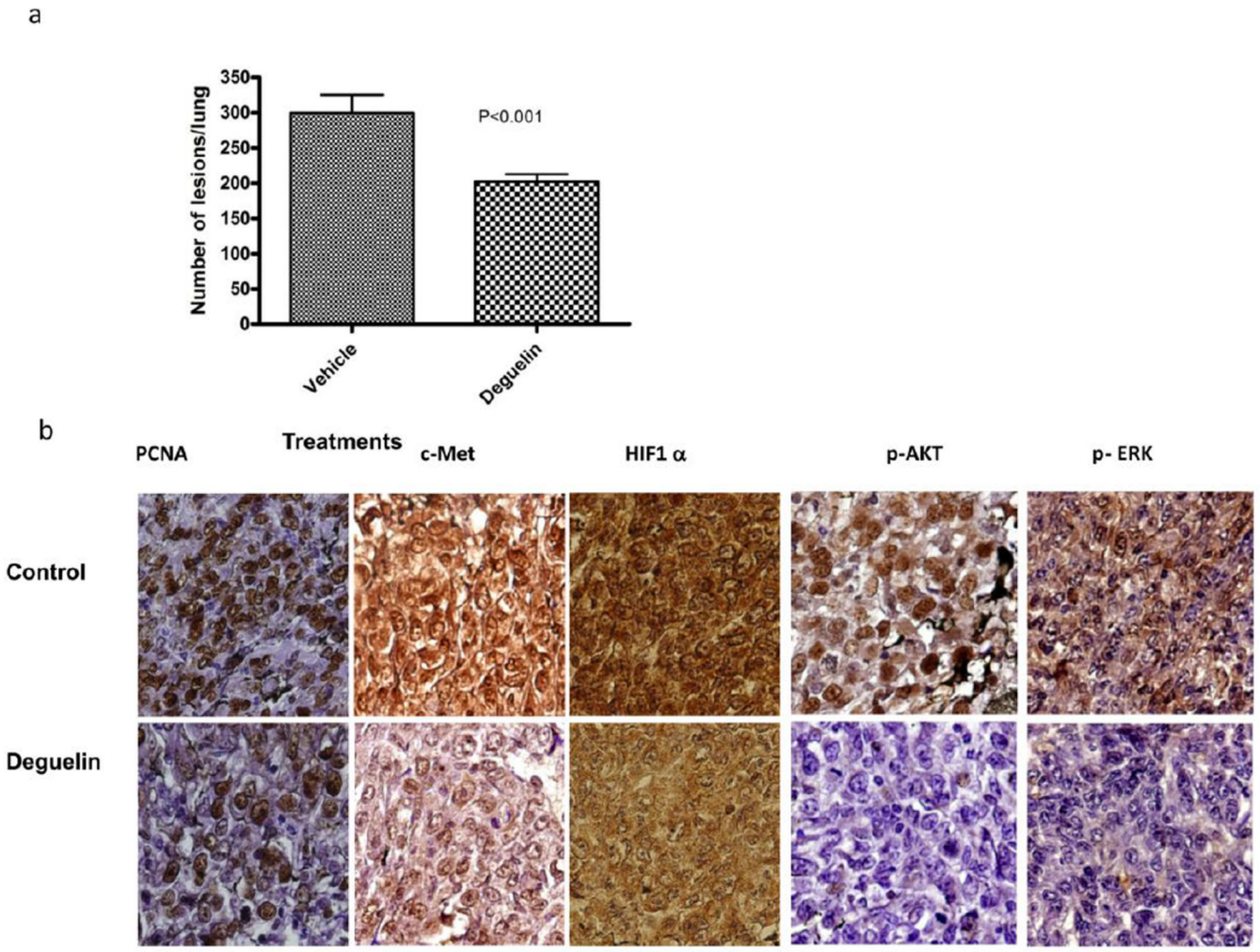


Fig. 4.

a Number of metastatic lesions in the lungs in Deguelin treated and vehicle only treated animals. 4T1 cells (10,000/animal) were injected in to Balb/c mice through tail vein, animals received vehicle or 2mg/kg body weight Deguelin daily by i.p. route for 21 days as described above. At termination, metastatic lesions in the lungs were counted following India ink infusion in the lungs. Data represent mean+ SE (numbers) of lesions/lung.
b Effect of Deguelin on various biomarkers in lung lesions. Lungs from vehicle and Deguelin (2mg/kg body weight) treated animals were fixed in formalin, dehydrated, paraffin embedded and processed for paraffin sections. Sections were processed for immunohistochemical staining of various biomarkers as indicated. images were taken at 40x (cropped) objective lens; representative images from each group and parameter are shown.

Table 1

Body and organ weights in control and Deguelin (6mg/kg body weight treated animals at termination of the experiment

	Control	Deguelin treated
Body weight (g)	19.92 ± 0.49	19.62 ± 0.31
Liver (g)	0.93 ± 0.03	1.06 ± 0.02
Spleen (g)	0.19 ± 0.01	0.19 ± 0.10