4-[6-(4-Isopropoxyphenyl)Pyrazolo[1,5-a]Pyrimidin-3-yl]Quinoline) Suppresses Metastasis Through HIF-1α/MCT-4-Mediated Lactate Excretion in Colorectal Cancer Cells

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Abstract

DMH1 (D4-[6-(4-isopropoxyphenyl)pyrazolo[1,5-a]pyrimidin-3-yl]quinoline) is a bone morphogenetic protein antagonist. However, the influence of DMH1 on colorectal cancer metastasis remains unknown. Hence, this study aimed to investigate the inhibitory effect of DMH1 on the migration of cancer cells. *In vitro* incubation with DMH1 (1 mM) inhibited the high protein level of HIF-1α in CT-26 cells. Transwell assay confirmed that DMH1 suppresses CT-26 cells migration via the HIF-1α under hypoxia. The accumulation of lactate in culture medium down-regulated the extracellular pH, then stimulated the migration of CT-26 cells.

DMH1 suppressed the migration of CT-26 cells through HIF-1α/MCT-4 signaling pathway mediate the excretion of lactate. The data demonstrated that DMH1 downregulated the high intracellular pH levels via the HIF-1α/MCT-4 signaling pathway to mediate lactate excretion. This study indicated that DMH1 might serve as a potential drug for treating tumor migration by minimizing the intracellular pH via the HIF-1α/MCT-4 signaling pathway.

Introduction

Cancer metastasis is the primary cause of high mortality in patients with colorectal cancer [1]. It is considered one of the most important factors for patient survival [2].

Hypoxia is a common characteristic of tumors. The hypoxic response is primarily attributed to the hypoxia-inducible factor (HIF). HIF is composed of α- and β-subunits and plays a central role in adapting and responding to low oxygen levels. Studies have indicated that HIF-1α regulates the expression of several genes involved in cell metastasis under hypoxia conditions [3].

(4-[6-(4-isopropoxyphenyl)pyrazolo[1,5-a]pyrimidin-3-yl]quinoline) (DMH1) is a low-molecular-weight chemical used as an inhibitor of bone morphogenetic protein (BMP) [4]. However, compared with other BMP inhibitors, DMH1 does not significantly inhibit the transforming growth factor-β (TGF-β) receptors [5]. Studies confirmed that DMH1 could reduce tumor growth in subcutaneous xenograft mouse models probably by blocking BMP signaling. DMH1 was recently shown to inhibit breast-to-lung metastases, reduce primary growth in mice, and suppress tumor growth in a human lung cancer xenograft model [6].

This study examined the effects of DMH1 on the expression of HIF-1α in CT-26 cells *in vivo* and *in vitro*, the effects of DMH1 on CT-26 cell migration, and the underlying mechanism. The findings indicated that DMH1 inhibited cancer metastasis, thus providing preclinical evidence for using DMH1 to impede colon cancer cell migration.

Materials And Methods

Cell culture and cell transfection
CT-26 cells were maintained in RPMI-1640 medium (HyClone, UT, USA) supplemented with 10% fetal bovine serum (Gibco, CA, USA), 100 U/mL penicillin, and 100 mg/mL streptomycin (Gibco, CA, USA). All cell lines were cultured at 37°C in 5% CO₂ environment. The hypoxic condition was achieved by placing cancer cells in a sealed hypoxia chamber equilibrated with a triple gas mixture of 1% O₂, 5% CO₂ and 94% N₂.

For siRNA transfection, CT-26 cells were plated at 3×10⁵ cells/mL in OPTI-MEM serum-free medium and transfection of siRNA duplex using Lipofectamine RNAiMAX Reagent Agent (Life Technologies) according to the manufacturer’s instructions. Mouse MCT-4 siRNA (Cat#sc-40120) was purchased from Santa Cruz (Biotechnology, USA); mouse HIF-1α siRNA (Cat#s67530) purchased from Invitrogen (USA).

**Cell viability assay**

CT-26 cells were seeded into 96-well plates (5×10⁵ cells/well) and incubated at 37°C for 24 h. On reaching 90% confluence, the cells were treated with different concentrations of DMH1 for 24 h. 20 μL of MTT (pH 4.7) was added to each well, and then the cells were incubated for another 4 h. 100 μL of 10% SDS 0.01 M HCl was added, and the cells were incubated at 37°C overnight to dissolve the formazan crystals. Absorbance was measured at 570 nm.

**Western blot**

Briefly, the samples were subjected to electrophoresed in 10% SDS-PAGE and separated proteins were transferred to nitrocellulose membrane. The membrane were blocked in 5% non-fat milk overnight at 4°C. The appropriate primary antibodies HIF-1α (Cat#WL01607) and GAPDH (Cat#WL01114) were purchased from Wanleibio (China); MCT-4 (Cat#22787-1-AP) was purchased from Proteintech (USA). The appropriate primary (at dilution 1:200 in phosphate-buffer saline) were incubated with fluorescence-conjugated goat anti-rabbit IgG secondary antibody at dilution 1:1000 which purchased from Invitrogen Corporation (Invitrogen, USA). Odyssey v3.0 software was used to analysed protein bands.

**Transwell assay**

Cell migration assays were performed using 8.0 μm pore size coated with 0.5% gelatin (Costar). The lower chamber contained 15% serum as a chemoattractant. Cancer cells were prepared in serum-free medium, and 5×10⁴ cells were added to the upper chamber in migration buffer (M199 containing 0.1% BSA). After 4 h of incubation at 37°C, cells were removed from the upper surface of the membranes with a cotton swab, and cells that migrated to the lower surface were fixed with 4% paraformaldehyde for 30 mins and then stained with 0.1% crystal violet for 10 mins. Migrated cells were counted under a microscope.

**Lactate concentration**
The concentration of lactate in culture media were detected by using assay kits (Cat#A019-2-1, Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions.

Statistics

All data were expressed as mean ± standard error of the mean. Data were assessed with SPSS 15.0 evaluation version (SPSS, Chicago, IL, USA) statistical programme with one-way analysis of variance. P < 0.01 was considered indicate a significant.

Results

DMH1 effectively inhibited CT-26 cell migration under hypoxia conditions

The in vitro experiments revealed that DMH1 stimulated CT-26 cell apoptosis at a concentration of more than 5mM after 24 h. However, it had no effect on the apoptosis of CT-26 cells at a concentration of less than 1mM (Fig. 1a) with or without serum. Hence, 1mM DMH1 was used to examine whether DMH1 inhibited the migration of CT-26 cells.

The results indicated that the number of CT-26 cells that migrated through the chamber membrane markedly reduced after treatment with DMH1 under hypoxic conditions (P < 0.01, Fig. 1b). But DMH1 had no effect on the migration of CT-26 cells under normxia (Fig. 1b).

DMH1 effectively inhibited CT-26 cell migration via HIF-1α

Previous studies demonstrated that DMH1 suppressed migration [7]. However, the underlying mechanism was still unknown.

HIF-1α regulates the expression of several genes involved in cancer cells migration under hypoxia conditions [3]. In our studies, we found that treatment with DMH1 significantly downregulate the high level of HIF-1α under hypoxic conditions (P < 0.01, Fig. 2a). And hence, we wondered that DMH1 suppress migration of CT-26 through HIF-1α mediate signaling pathway.

Incubating CT-26 cells with CoCl2 (the specific inducer of HIF-1α, 150μM) significantly increased the protein levels of HIF-1α (Fig. 2b), but not those of HIF-1β (data not shown). The transwell assay results showed that treatment with CoCl2 stimulated CT-26 cell migration. However, co-application of CoCl2 and DMH1 inhibited the CoCl2-induced migration of CT-26 cells (P < 0.01, Fig. 2c-d). Next, the HIF-1α siRNA (siHIF-1α) was used to test the hypothesis. The protein level of HIF-1α decreased in CT-26 cells after the transfection of HIF-1α siRNA, but not that of HIF-1β siRNA (data not shown), compared with that in the non-transfection group (P < 0.01, Fig. 3a). These data indicated that the experimental conditions were reliable.

Next, the Transwell assay was used to clarify the effects of DMH1 incubation on HIF-1α-induced cell migration. The results revealed that CoCl2 stimulated the migration and DMH1 inhibited the migration of
CT-26 cells in the negative control (NC) group. However, CoCl$_2$ did not stimulate the migration of CT-26 cells after transfection of HIF-1α siRNA. Also, siRNA-mediated HIF-1α knockdown almost completely abrogated the inhibition of migration induced by incubation with DMH1 ($P < 0.01$, Fig. 3b). These results accorded with the results obtained after culturing CT-26 cells transfected with HIF-1α siRNA under hypoxia condition ($P < 0.01$, Fig. 3c). And hence, those results indicated that DMH1 inhibit the migration of CT-26 cells via HIF-1α.

**DMH1 suppressed lactate-mediated CT-26 cell migration**

Recently, some studies confirmed that the accumulation of lactate reduced the pH in the tumor microenvironment, which induced cancer cell migration [9]. Hence, it was hypothesized that increased lactate levels in culture media, which reduced the extracellular pH (pHe), might stimulate the migration of cancer cells.

In our studies, we found that the CT-26 cells cultured at pH 6.8 migrated much more compared with cells grown at pH 7.4 (tested at pH 6.8). Accordingly, CT-26 cells grown at pH 7.4 migrated less compared with those cultured at pH 6.8 (tested at pH 7.4). These findings supported that low pH (acidic environment) stimulated the migration of cancer cells (Fig. 4a).

Furthermore, it was found that exposure to 20mM lactate for 24 h was not cytotoxic, irrespective of the presence or absence of serum. Hence, 20mM lactate was chosen for the subsequent experiments (Fig. 4b).

After incubation with lactate for 24 h, it was found that the pH in the upper compartment was lower than that in the lower compartment when lactate was added to the upper compartment. Also, the pH in the lower compartment was lower than that in the upper compartment when lactate was added to the lower compartment. These results confirmed that lactate could not enter the membrane from upper to lower (or lower to upper) compartments (Fig. 4c). The Transwell assay showed that 20mM lactate delivered into the upper compartment significantly stimulated CT-26 cell migration, but lactate did not act as a chemoattractant (Fig. 4c) [10].

The observed migratory changes could be exclusively not due to an unspecific cellular response. Hence, the migratory effects were tested by adding D-lactate, NaCl, or pyruvate. Initially, adding D-lactate (20mM) did not affect CT-26 cell migration. It was verified that the changes in cell migration could not be attributed to the changes in osmolarity after adding sodium chloride (NaCl, 20mM), similar to D-lactate. Pyruvate (20mM) also did not promote CT-26 cell migration (Fig. 4d). Therefore, the results confirmed that L-lactate promoted CT-26 cell migration.

**DMH1 inhibited CT-26 cell migration through HIF-1α/MCT-4 signaling pathway**

Our previous studies indicated that 1) low extracellular pH stimulate the migration of CT-26 cells which induce by lactate; 2) DMH1 inhibit the migration of CT-26 cells via HIF-1α. But, whether DMH1 inhibited
the migration of CT-26 cells via HIF-1α induce the excretion of lactate was still unknown.

In our experiments, the lactate concentration in the culture media was upregulated over time ($P < 0.01$; Fig. 5a). However, the lactate level was downregulated in the culture media with CT-26 cells after treatment with DMH1 under hypoxic conditions and treatment with CoCl$_2$. The same results were also obtained after transfection of HIF-1α siRNA ($P < 0.01$, Fig. 5a).

Our study indicated that DMH1 inhibited lactate excretion in CT-26 cells under hypoxic conditions. However, whether DMH1 affected lactate-induced extracellular pH alteration (pHe) remained unknown. The CT-26 cells underwent extracellular acidification within 24 h after hypoxia exposure and treatment with CoCl$_2$ ($P < 0.01$, Fig. 5b). However, treatment with DMH1 significantly reversed the low extracellular pH which induce by hypoxia and CoCl$_2$. The same results were also obtained after transfection of HIF-1α siRNA.

Studies indicated that MCT-4 regulated the excretion of lactate in the tumor microenvironment, which always accompanied extracellular acidification [8]. And the Long-term exposure of tumor cells under extracellular acidification leads to migration [16]. Under normoxia, DMH1 had no effect on the expression of MCT-4(Fig 5c). However, the protein levels of MCT-4 was upregulated under hypoxia and treatment with CoCl$_2$, and DMH1 downregulated the high protein level of MCT-4 under hypoxia and CoCl$_2$ ($P < 0.01$, Fig. 5d-e). Surely, the transfection of HIF-1α siRNA suppressed the high level of MCT-4 under hypoxia ($P < 0.01$, Fig. 5d-e). The same results were also obtained after transfection of HIF-1α siRNA.

Studies confirmed that MCT-4 is one of the downregulators of HIF-1α [8]. And our results also indicated that DMH1 inhibited the extracellular acidification through HIF-1α/MCT-4 induced excretion of lactate in CT-26 cells. But, whether DMH1 inhibited the migration of CT-26 cells via MCT-4 induce the excretion of lactate was still unknown. With RNAi assay, we found MCT-4 levels decreased in cultured CT-26 cell transfected with MCT-4 siRNA compared with that in the non-transfection group ($P < 0.01$, Fig. 6a).

In our studies, we also observed that CT-26 cells underwent extracellular acidification within 24 h after hypoxia exposure and treatment with CoCl$_2$ ($P < 0.01$, Fig. 6b-c). Transfection of MCT-4 siRNA reversed the low extracellular pH which induce by hypoxia and CoCl$_2$.

The transwell assay indicated that the transfection of MCT-4 siRNA inhibited CT-26 cell migration after treatment with CoCl$_2$ ($P < 0.01$, Fig. 7a). The same results were also observed under hypoxic conditions ($P<0.01$, Fig. 7b).

**Discussion**

Migration is one of the most important aspects of the malignant cancer phenotype. Hence, clarifying the mechanisms of migration in cancer cells is important for developing new therapeutic strategies for cancer. The present study demonstrated the following: (1) DMH1 inhibited CT-26 cell migration via the
HIF-1α/MCT-4 signaling pathway. (2) DMH1 reversed the extracellular acidification under hypoxic conditions, which was always associated with cancer migration.

Cancer cells deploy several strategies for migration and metastasis, which suggests that cancer cells need a special microenvironment for metastasis [11]. Protein analyses in animal models suggested that tumors expressed high levels of HIF-1α, indicating that they are in a sustained hypoxic environment. The results indicated that hypoxia was an important stimulant to initiate migration and metastasis [3]. Also, DMH1 inhibited migration in CT-26 cells after incubation with CoCl₂ and under hypoxic conditions in vitro. These results indicated that DMH1 suppressed tumor migration via HIF-1α expression. It was found that DMH1 suppressed the high protein level of HIF-1α induced by CoCl₂. DMH1 was found to regulate the expression of HIF-1α. However, the exact mechanism is still unknown. Some reports provided evidence that the negative feedback existed between PHD-2 and HIF-1α in cancer cell lines, indicating that PHD-2 might be one of the upstream regulators of HIF-1α [12]. However, several pathways have also been shown to control HIF-1α stability and transcriptional activity via post-translational modifications under hypoxic conditions [13]. Therefore, further studies into the molecular mechanisms associated with the effects of DMH1 on the migration of cancer cells should explore its effect on the upstream regulation.

The present study also indicated that DMH1 did not induce apoptosis at lower doses but inhibited the migration of cancer cells at low doses in vitro. Hence, DMH1 was not a cytotoxic agent and might suppress the migration of cancer cells at lower doses. Also, it was speculated that DMH1 might serve as a potential anti-cancer agent. Some reports showed that treatment with DMH1 dramatically reduced lung tumor growth in subcutaneous xenograft mouse models [14]. They demonstrated that DMH1 increased the efficacy of anti-tumor drugs via blocking BMP signal [6]. However, studies indicated that the effects of DMH1 in attenuating tumor growth and migration might be mediated by multiple mechanisms. Hence, the possibility that DMH1 inhibited the migration of cancer cells through BMPs could not be excluded. Future studies should focus on whether DMH1 treatment may similarly disrupt the microenvironment required for tumor growth and migration [15].

Studies indicated that the excretion of lactate in the MCT-4-induced cancer microenvironment was always accompanied by extracellular acidification [16], and the long-term exposure of tumor cells under extracellular acidification led to migration [17]. Hence, it was proposed that the high lactate levels in tumors reflected the extent of the tumor cell adaptation to migration. The results indicated that the lactate concentration in cancer cell microenvironment increased, which was associated with a lower pHe after culture for 24 h.

The results indicated that the high level of lactate may induce the migration of CT-26 cells. However, lactate did not function as a chemoattractant in the studies. Many factors might affect the migration of cancer cells. Studies only showed that L-lactate, but not D-lactate, induced the migration of cancer cells osmotic effects, or metabolic activation [10]. Studies demonstrated the enhancement of the metastatic potential of tumors generally exhibiting pHe values of 6.7–6.8 [18]. Hence, it was speculated that the alteration of pHe and pHi might be one of the potential mechanisms that induced the migration of cancer
cells [20]. Studies confirmed that tumor cells were highly efficient at maintaining plasmalemmal pH gradients, in which the extracellular pH (pHe) might be significantly lower than the pHi [19]. DMH1 reversed the extracellular acidification. The results indicated that the extracellular environment was more acidic than the intracellular environment (data not shown), leading to a pH gradient across the cell membrane. The results accorded with the previous finding that pH regulation was crucial in influencing cancer cell metastasis [20]. The pHe/pHi gradient was downregulated (intracellular acidification occurred to lower the pH), which was dangerous to cancer development [21]. The pH alterations stimulated the migration of CT-26 cells; however, conflicting results were reported in vitro [22]. Some studies found that the low pHe could stimulate the migration of cells [23]. Hence, the question arose whether the low pHe–induced modifications of the microenvironment regulated by signal transduction might also affect the actin polymerization. Therefore, further studies should be conducted to detect (1) whether DMH1 regulates the pH of the tumor microenvironment and (2) whether DMH1 regulates the alteration of pH in the tumor microenvironment.

**Conclusion**

In general, the present study suggested that a better understanding of the migration of cancer cells might allow the development of novel therapeutic concepts. It was proposed that DMH1 regulated lactate excretion in primary tumors and might serve as a novel target for improving therapy. Furthermore, (1) proton-pump and MCT-1 inhibitor (AZD3965) might also affect metastasis by regulating the pH in the tumor microenvironment. Experimental studies should investigate this possibility. (2) For clinical application, combined use of DMH1 with other targeted therapies or chemotherapy might be used to test whether DMH1 enhances the sensitivity to anti-cancer effect.

**Declarations**

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**Conflict of Interest** The authors declare that they have no competing interests.

**Disclosure Summary** The authors declare that they have no conflict of interest.

**Ethics approval** All the experiments were performed by Using commercially available cell line from ATCC company. Neither human's nor animal's body material was used in this research.

**Availability of data and material** The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figures
DMH1 suppressed the migration of CT-26 under hypoxic conditions. a DMH1 induced apoptosis of CT-26 at a concentration of more than 5mM. ** P < 0.01 versus the control group. b Incubation with DMH1 (1mM) inhibited the migration of CT-26 cells under hypoxic conditions after 24 h. ** P < 0.01 versus the control group; ## P < 0.01 versus hypoxia. The results are representative of three independent experiments run in triplicate. Data represent mean ± SD.
Figure 2

DMH1 inhibited the migration of CT-26 cells through HIF-1α. a DMH1 suppressed the expression of HIF-1α under hypoxia conditions in CT-26 cells, **p < 0.01 versus normoxia; ## P < 0.01 versus hypoxia. b Treatment with CoCl2 induced the expression of HIF-1α in CT-26 cells. ** P < 0.01 versus the control group. c DMH1 suppressed the expression of HIF-1α in CT-26 cells after treatment with CoCl2. ** P < 0.01 versus the control group, ## P < 0.01 versus CoCl2. d Co-application of DMH1 and CoCl2 inhibited the migration of CT-26 cells, ** P < 0.01 versus the control group, ## P < 0.01 versus CoCl2. Hypoxia, Hypo. Magnification, 200×. The results are representative of three independent experiments run in triplicate. Data represent mean ± SD.
DMH1 inhibited migration through HIF-1α in CT-26 cells. a HIF-1α siRNA suppressed the expression of HIF-1α in CT-26 cells, ** P < 0.01 versus the control group. b DMH1 inhibited the migration of CT-26 cells through HIF-1α. ** P < 0.01 versus NC; ## P < 0.01 versus NC + CoCl2. c DMH1 inhibited the migration of CT-26 cells through HIF-1α under hypoxic conditions. ** P < 0.01 versus NC. ## P < 0.01 versus NC + DMH1. Hypoxia, Hypo. Magnification, 200×. The results are representative of three independent experiments run in triplicate. Data represent mean ± SD.
Figure 4

Lactate stimulated the migration of CT-26 cells. a Decreased pH e enhanced the migration of cancer cells. b Exposure to 20mM lactate for 24 h had no effect on CT-26 cell activity with or without serum. ** P < 0.01 versus the control. c Lactate stimulated the migration of cancer cells but did not act as a chemoattractant, ** P < 0.01 versus the control. d Lactate specifically influenced the migration of cancer cells, but not osmotic effects or metabolic activation. ** P < 0.01 versus the control. Hypoxia, Hypo. Magnification, 200×. The results are representative of three independent experiments run in triplicate. Data represent mean ± SD.
DMH1 suppressed MCT4 mediated excretion of lactate in cancer cells. a-b DMH1 suppressed the excretion of lactate through HIF-1α in CT-26 cells after 0 and 24 h under hypoxia and treatment with CoCl2, ** P < 0.01 versus normoxia; ## P < 0.01 versus hypoxia or CoCl2. c DMH1 had no effect on the expression of MCT4 in CT-26 cells. d DMH1 suppressed the expression of MCT-4 through HIF-1α under hypoxia in CT-26 cells, ** P < 0.01 versus the control group; ## P < 0.01 versus hypoxia. e DMH1 suppressed the expression of MCT-4 through HIF-1α after treatment with CoCl2 in CT-26 cells, ** P < 0.01 versus the control; ## P < 0.01 versus CoCl2. The results are representative of three independent experiments run in triplicate. Data represent mean ± SD.
Figure 6

DMH1 suppressed MCT4 mediated excretion of lactate in cancer cells. 

**a** MCT-4 siRNA suppressed the expression of MCT-4 in CT-26 cells, **P < 0.01** versus the control group. 

**b** Transfection of MCT-4 siRNA reversed the low extracellular pH induced by hypoxia and CoCl2 after 0 and 24 h. **P < 0.01** versus normoxia; ## P < 0.01 versus hypoxia or CoCl2. 

**c** Transfection of MCT-4 siRNA suppressed the excretion of lactate in CT-26 cells after 0 and 24 h under hypoxia and treatment with CoCl2, **P < 0.01** versus normoxia; ## P < 0.01 versus hypoxia or CoCl2.
DMH1 inhibited migration through HIF-1α-mediated MCT4 expression in CT-26 cells. a DMH1 inhibited the migration of CT-26 cells through MCT-4 after treatment with CoCl2. ** P < 0.01 versus CoCl2. b DMH1 inhibited the migration of CT-26 cells through MCT-4 under hypoxia conditions.** P < 0.01 versus hypoxia. Hypoxia, Hypo. Magnification, 200×. The results are representative of three independent experiments run in triplicate. Data represent mean ± SD.