



## Original research article

## DMH4, a VEGFR2 inhibitor, effectively suppresses growth and invasion of lung cancer cells

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## ABSTRACT

Non-small-cell lung cancer (NSCLC), the most common type of lung cancer, remains the leading cause of cancer death worldwide. Blocking vascular endothelial growth factor (VEGF) signalling is an effective approach to the treatment of NSCLC. Small molecules have been proven to be good resource for discovery of inhibitors of VEGF signalling. DMH4 is a small molecule that we previously developed and demonstrated to have the property of selectively inhibiting VEGF signalling by targeting VEGF receptor 2 (VEGFR2). In this study, we reported that DMH4 can effectively block phosphorylation of VEGFR2 in both H460 and A549 NSCLC cells, which resulted in significant reduction of NSCLC cell viability in a dose-dependent manner, and the growth inhibition (GI50) of DMH4 against H460 and A549 cell lines were 13.27 and 2.75  $\mu$ M respectively at 24 h. Our further studies demonstrated that DMH4 significantly suppressed migration and invasion of A549 and H460 cells, and induced apoptosis in those cells. Therefore, DMH4 as a small molecular VEGFR2 inhibitor may represent a new valuable drug lead for NSCLC treatment.

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## Introduction

Lung cancer remains the leading cause of cancer death worldwide (DeSantis et al., 2014; Siegel et al., 2014, 2015). Overall lung cancer survival rate is abysmally low, with the 5-year survival rate of an approximately 15%. Approximately 80% of all lung cancers are non-small-cell lung cancer (NSCLC) including squamous cell carcinomas, adenocarcinoma, and large cell carcinoma (DeSantis et al., 2014; Sholl, 2014; Siegel et al., 2015). Despite recent improvements in diagnosis and treatment, there is still an imperative need of new therapies for NSCLC.

Vascular endothelial growth factor (VEGF) signalling can promote tumour progression via both paracrine angiogenic model and autocrine model (Chatterjee et al., 2013; Herbst and Sandler, 2004; Lichtenberger et al., 2010). In the paracrine model, VEGF

expressed by tumour cells binds to its cognate receptors such as VEGF receptor 2 (VEGFR2) on endothelial cells, thus stimulating vessel formation to supply oxygen and nutrient for tumour growth and metastasis (Carmeliet, 2000). On the other hand, some recent studies have demonstrated that VEGF and its receptor VEGFR2 are expressed in primary tumour cells to promote tumour growth in an autocrine mode (Fan et al., 2005; Lee et al., 2007; Lichtenberger et al., 2010; Vincent et al., 2005). For instance, both VEGF and VEGFR2 were reported to express in NSCLC cells and the autocrine VEGF/VEGFR2 feed-forward loop was identified in NSCLC cells to amplify VEGF secretion which is required for development of fully angiogenic tumour *in vivo* (Chatterjee et al., 2013; Wang et al., 2013; Yang et al., 2011). Thus, VEGF signalling is an important therapeutic target for cancer treatment, and significant efforts have been made to develop drugs that target the VEGF cascade in the past (Falchook et al., 2013; Hurwitz, 2004; Liang et al., 2014; Sandler et al., 2006).

Using an *in vivo* zebrafish phenotype-based screening, we have previously developed a small novel molecule that was named DMH4. DMH4 can effectively disrupt zebrafish intersomitic vessel formation which is known to be regulated by VEGF pathway, and further study confirmed that DMH4 specifically inhibits VEGF

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signalling by targeting VEGFR2 (Hao et al., 2010). Hence, in this study we tested the effects of DMH4 on inhibiting NSCLC cell viability, migration and invasion, and inducing NSCLC cell apoptosis.

## Materials and methods

### Materials and cell culture

A549 and H460 cell lines purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) were cultured in RPMI-1640 (Cyclone, Logan, Utah, USA) supplemented with 10% FBS (Cyclone, Logan, Utah, USA) and 1% penicillin-streptomycin (Sigma, St. Louis, MO, USA). DMH4 (6-[4-[2-(4-Morpholinyl)ethoxy]phenyl]-3-phenylpyrazolo[1,5-a]pyrimidine), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), DMSO (dimethyl sulfoxide), and penicillin were purchased from Sigma (St. Louis, MO, USA).

### Cell proliferation assay

$6 \times 10^3$  cells were plated on each well of 96 well-plates for overnight. DMH4 was then administrated into the cells for incubation of 24 h at concentrations of 0.625  $\mu\text{M}$ , 1.25  $\mu\text{M}$ , 2.5  $\mu\text{M}$ , 5  $\mu\text{M}$ , 10  $\mu\text{M}$ , 20  $\mu\text{M}$  and 40  $\mu\text{M}$ . MTT (Sigma, St. Louis, MO, USA) was then added into each well of post-treated cells followed by 4 h incubation. The medium was then aspirated and DMSO was added to dissolve the formazan. Then absorbance of the medium was measured at 570 nm using an ELISA plate reader (BioTek, Winooski, VT, USA).

### Scratch wound healing assay

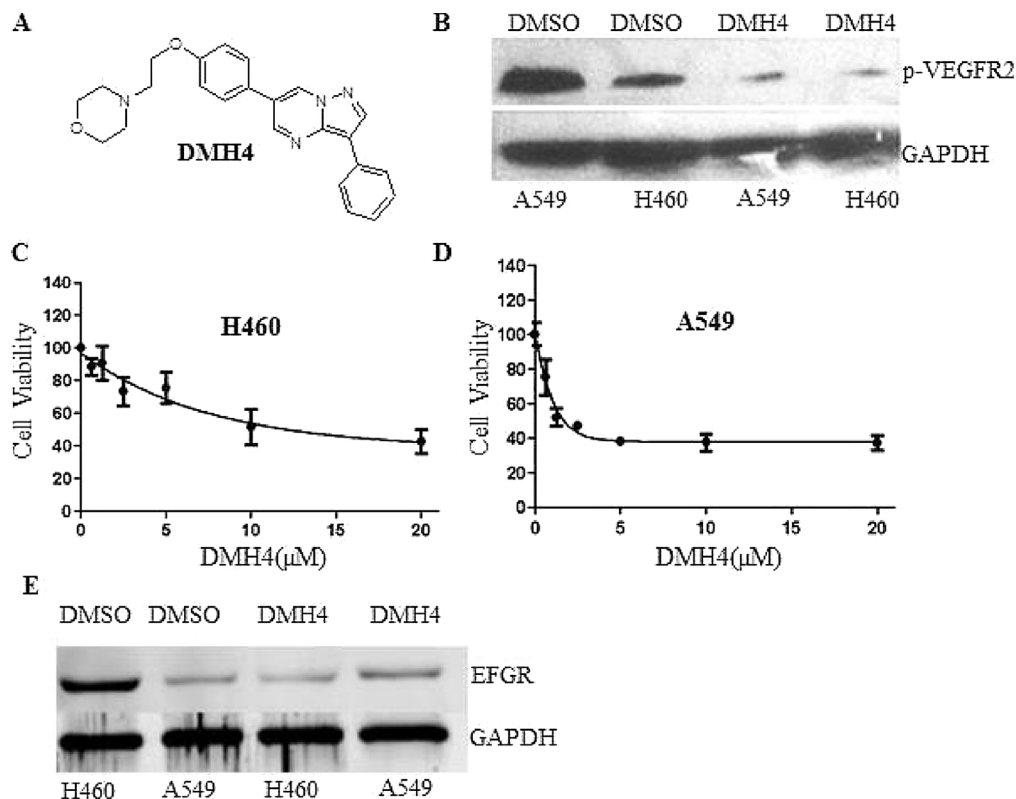
$4 \times 10^5$  cells were cultured in 6 well-plates overnight to form monolayer. On the following day, wounds were created by a straight scratch with a 1 ml pipette tip in the center of the culture. The cells were washed with PBS, and treated with 3  $\mu\text{M}$  DMH4 for 24 h. Photographs were taken when wounds were created and after 24 h incubation using phase-contrast microscopy (Nikon, Melville, NY, USA), and gap distances were quantitatively evaluated using software ImageJ (NIH). The gap distances after 24 h incubation were normalized with the gap distance at 0 h as the migration rates.

### Modified boyden chamber assay

Cell invasion was measured using a 24-Multiwell Insert System (8  $\mu\text{m}$  membrane, BD Biosciences) according to the manufacture instruction. The cell culture inserts were coated with matrigel (BD Biosciences). Both A549 and H460 cells were seeded at a concentration of  $3 \times 10^5$  cells/chamber respectively. After 24 h incubation with or without DMH4 (1  $\mu\text{M}$  and 3  $\mu\text{M}$ ), cells that had not moved to the lower wells were removed from the upper face of the filters using cotton swabs, and the cells that invaded through the matrigel-coated-inserts were counted.

### Cell apoptosis assay

The FITC Annexin V/Dead Cell Apoptosis Kit (Invitrogen, Eugene, OR, USA) with FITC annexin V and Propidium Iodide (PI) for flow cytometry provided a rapid and convenient assay for cell apoptosis. Briefly, cells were treated with 2.5  $\mu\text{M}$  DMH4 for



**Fig. 1.** DMH4 blocked VEGFR2 phosphorylation and dramatically attenuated viabilities of NSCLC cells. (A) Structure of DMH4; (B) Western blotting showed that 2.5  $\mu\text{M}$  DMH4 effectively blocked phosphorylation of VEGFR2 in both H460 and A549 cells; (C) and (D) A549 and H460 cells were treated with DMH4 at various concentrations for 24 h followed by the MTT assay and the results demonstrated that DMH4 treatment significantly reduced viability of A549 and H460 cells in a dose-dependent manner ( $P < 0.05$ ). Measurements were performed in triplicate for each point from three independent experiments; (E) Western Blot showing that EGFR expressing level was reduced significantly in H460 cells, but not in A549 cells.

24 h and harvested after cold PBS wash. Then cells were stained by FITC annexin V and PI working solution followed by flow cytometry analysis.

#### Western blot analysis

Briefly, after 24 h treatment with 2.5  $\mu$ M DMH4, cells were lysed with RIPA buffer (Sigma), and the cell lysates were then separated by 10% SDS-PAGE gels and transferred to a PVDF membrane. The membrane was blocked with Tris-buffered saline (TBS) with 5% skim milk for 1 h at room temperature, followed by primary antibody incubation at 4 °C overnight. The membrane was then washed with TBS with 0.1% Tween-20 before 2 h incubation with secondary antibodies at room temperature. The primary antibodies included the rabbit anti-human phospho-VEGF Receptor 2 antibody (1:1000 dilution, Cell Signalling, Danvers, MA, USA), mouse anti-human EGFR antibody (1:1000 dilution, Cell Signalling, Danvers, MA, USA) and mouse anti-human GAPDH antibody (1  $\mu$ g/ml, R&D, Minneapolis, MN, USA). Two secondary antibodies used were the goat anti-rabbit HRP-conjugated antibody (Cell Signalling, Danvers, MA, USA) anti-mouse HRP-conjugated antibody (R&D, Minneapolis, MN, USA).

#### Results

DMH4 effectively blocks VEGFR2 phosphorylation in NSCLC cells and suppresses NSCLC cell proliferation.

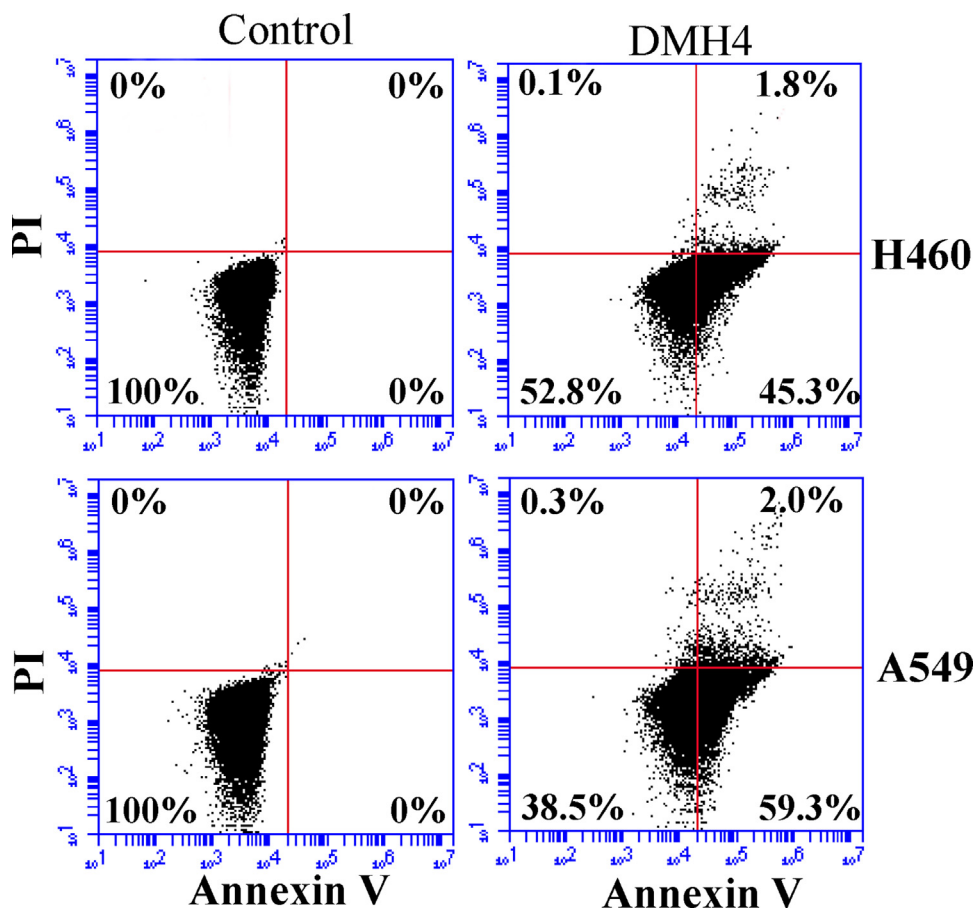
Phosphorylation of VEGFR2 is a critical step for VEGF signalling activation, and DMH4 (Fig. 1A) was previously shown to block

phosphorylation of VEGFR2 in endothelial cell lines. To confirm its effects on NSCLC cells, we treated H460 and A549 NSCLC cells with DMH4 and DMSO as vehicle control respectively and examined phosphorylation of VEGFR2 (phospho-VEGFR2) levels by Western Blotting. Our result showed that both H460 and A549 NSCLC cell types displayed high basal expression of phospho-VEGFR2, and DMH4 treatment effectively suppressed phospho-VEGFR2 expression. It is suggested that DMH4 can block VEGF signalling in NSCLC cells (Fig. 1B). As a tyrosine inhibitor, DMH4 may affect or targeted EGFR expression. The Western Blotting result shows that EGFR protein level is reduced by DMH4 in H460 cell line, but not in A549 cell line (Fig. 1E). DMH4 induced both VEGFR and EGFR expression, and VEGFR result is more correlated to cell viability result (Fig. 2).

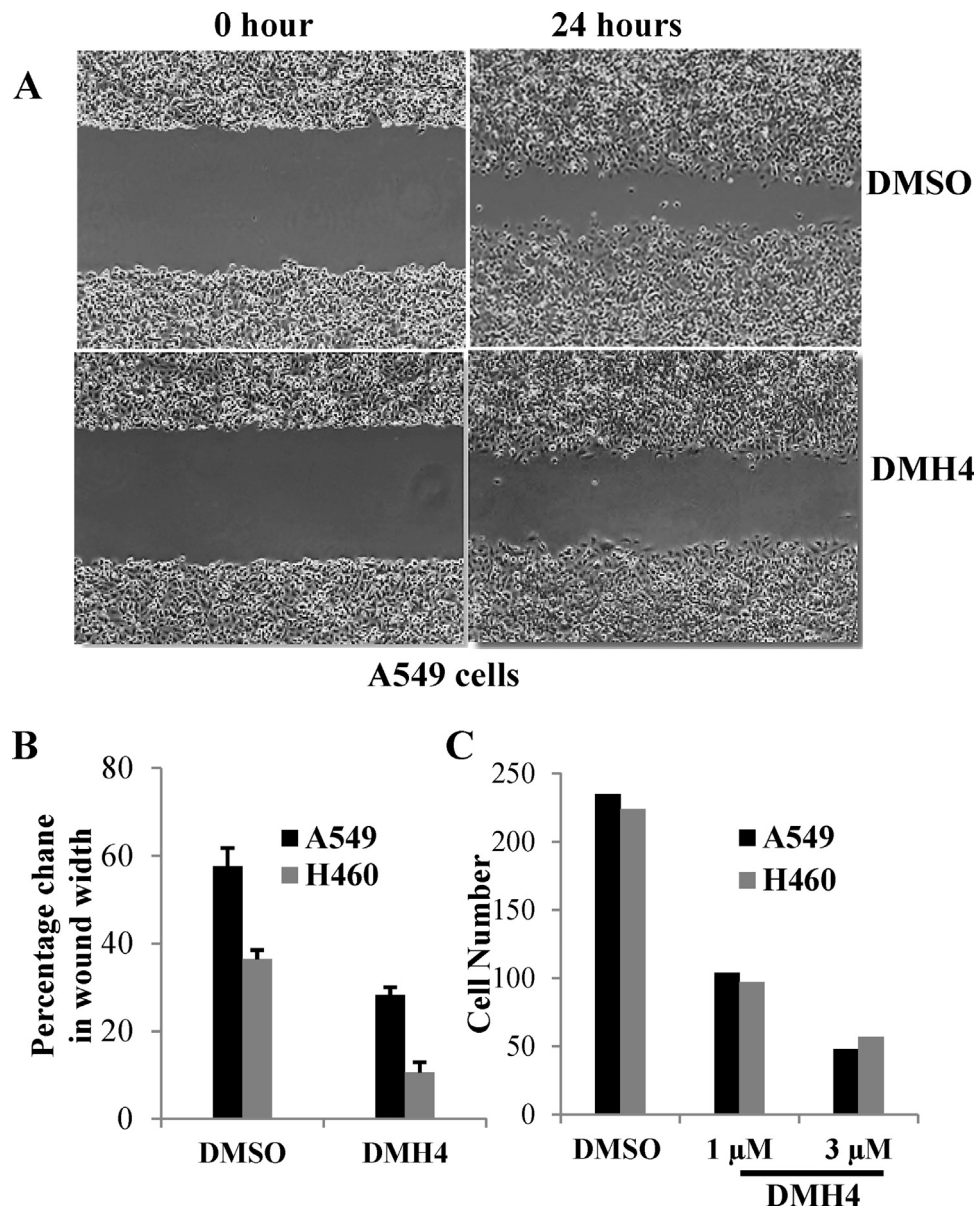
Next we examined the effect of DMH4 on NSCLC cell viability by the MTT assay. The NSCLC A549 and H460 cells were treated with DMH4 for 24 h at various concentrations. The MTT assay results demonstrated that DMH4 significantly reduced viability of A549 and H460 cells respectively in dose-dependent manners ( $P < 0.05$ , Fig. 1C and 1D), and the GI50 of DMH4 were 13.27 and 2.75  $\mu$ M after 24 h treatment for H460 and A549 cell line. Our result showed that A549 cell line was 5 times more sensitive to DMH4 than Bevacizumab (Xiao et al., 2016).

#### DMH4 dramatically decreases migration and invasion of NSCLC cells

Since cell migration and invasion play an important role in cancer progression, we then examined the effects of DMH4 on NSCLC cell migration and invasion *in vitro*. For cell migration assay, we first created wound gaps in the cultured A549 and H460 cells



**Fig. 2.** DMH4 treatment dramatically induced NSCLC cell apoptosis. A549 and H460 cells were treated with DMSO (control) and 2.5  $\mu$ M DMH4 for 24 h followed by double stained with FITC annexin V and PI. Flow cytometry analysis showed that DMH4 significantly induced NSCLC cell apoptosis.



**Fig. 3.** DMH4 downregulates NSCLC cell migration and invasion. (A) Representative images from scratch-wound assays performed in the cultured A549 cells treated with DMSO and DMH4 (3  $\mu$ m) for 24 h; (B) Cell migrations were quantified by the gap distances after 24 h treatment normalized with the initial gap distances for both A549 and H460 cells. Measurements were performed in triplicate for each point from two independent experiments; (C) The effect of DMH4 (1  $\mu$ m and 3  $\mu$ m) on invasion of A549 and H460 cells was determined using modified Boyden chamber assay in a 24-Multiwell Insert System (8  $\mu$ m membrane, BD Biosciences) coated with matrigel.

followed by treatment with vehicle DMSO and 3  $\mu$ m DMH4 for 24 h respectively. The gap distances were then normalized with the initially measured distances. As shown in Fig. 3A and B, DMH4 dramatically slowed down cell migration in contrast to the control of vehicle DMSO in A549 and H460 cells.

Moreover, we examined DMH4 on cell invasion by using modified Boyden chamber assay. Both A549 and H460 cells were seeded on matrigel-coated chambers followed by 24 h incubation with vehicle DMSO or DMH4 (1  $\mu$ m and 3  $\mu$ m). DMH4 dramatically reduced the cell invasion through matrigel-coated membranes by about 56% at 1  $\mu$ m and 75%–79% at 3  $\mu$ m for A549 and H460 cells in comparison with the vehicle controls (Fig. 3C).

#### DMH4 significantly induces NSCLC cell apoptosis

Compared with normal live cells, phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma

membrane in apoptotic cells, thus exposing PS to the external cellular environment. FITC annexin V has a high affinity to PS, and propidium iodide (PI) can stain dead cells with red fluorescence via binding to the nucleic acids. The flow cytometric analysis results indicated that DMH4 treatment promoted 45.3% cell apoptosis in A549 cells and 59.3% in H460 cells (Fig. 2).

#### Discussion

VEGF is an essential growth factor for vascular endothelial cells, and its contributions to angiogenesis for cancer growth and metastasis via a paracrine effect have been well documented. Blocking VEGF signalling can disrupt angiogenesis of tumour capillaries and to suppress cancer growth due to oxygen and nutrient deprivation. In addition, VEGF and VEGF receptors are expressed on many non-endothelial cells including cancer cells to promote cancer progression through autocrine signalling. For



instance, an autocrine feed-forward loop was reported in lung cancer cells in which cancer-derived VEGF stimulates VEGF signalling via VEGFR2 activation, and inhibition of cancer cell VEGFR2 dramatically slowed down lung cancer growth (Chatterjee et al., 2013). To date, two antibodies have been successfully developed to inhibit VEGF signalling for NSCLC treatment. Comparing to Bevacizumab, a recombinant humanized monoclonal antibody targeting VEGF ligand, approved by FDA in 2006 to treat advanced non-squamous NSCLC in combination with carboplatin/paclitaxel chemotherapy, NSCLC cell line A549 is more sensitive to DMH4 (Cohen et al., 2007; Xiao et al., 2016). More recently, another monoclonal antibody drug, Ramucirumab, which targets VEGF receptor 2 (VEGFR2) was approved to treat the metastatic NSCLC in 2014 (Larkins et al., 2015). In comparison with the protein-based drugs like antibodies, small molecular drugs exhibit many advantages. For instance, small molecular drugs are generally easier to manufacture, and can be taken orally whereas protein-based drugs often require injection or other parenteral administration due to their poor stability. However, so far no small molecule VEGF inhibitor-based drugs are available to treat NSCLC in clinic.

We previously developed a small molecule named DMH4 in a zebrafish-based *in vivo* structure/activity relationship study and DMH4 inhibits VEGF signalling by selectively targeting VEGFR2. Here we demonstrated that DMH4 effectively inhibited NSCLC cell viability, migration and invasion, and induced NSCLC cell apoptosis, suggesting therapeutic potential of DMH4 as a VEGFR2 inhibitor in NSCLC treatment. Future studies of potencies of DMH4 or its analogues in *in vivo* animal models will be critical in evaluation of DMH4 clinical application.

## Conclusion

DMH4, a small molecule VEGFR2 inhibitor developed in our previous zebrafish-based screening, can significantly reduce NSCLC cell viability, dramatically suppress migration and invasion of NSCLC cells, and induce apoptosis in those cells. Therefore, DMH4 may represent a new drug lead for NSCLC treatment.

## Conflict of interests

The authors have no conflict of interest to disclose.

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