

Sepiapterin inhibits cell proliferation and migration of ovarian cancer cells via down-regulation of p70^{S6K}-dependent VEGFR-2 expression

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Abstract. Tetrahydrobiopterin (BH₄) is known to be an essential cofactor for the aromatic amino acid hydroxylases, which are involved in the production of neurotransmitters, and for nitric oxide (NO) synthase. In the present study, we report that sepiapterin, the more stable form of the BH₄ precursor, modulates ovarian cancer cell proliferation and migration by NO-dependent and -independent mechanisms. Sepiapterin induction of cell proliferation and migration in SKOV-3 cells is accompanied by ERK, Akt and p70^{S6K} activation. These stimulatory effects of sepiapterin are reversed by pretreatment with NO synthase inhibitor. We also show that sepiapterin significantly inhibits vascular endothelial growth factor-A (VEGF-A)-stimulated cell proliferation and migration. Pretreatment with NO synthase inhibitor does not alter the ability of sepiapterin to inhibit VEGF-A-induced cell proliferation and migration, indicating that the suppressive effects of sepiapterin on VEGF-A-induced responses are mediated by a NO-independent mechanism. Finally, we demonstrate that sepiapterin markedly suppresses VEGF-A-induced p70^{S6K} phosphorylation and VEGFR-2 expression, resulting in inhibition of VEGF-A-induced cell proliferation and migration. Collectively, these findings represent a biphasic effect of sepiapterin on cellular fates, depending on the presence of growth factors, and support further development and evaluation of sepiapterin for the treatment of cancers overexpressing VEGFR-2.

Introduction

Tetrahydrobiopterin (BH₄), which is an essential cofactor for nitric oxide (NO) synthase as well as aromatic amino acid hydroxylases, is required for various biological processes in most cells or tissues of higher organisms (1-3). BH₄ is synthesized via two different mechanisms, *de novo* pathway and the salvage pathway. The pathway for the *de novo* biosynthesis of BH₄ from GTP involves GTP cyclohydrolase I, 6-pyruvoyl-tetrahydropterin synthase and sepiapterin reductase (1). Alternatively, in the salvage pathway, BH₄ can be synthesized from the exogenous pterin precursor sepiapterin, utilizing sepiapterin reductase and dihydrofolate reductase (2).

When BH₄ level is limiting, NO synthase becomes uncoupled and prefers to generate superoxide, resulting in various pathophysiological conditions such as vascular injury and inflammation (2). BH₄ has been known to stimulate cell proliferation in a variety of cell types including endothelial and PC12 cells (4-7). Sepiapterin, a stable form of BH₄ precursor, improves endothelial dysfunction in small mesenteric arteries from diabetic (*db/db*) mice, and induces angiogenesis via increase of endothelial cell proliferation and migration (8). In addition, sepiapterin attenuates 1-methyl-4-phenylpyridinium (MPP⁺, an ultimate toxic metabolite of a mitochondrial neurotoxin)-induced apoptosis in neuroblastoma cells by restoring NO synthase activity (9).

Vascular endothelial growth factor (VEGF) has been reported to play the pivotal roles in tumor angiogenesis, and VEGF-VEGF receptor (VEGFR) signaling pathways are widely appreciated as the therapeutic targets for cancer progression (10). VEGFRs are overexpressed in some cancer cells including ovarian cancer as well as endothelial cells (11-14). Ovarian cancer is the fifth leading cause of cancer death among women in United States and its incidence rate is highest in Western industrialized countries. The lack of potent therapeutics and strategies that are highly effective against metastatic and recurrent ovarian cancer is a critical obstacle to the treatment of ovarian cancer. In the present study, we evaluated the efficacy and molecular mechanisms of sepiapterin on cell proliferation and migration in ovarian cancer SKOV-3

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cells in the absence or presence of VEGF-A. Our results demonstrate that sepiapterin induces cell proliferation and migration through NO-dependent activation of ERK, Akt, and p70^{S6K} signaling pathways. In contrast, sepiapterin markedly inhibits VEGF-A-induced cell proliferation and migration through down-regulation of p70^{S6K}-dependent VEGFR-2 expression, independently of NO synthesis.

Materials and methods

Cell culture conditions. Human ovarian carcinoma cells (SKOV-3) from the American Tissue Culture Collection (Manassas, VA) were grown in 10% fetal bovine serum-Dulbecco's modified Eagle's medium (FBS-DMEM) (HyClone Laboratories, Logan, UT).

Reagents. L-Sepiapterin [S-(-)-2-amino-7,8-dihydro-6-(2-hydroxy-1-oxopropyl)-4(1H)-pteridinone], N ω -nitro-L-arginine (L-NNA), and rapamycin were obtained from Sigma-Aldrich (St. Louis, MO). The vascular endothelial growth factor-A (VEGF-A) was obtained from Millipore (Billerica, MA). The following antibodies were purchased from commercial sources: anti-phospho-ERK (T202/Y204), anti-phospho-Akt (Ser473), and anti-phospho-p70^{S6K} (T421/S424) (Cell Signaling, Beverly, MA); anti-ERK, anti-Akt, anti-p70^{S6K}, anti-Cdk4, anti-Cdk2, anti-cyclin D, anti-cyclin E, anti-VEGFR-2, anti-actin antibodies, and mouse and rabbit IgG-horseradish peroxidase conjugates (Santa Cruz Biotechnology, Santa Cruz, CA).

Cell growth assay. SKOV-3 cells, plated on 6-well plates (2x10⁴ cells/well), were grown in basal DMEM without serum for 24 h to synchronize cells in the G₁/G₀ phase of cell cycle, replaced with fresh DMEM, and further incubated with sepiapterin at different concentrations (0.1-10 μ M) for 24 h. Where indicated, cells were pretreated with or without NNA (100 μ M) for 20 min, and followed by treatment with sepiapterin alone, or sepiapterin plus vascular endothelial growth factor-A (VEGF-A, 50 ng/ml) for 24 h. Following culture for 24 h, the cell numbers were quantified using the trypan blue exclusion method. The results from triplicate determinations (mean \pm standard deviation) are presented as the numbers of cells per culture or the fold-increase of the untreated controls.

Migration assay. Cell migration was quantified in the *in vitro* wound-healing assay as previously described (15). After cells were plated on 48-well plates and grown to confluence, a single wound was created in the center of the cell monolayer by the gentle removal of the attached cells with a sterile plastic pipette tip. Cells were pretreated with or without NNA (100 μ M) for 20 min, followed by treatment with sepiapterin alone, or sepiapterin plus VEGF-A for 16 h. Cells were fixed with methanol, and then stained with 0.04% Giemsa staining solution (Sigma-Aldrich). The migration of the cells into the wound was observed with still images taken at the indicated time point.

Western blot analysis. Subconfluent cells in 100-mm dishes (BD Biosciences) were serum-starved for 24 h in DMEM and replaced with fresh media, followed by treatments for different time points, as indicated, at 37°C. Cells were rinsed twice with

ice-cold phosphate-buffered saline (PBS) and lysed by incubation in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 100 μ g/ml 4-(2-aminoethyl) benzenesulfonyl fluoride, 10 μ g/ml aprotinin, 0.5 μ g/ml leupeptin, 1 μ g/ml pepstatin A, 80 mM β -glycerophosphate, 25 mM NaF and 1 mM sodium orthovanadate for 30 min at 4°C. Cell lysates were clarified at 13,000 x g for 20 min at 4°C, and the supernatants were subjected to Western blot analysis as previously described (16,17).

NO measurements. Cells were serum-starved for 24 h in DMEM and replaced with fresh phenol red-free DMEM, followed by treatment with or without NNA, sepiapterin, or VEGF-A, as indicated, and NO was determined by measuring the concentration of nitrite in the culture medium using the Griess reagent system (Promega, Madison, WI), according to the manufacturer's instructions.

Statistical analysis. Statistical analysis was performed using the Student's t-test, and was based on at least three different experiments. The results were considered to be statistically significant when $p < 0.05$.

Results

Sepiapterin induction of cell proliferation and migration is associated with NO-dependent mitogenic signaling pathways. NO promotes or suppresses cancer progression and metastasis, depending on the dose and duration of NO exposure and on the cellular sensitivity to NO (18). We first examined the ability of sepiapterin, a stable precursor of BH₄, which is an essential cofactor for NO synthase, to regulate cell proliferation and migration in SKOV-3 cells. Sepiapterin treatment induced cell proliferation in a dose-dependent manner in concert with marginal up-regulation of cell cycle-related proteins such as cyclin D and cyclin E (Fig. 1A and B). In contrast, the expression of cyclin-dependent kinases (Cdks) was not altered by treatment with sepiapterin. To examine the effect of sepiapterin on cell migration, we next performed a wound-healing assay using SKOV-3 cells. As shown in Fig. 1C, sepiapterin, significantly induced cell migration at a concentration (50 μ M) higher than that required to increase cell proliferation (Fig. 1A). Enhanced cell proliferation and migration in response to sepiapterin was abrogated by pretreatment with NNA (100 μ M), an inhibitor of NO synthase (Fig. 2A and B). In addition, the sepiapterin-induced NO release was significantly blocked by NNA (Fig. 2C). These findings suggest that sepiapterin-induced cell proliferation and migration are dependent on NO synthesis. To further investigate the molecular mechanism by which sepiapterin induces cell proliferation and migration, we examined the changes in activation of extracellular signal-regulated kinase (ERK), phosphatidylinositol 3-kinase (PI3K)/Akt, and mammalian target of rapamycin (mTOR)/p70^{S6K} which were central to the coordinated control of cell mitogenesis (19-21). Sepiapterin treatment for 15 min markedly induced the phosphorylation/activation of ERK, Akt, and p70^{S6K} (Fig. 2D), and this activation was sustained until the end of these experiments (24 h) (data not shown). Pretreatment with NNA completely blocked the phosphorylation/activation of ERK, Akt, and p70^{S6K}, consistent with previous observations

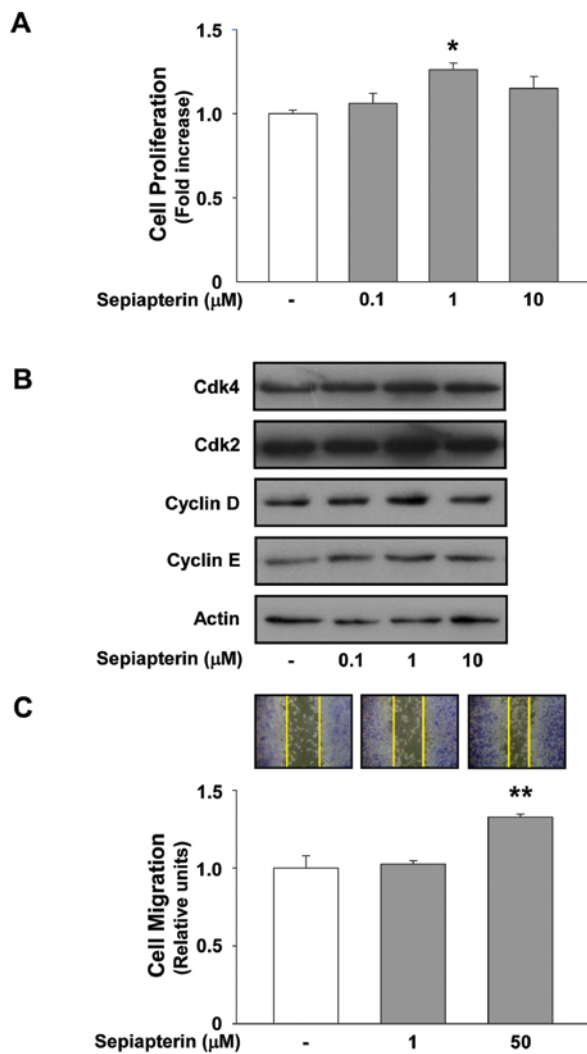


Figure 1. Sepiapterin induces cell proliferation and migration in SKOV-3 cells. (A) Quiescent cells were treated with or without sepiapterin at different concentrations ranging from 0.1 to 10 μ M for 24 h. Cell proliferation results from three independent experiments (mean \pm SD) were presented as the fold-increase of the untreated controls. Statistical significance is indicated (* p <0.05, compared with untreated control cells). (B) Cell lysates were immunoblotted with anti-Cdk4, anti-Cdk2, anti-cyclin D, anti-cyclin E or anti-actin antibodies. Results shown are representative of three independent experiments. (C) Cells were grown to confluence, and a wound was introduced by scratching the monolayer with a pipette tip as described in Materials and methods. Cells were treated with or without sepiapterin for 16 h. Migration into the wounded area was quantified by measuring the migration distance of cells from wound edge. Results from six independent experiments (mean \pm SD) are represented as the fold-increase of untreated controls. Statistical significance is indicated (** p <0.01, compared with untreated control cells).

on cell proliferation and migration (Fig. 2). Collectively, these findings suggest that sepiapterin-induced cell proliferative and migratory responses are mediated by NO-dependent activation of related signaling pathways.

Sepiapterin inhibits VEGF-A-induced cell proliferation and migration through NO-independent mechanism. Vascular endothelial growth factor-A (VEGF-A) is one of the key regulators of physiological and pathological angiogenesis, acting through cognate receptors such as VEGFR-1, VEGFR-2, and neuropilin (22). Many investigations demonstrate that

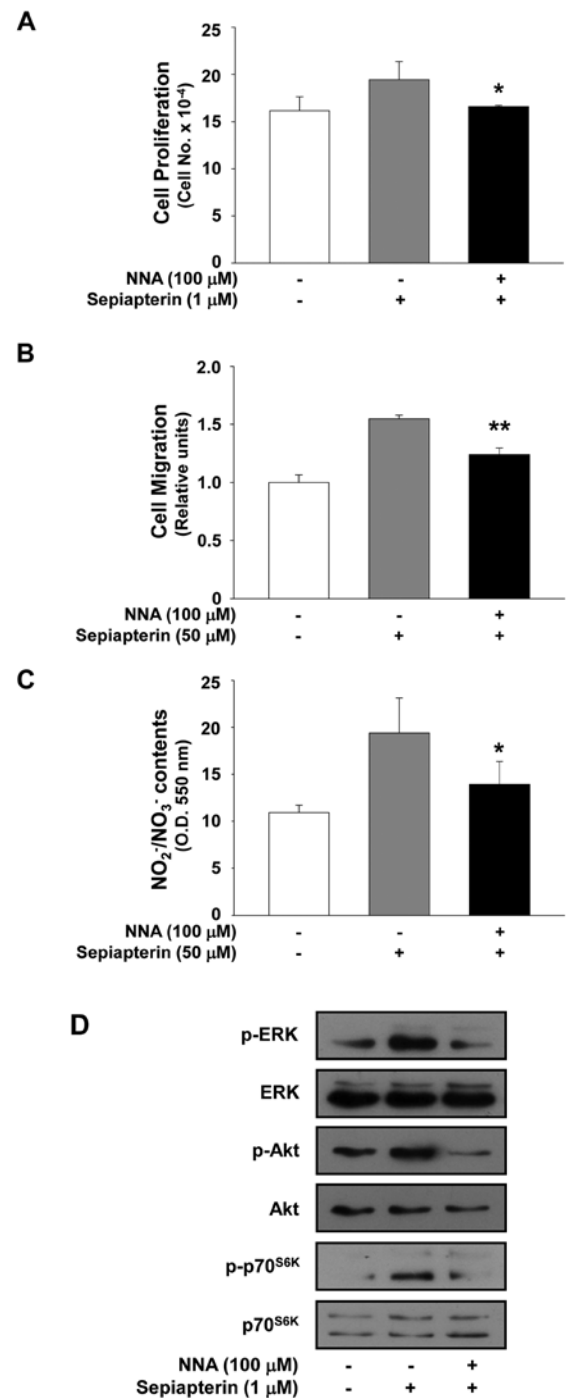


Figure 2. Sepiapterin induction of cell proliferation and migration is mediated by NO-dependent activation of signaling pathways. (A) Quiescent cells were pretreated with or without an NO synthase inhibitor NNA for 20 min, followed by sepiapterin for 24 h. Cell proliferation results from three independent experiments (mean \pm SD) are presented as the numbers of cells per culture. Statistical significance is indicated (* p <0.05, compared with sepiapterin-treated cells). (B) Cells were pretreated with or without NNA, and further incubated with sepiapterin for 16 h. Values represent the mean \pm SD of six independent experiments. Statistical significance is indicated (** p <0.01, compared with sepiapterin-treated cells). (C) Cells were pretreated with or without NNA, followed by sepiapterin for 24 h. NO production was determined by measuring the concentration of NO₂⁻ and NO₃⁻ in the culture medium. Values represent the mean \pm SD of six independent experiments. Statistical significance is indicated (* p <0.05, compared with sepiapterin-treated cells). (D) Cells were pretreated with or without NNA, followed by sepiapterin for 15 min. Cell lysates were Western-blotted with anti-phospho-ERK, anti-ERK, anti-phospho-Akt, anti-Akt, anti-phospho-p70^{S6K}, or anti-p70^{S6K} antibodies. Results shown are representative of three independent experiments.

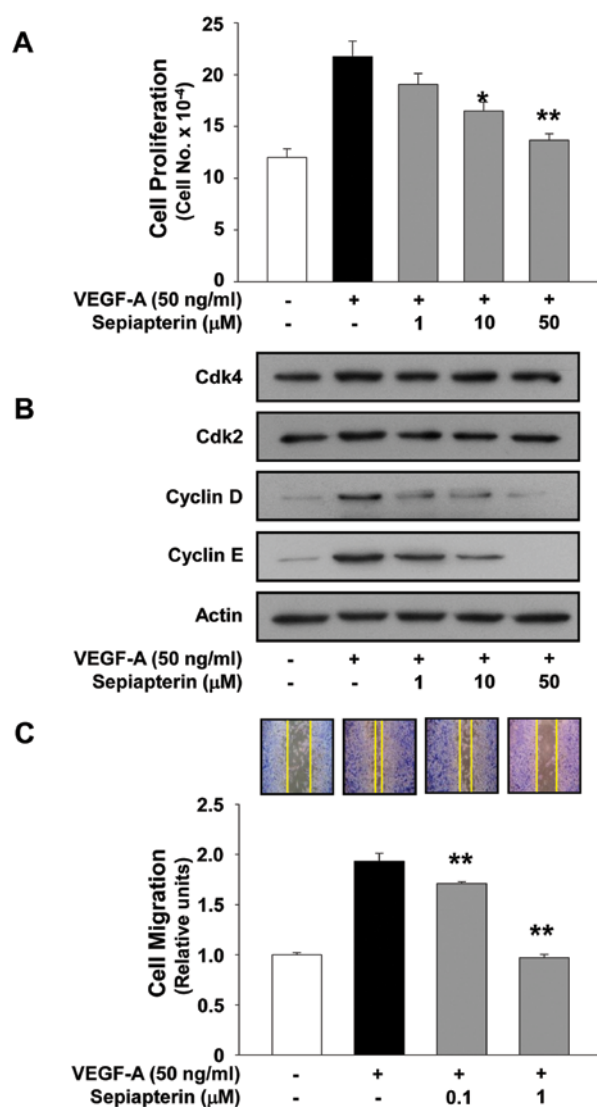


Figure 3. Sepiapterin inhibits VEGF-A-induced cell proliferation and migration. (A) Quiescent cells were pretreated with or without sepiapterin for 20 min, followed by VEGF-A stimulation for 24 h. Values represent the mean \pm SD of three independent experiments. Statistical significance is indicated (* p <0.05, ** p <0.01, compared with VEGF-A-treated cells). (B) Cell lysates were immunoblotted with anti-Cdk4, anti-Cdk2, anti-cyclin D, anti-cyclin E or anti-actin antibodies. Results shown are representative of three independent experiments. (C) Cells were pretreated with or without sepiapterin for 20 min, followed by VEGF-A stimulation for 16 h. Values represent the mean \pm SD of six independent experiments. Statistical significance is indicated (** p <0.01, compared with VEGF-A-treated cells).

VEGF and VEGFR are overexpressed in a variety of human cancers including ovarian cancer (11,12,23,24). Therefore, we next examined the effects of sepiapterin on cell proliferation and migration in response to VEGF-A. Pretreatment with sepiapterin markedly inhibited VEGF-A-induced cell proliferation in a dose-dependent manner, and this anti-proliferative effect was found to be mediated by dramatic down-regulation of cyclin D and cyclin E (Fig. 3A and B). In addition, sepiapterin markedly inhibited VEGF-A-induced cell migration at low concentrations (0.1, 1 μ M) (Fig. 3C) as well as high concentration (50 μ M) (data not shown). However, sepiapterin inhibition of VEGF-A-induced cell proliferation and migration was not ablated by pretreatment with NNA (Fig. 4A and B). We also examined the changes of NO release associated with inhibi-

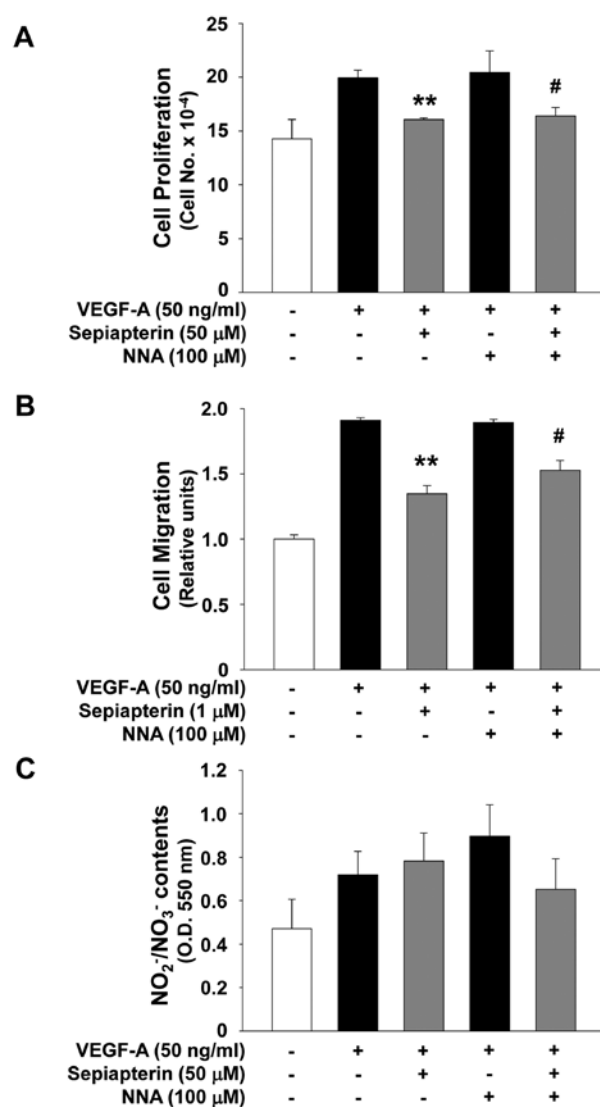


Figure 4. Inhibition of NO synthase activity does not reverse the anti-proliferative and anti-migratory activities of sepiapterin in SKOV-3 cells. Quiescent cells were pretreated with or without NNA for 20 min, followed by sepiapterin treatment for 20 min prior to VEGF-A stimulation for 24 h (A and C) or 16 h (B). Values represent the mean \pm SD of at least three independent experiments. Statistical significance is indicated (** p <0.01, compared with VEGF-A-treated cells; # p <0.05, compared with VEGF-A plus NNA-treated cells).

tion of VEGF-A-induced cell proliferation and migration. As shown in Fig. 4C, NNA pretreatment prior to VEGF-A stimulation resulted in a slight increase in NO release, however, this effect appeared to be irrelevant to VEGF-A-induced proliferative and migratory responses (Fig. 4A and B). In addition, sepiapterin did not alter VEGF-A-induced NO release in the presence or absence of NNA (Fig. 4C). These findings suggest the existence of an alternative mechanism to regulate VEGF-A-induced cell proliferation and migration, independently of NO synthesis. Thus, we examined the effect of sepiapterin on the phosphorylation of ERK, Akt, and p70^{S6K} in VEGF-A-treated SKOV-3 cells (Fig. 5A). As expected, VEGF-A stimulation for 15 min markedly increased the phosphorylation of ERK, Akt, and p70^{S6K} when compared with untreated controls. However, sepiapterin significantly inhibited the phosphorylation of VEGF-A-induced p70^{S6K}, but not that of ERK

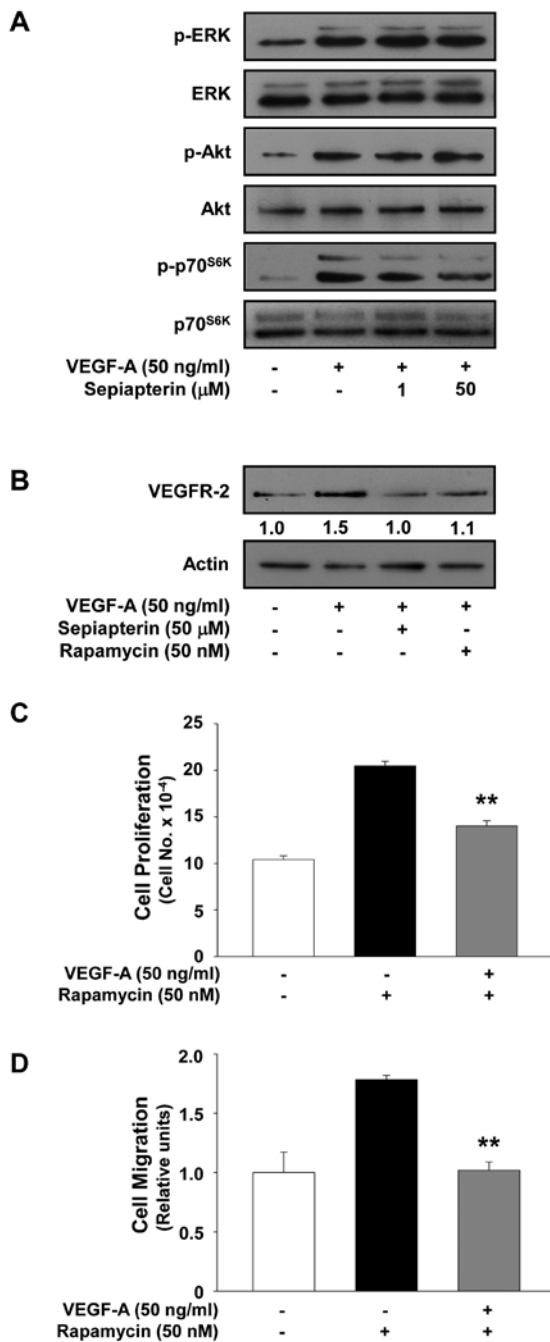


Figure 5. Sepiapterin inhibition of VEGF-A-induced cell proliferation and migration is associated with down-regulation of p70^{S6K}-dependent VEGFR-2 expression. (A) Quiescent cells were pretreated with or without sepiapterin for 20 min, followed by VEGF-A stimulation for 15 min. (B) Cells were pretreated with or without rapamycin for 20 min, followed by sepiapterin treatment for 20 min prior to VEGF-A stimulation for 24 h. Cell lysates were immunoblotted with anti-phospho-ERK, anti-ERK, anti-phospho-Akt, anti-Akt, anti-phospho-p70^{S6K}, anti-p70^{S6K}, anti-VEGFR-2, or anti-actin antibodies. The band intensities were determined using the NIH ImageJ software. The integrated band intensities for the VEGFR-2 were normalized to untreated controls. Results shown are representative of at least three independent experiments. Cells were pretreated with or without rapamycin for 20 min, followed by VEGF-A stimulation for 24 h (C) or 16 h (D). Values represent the mean \pm SD of at least three independent experiments. Statistical significance is indicated (** $p < 0.01$, compared with VEGF-A-treated cells).

or Akt. Pretreatment with NNA did not alter VEGF-A-induced phosphorylation of ERK, Akt, and p70^{S6K} in the presence or absence of sepiapterin (data not shown), consistent with

cell proliferation and migration (Fig. 4A and B). To further investigate the molecular mechanism of sepiapterin inhibition of VEGF-A-induced cell proliferation and migration, we next analyzed the changes in the expression of VEGFR-2 in SKOV-3 cells. Recent studies demonstrate that overexpression of VEGFR-2 in ovarian cancers contributes to cell migration and invasion associated with the tumor aggressiveness (11,12,14). As shown in Fig. 5B, sepiapterin significantly inhibited VEGF-A-induced expression of VEGFR-2 through the down-regulation of p70^{S6K} activity, as evidenced by using the mTOR/p70^{S6K} inhibitor rapamycin. This sepiapterin inhibition of VEGFR-2 expression in response to VEGF-A was also not affected by NNA pretreatment (data not shown). Finally, rapamycin pretreatment markedly inhibits VEGF-A-stimulated cell proliferation and migration (Fig. 5C and D), demonstrating that anti-proliferative and anti-migratory activities of sepiapterin might be attributed to the down-regulation of p70^{S6K}-dependent VEGFR-2 expression.

Discussion

In the present study, we demonstrate a mechanism in which sepiapterin, a stable form of BH₄ precursor, modulates ovarian cancer cell proliferation and migration through a NO-dependent and NO-independent fashion. Sepiapterin treatment induces cell proliferation and migration by NO-dependent activation of ERK, Akt, and p70^{S6K} signaling pathways, as demonstrated by pretreatment with the NO synthase inhibitor NNA. In contrast, sepiapterin markedly inhibits VEGF-A-stimulated cell proliferation and migration. These suppressive effects of sepiapterin on VEGF-A-induced responses are mediated by p70^{S6K}-dependent down-regulation of VEGFR-2 expression, independently of NO synthesis.

BH₄, a cofactor for aromatic amino acid hydroxylases, has been known to be used in the biosynthesis of biological neurotransmitters including catecholamines and serotonin (1). Thus, dysregulation of BH₄ levels are often associated with pathophysiological conditions such as neurological diseases as well as phenylketonuria (PKU). Sapropterin, a commercial form of BH₄, is currently being used as a treatment for PKU patients (25,26). In addition, BH₄ is an essential cofactor of NO synthase which converts L-arginine to L-citrulline and NO. NO, a gaseous free radical, positively or negatively modulates cancer progression and metastasis (18). Several studies demonstrate that NO plays an important role in ovarian cancer progression (27-29). Furthermore, the intron 4 polymorphism of NO synthase in ovarian cancer patients is associated with advanced tumor stage and a high rate of pelvic lymph node metastasis (30). It has recently been reported that expression of NO synthase and cyclooxygenase-2 is correlated with a shorter survival and disease-free interval, and metastatic stages (31). However, pharmacological efficacy and molecular mechanisms of NO synthase cofactor BH₄ in ovarian cancer progression remain unexplored.

VEGF-A is a potent stimulator of tumor angiogenesis. High expression of VEGF in ovarian cancers has been associated with poor survival *in vivo* (23,24,32-34). In addition, VEGFR expression in ovarian cancers correlates with tumor grade and progression (11-14,35). To investigate the role and mechanism of BH₄ in ovarian cancer, we examined the changes for

signaling pathways and cellular responses using sepiapterin, a stable precursor of BH₄, in the presence or absence of VEGF-A.

Alone sepiapterin treatment induced cell proliferation and migration in a dose-dependent manner (Fig. 1), and these effects were found to be mediated by activation of ERK, Akt, and p70^{S6K} signaling pathways (Fig. 2D). Pretreatment with the NO synthase inhibitor NNA completely ablated the ability of sepiapterin to induce cell proliferation and migration as well as mitogenic signaling pathways (Fig. 2A and B), demonstrating that sepiapterin induction of proliferative and migratory responses are dependent on NO synthesis, as confirmed by determination of NO release (Fig. 2C). However, sepiapterin pretreatment significantly inhibited VEGF-A-induced cell proliferation and migration, and this anti-proliferative effect was found to be mediated by down-regulation of cyclin D and cyclin E (Fig. 3). NNA pretreatment did not interfere with the ability of sepiapterin to inhibit VEGF-A-induced responses (Fig. 4A and B). Finally, sepiapterin inhibition of VEGF-A-induced p70^{S6K} activation, but not ERK or Akt (Fig. 5A), contributed to down-regulation of VEGFR-2 expression (Fig. 5B) associated with cell proliferation and migration (Fig. 5C and D).

In conclusion, our study is the first demonstration of a biphasic effect of sepiapterin on cell growth and migration. Sepiapterin-induced cell growth- and migration-promoting effects are mediated by activation of ERK, Akt, and p70^{S6K} signaling pathways, and these effects are dependent on NO synthesis. However, sepiapterin inhibits VEGF-A-induced cell proliferation and migration through down-regulation of p70^{S6K}-dependent VEGFR-2 expression, independently of NO synthesis. These findings provide important insights into the role and molecular mechanism of sepiapterin in the regulation of cell proliferation and migration, and support the development of sepiapterin as an anti-cancer agent that may complement ovarian cancer therapies already in clinical use or in preclinical trials.

Acknowledgements

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