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Anti-angiogenic action of 5,5-diphenyl-2-thiohydantoin-N10 (DPTH-N10) ☆

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Abstract

Previously, we demonstrated that 5,5-diphenyl-2-thiohydantoin (DPTH) exerts an anti-proliferation effect on subcultured human umbilical vein endothelial cells (HUVEC). In the present study, we show that 2(naphthalen-2-ylmethylsulfanyl)-5,5-diphenyl-1,5-dihydro-imidazol-4-one (DPTH-N10), a derivative compound of DPTH, exerts a 5 times stronger inhibition of [3H]thymidine incorporation into HUVEC as compared with DPTH and at very low concentrations (0–20 μM) inhibited DNA synthesis and decreased cell number in cultured HUVEC in a concentration- and time-dependent manner, but not in human fibroblasts. [3H]thymidine incorporation analysis demonstrated that treatment of HUVEC with DPTH-N10 arrested the cell at the G0/G1 phase of the cell cycle. Western blot analysis revealed that the protein level of p21 in HUVEC increased after DPTH-N10 treatment. In contrast, the protein levels of p27, p53, cyclins A, D1, D3 and E, cyclin-dependent kinase (CDK)2, and CDK4 in HUVEC were not changed significantly after DPTH-N10 treatment. Immunoprecipitation showed that the formation of the CDK2–p21 complex, but not the CDK2–p27, CDK4–p21, and CDK4–p27 complex, was increased in the DPTH-N10-treated HUVEC. Kinase assay further demonstrated that CDK2, but not CDK4, kinase activity was decreased in the DPTH-N10-treated HUVEC. Pretreatment of HUVEC with

Abbreviations: DPTH-N10, 5,5-diphenyl-2-thiohydantoin-N10; DPT, 5,5-diphenylhydantoin; HUVEC, human umbilical vein endothelial cells; CDK, cyclin-dependent kinase; CAM, chick embryo chorioallantoic membrane; SDS, sodium dodecyl sulfate; ECGS, endothelial cell growth supplement; M199, medium 199; FBS, fetal bovine serum; CKI, CDK-inhibitory protein; FACS, fluorescence-activated cell sorter.

☆ Inhibition of HUVEC proliferation by DPTH-N10.

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a p21, but not p27, antisense oligonucleotide reversed the DPTH-N10-induced inhibition of [3H]thymidine incorporation into HUVEC. Taken together, these data suggest that DPTH-N10 inhibits HUVEC proliferation by increasing the level of p21 protein, which in turn inhibits CDK2 kinase activity, and finally interrupts the cell cycle. Capillary-like tube formation, aortic ring culture, and chick embryo chorioallantoic membrane (CAM) assays further demonstrated the anti-angiogenic effect of DPTH-N10.

Keywords: Angiogenesis; p21; Proliferation; Endothelial cells; Cyclin-dependent kinase

1. Introduction

Angiogenesis, the generation of new blood vessels which occurs when a vascular bed is extended, is a complex process regulated by multiple stimulatory and inhibitory factors [1]. Normally, vascular proliferation occurs only during embryonic development, the female reproductive cycle and wound healing. However, many pathological conditions (e.g., development of atherosclerosis, diabetic retinopathy, and tumor formation) are characterized by persistent, unregulated angiogenesis [2]. The dependence of angiogenesis in the growth and metastasis of solid tumors is well recognized [3,4]. Lately, application of anti-angiogenic therapy has been suggested to be a potential therapeutic strategy against cancer development and metastasis.

Proliferation of vascular endothelial cells is one of the major events essential for angiogenesis. In our study of medicinal agents capable of inhibiting the cell cycle in the vascular endothelial cells, we showed previously that DPTH, an analogue of anti-epileptic drug phenytoin (5,5-diphenylhydantoin, DPH), exerts an anti-proliferation action on cultured HUVEC [5], suggesting the potential of DPTH as an anti-angiogenic agent. Searching further for an even more powerful agent, we found that a series of alkyl and aryl congeners, in particular one with 2-methylnaphthalenyl at the 2-thio position of the parent DPTH demonstrated the strongest inhibition on the growth of HUVEC in culture [6]. This derivative, 2(naphthalen-2-ylmethylsulfanyl)-5,5-diphenyl-1,5-dihydro-imidazol-4-one, has the abbreviation DPTH-N10 (Fig. 1).

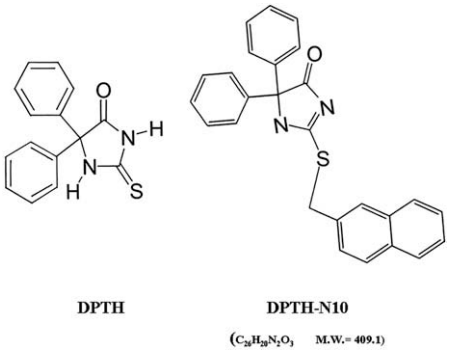

Fig. 1. Chemical structure of 5,5-diphenyl-2-thiohydantoin (DPTH) and 2(naphthalen-2-ylmethylsulfanyl)-5,5-diphenyl-1,5-dihydro-imidazol-4-one (DPTH-N10).

In the present investigation, we studied the anti-angiogenic action of DPTH-N10 and examined the underlying cellular and molecular mechanisms. We demonstrate that DPTH-N10 at concentrations as very low as 20 μM concentration-dependently inhibited the growth of HUVEC, capillary-like tube formation and growth of capillary. The DPTH-N10-induced cell cycle arrest at the G0/G1 phase in HUVEC occurred when the CDK2 activity was inhibited just as the level of p21 protein increased.

2. Methods and materials

2.1. Materials

DPTH-N10 was synthesized as previously described [7]. Briefly, SDil-O (1 mmol) was dissolved in 95% EtOH at 25 °C. To which was added more than 2 equiv. of aqueous NaOH to give a light yellow solution. Proper halide (more than 1.2 equiv.) was added and the reaction mixture was stirred at room temperature until starting material disappeared (Thin Layer Chromatography evidence, SiO_2, CH_2Cl_2) or a mass of white precipitate deposited. The reaction mixture was evaporated to dryness and re-suspended in water before suction filtration. The white crude product was washed with water and then re-crystallized with 95% EtOH.

4-(2-Hydroxy-ethyl)-1-piperazineethanesulfonic acid (HEPES), glycerol, phenylmethylsulphonyl

fluoride (PMSF), sodium dodecyl sulfate (SDS), Nonident P-40 (NP-40), and ECGS were purchased from Sigma Chem. M199, trypsin–EDTA, and kanamycin were purchased from Life Technologies. FBS was purchased from HyClone. Antibodies specific for cyclins, CDKs, and CKIs were purchased from Transduction Laboratories. An antibody specific for G3PDH was purchased from Biogenesis. Anti-mouse IgG conjugated alkaline phosphatase was purchased from Jackson Immuno Research Laboratories. [3H]thymidine was purchased from Amersham Biosciences. Glutathione *s*-transferase/retinoblastoma (Gst-Rb) was purchased from Santa Cruz.

2.2. Cell culture

HUVEC were grown in M199 containing 10% FBS, endothelial cell growth supplement (ECGS, 0.03 mg ml^{-1}) and kanamycin (50 U ml^{-1}) in a humidified 5% CO_2 incubator at 37 °C. After the cells had grown to confluence, they were disaggregated in trypsin solution, washed with M199 containing 10% FBS, centrifuged at 125g for 5 min, re-suspended, and then subcultured according to standard protocols. Cells from passages 5–10 were used.

2.3. [3H]thymidine incorporation

The [3H]thymidine incorporation was performed as previously described [8,9]. Briefly, HUVEC were applied to 24-well plates in growth medium (M199 plus 10% FBS and 0.03 mg ml^{-1} ECGS). After the cells had grown to 70–80% confluence, they were rendered quiescent by incubation for 24 h in M199 containing 2% FBS. M199 supplemented with 10% FBS and 0.05% DMSO (control) or various concentrations of DPTH-N10 were added to the cells and the cultures were allowed to incubate for 24 h. To test the effect of DPTH-N10 on the VEGF-induced thymidine incorporation, the cells were treated with VEGF (10 ng/ml) and DPTH-N10 (20 μM) or vehicle for 24 h. During the last 3 h of the incubation, [3H]thymidine was added at 1 μCi ml^{-1} (1 μCi = 37 kBq). The cells were incubated with 500 μl of 10% TCA (trichloroacetic acid) at 4 °C for overnight and incorporated [3H]thymidine was then extracted in 0.2 N NaOH and measured in a liquid scintillation counter.

2.4. Cell counting

As a measurement of cell proliferation, the cells were seeded onto 6-well 1% gelatin-coated plates and grown in M199 supplemented with 10% FBS and ECGS. Media without (control) and with DPTH-N10 were changed daily until cell counting. At various times of incubation, cultures were treated with trypsin–EDTA and the released cells were counted in a Coulter apparatus.

2.5. Viability assay

Cell viability was estimated by a modified MTT [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay, which was conducted at 24 h after DPTH-N10 treatment, as previously described [10]. Four samples were analyzed in each experiment.

2.6. Protein preparation and Western blotting analysis

To determine the expression levels of cyclins, CDKs, CKIs, and G3PDH in HUVEC, the total proteins were extracted and Western blot analyses were performed as described previously [11,12]. Briefly, HUVEC were cultured in 10 cm Petri dishes. After reaching subconfluence, the cells were rendered quiescent, treated with various concentrations of DPTH-N10 for 24 h, and then incubated in a humidified incubator at 37 °C. After incubation, the cells were washed with PBS (pH 7.4), incubated with extraction buffer (Tris 50 mM, pH 7.5, NaCl 150 mM, PMSF 1 mM, NP-40 1%, 0.1% SDS, 10 μg/ml Aprotinin and EDTA 10 mM) on ice, and then centrifuged at 12,000g for 30 min. The cell extract was then boiled in a ratio of 3:1 with sample buffer (Tris–HCl 250 mM, pH 6.8, glycerol 40%, β-mercaptoethanol 20%, SDS 8% and bromophenol blue 0.04%). Electrophoresis was performed using 12% SDS–polyacrylamide gel (2 h, 110 V, 40 mA, 50 μg protein per lane). Separated proteins were transferred to PVDF membranes (1 h, 400 mA), treated with 5% fat-free milk powder to block the nonspecific IgGs, and incubated for 1 h with specific antibody for cyclins, CDKs, CKIs, or G3PDH. The blot was then incubated with anti-mouse or anti-rabbit IgG linked to alkaline phosphatase (1:1000) for 1 h. Subsequently, the blot was developed using the ECL (enhanced chemiluminescence) system

(Amersham). The intensity of each band was quantified by densitometry analysis using Image Pro-Plus 4.5 Software.

2.7. Immunoprecipitation

As previously described [5], CDK2 or CDK4 was immunoprecipitated from 200 μg of protein by using anti-CDK2 or anti-CDK4 antibody (2 μg/ml) and protein A agarose beads (1/10V). The precipitates were washed five times with washing buffer, re-suspended in sample buffer (250 mM Tris–HCl, pH 6.8, 8% SDS, 40% glycerol, 20% β-mercaptoethanol, and 0.04% bromophenol blue) and then incubated at 95 °C for 10 min before electrophoresis to release the proteins from the beads.

2.8. CDK kinase assay

As previously described [5], CDK2 or CDK4 immunoprecipitates from DPTH-N10-treated and control HUVEC were washed three times with lysis buffer and twice with kinase assay buffer [50 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$, and 1 mM dithiothreitol (DTT)]. Phosphorylation of histone H1 (for CDK2) and glutathione *s*-transferase/retinoblastoma (Gst-Rb) fusion protein (for CDK4) were measured by incubating the beads with 40 μl of "hot" kinase solution [0.25 μl (2.5 μg) histone H1, 0.5 μl [γ-^{32}P] ATP (10 mCi/ml), 0.5 μl 0.1 mM ATP, and 38.75 μl kinase buffer] at 37 °C for 30 min. The reaction was stopped by boiling the sample in SDS sample buffer for 5 min. The products were analyzed by 10% SDS–PAGE. The gel was dried and visualized by autoradiography.

2.9. Capillary-like tube formation assay

Capillary-like tube formation assay was performed as described previously [12] with minor modifications. The 96-well plates were coated with 50 μl Matrigel (10 mg/ml) (BD Bioscience Pharmigen) by incubating at 37 °C for 1 h. HUVEC were suspended in M200 (Cascade Biologics) supplemented with 10% FBS and ECGs, and plated onto a layer of Matrigel at a density of 4×10^4 cells/well without or with DPTH-N10 (10 μM). The plates were then incubated for a further 4 h at 37 °C, and capillary-like tube formation was observed under the microscope.

2.10. Aorta ring culture assay

Aorta ring assay was performed as described previously [13]. Briefly, male Sprague–Dawley rats of 6–8 weeks were purchased from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan). The use of animals was approved by the Institutional Animal Use and Care Committee of Taipei Medical University. The animals were anesthetized with urethane (1 g/kg B.W., i.p.), and the thoracic aortas were quickly harvested and rinsed with ice-cold normal saline containing 10 μg/ml gentamicin. The attached fibroadipose tissues were removed and the aortas were cut into rings with 1 mm thickness. A mixture of Matrigel solution and the EBM-II medium (1:1) with 2% FBS, 0.25 mg/ml amphotericin B, and 10 μg/ml getamicin was prepared and added at 100 μl to each well of a 48-well plate. The mixture was allowed to gel at 37 °C for 40 min. The aorta rings were then placed one ring per well onto the gelled matrix. The aorta rings were embedded in an additional 150 μl per well of the mixture solution, allowed to gel at 37 °C for 40 min, covered with 125 μl of the EGM-II medium, and then incubated overnight in a humidified 5% CO_2 incubator at 37 °C. After overnight incubation, 125 μl of the EGM-II medium with 2% FBS, 0.25 mg/ml amphotericin B, 10 μg/ml gentamycin, and DPTH-N10 (5 μM) or DMSO (control) was added. The capillary-like tube structure of the outgrown rat aorta endothelial cells was visualized by MTT staining after 5 days treatment with DPTH-N10 or DMSO. The medium on top of the matrix was removed from the established aorta ring cultures. Freshly prepared MTT solution of 5 mg/ml in normal saline was added to the cultures at 100 μl/well, and the cultures were incubated in a CO_2 incubator at 37 °C for 24 h. The stained cultures were then examined under a DMIRB inverted microscope from Leica Microsystems, Inc. (Wetzlar, Germany), and images were acquired using a digital imaging system, MagnaFire SP from Optronics (Goleta, CA, USA).

2.11. The chick embryo chorioallantoic membrane (CAM) assay

Angiogenesis was assessed by using the CAM assay method as previously described [12]. The eggs were opened on day 4 of incubation and each was cultured in a 10 cm-dish at 37 °C with 5% CO_2 in air and saturated humidity. Ten microliters of

SDil-N10 (10 μM) mixed with 1% methylcellulose were placed on Teflon plates and dried under laminar flow. The methylcellulose disks were placed on the CAM on day 7 and the response was assayed microscopically after 72 h. The density of blood vessel formation in each case was assessed by comparing with that of a control set. Images were photographed digitally at 10×.

2.12. Statistical analysis

Values represent means ± SE mean. Three to four samples were analyzed in each experiment. Comparisons were subjected to one way analysis of variance (ANOVA) followed by Fisher's least significant difference test. Significance was accepted at $p < 0.05$.

3. Results

3.1. Inhibition of [3H]thymidine incorporation and cell proliferation in HUVEC by DPTH-N10

Initially, we studied the anti-proliferative effect of DPTH-N10 on the vascular endothelial cells by examining changes in [3H]thymidine incorporation (a measurement of DNA synthesis) in subcultured HUVEC. As shown in Fig. 2a, treatment of HUVEC with DPTH and DPTH-N10 at a concentration of 20 μM induced a 15.8% and 71.7% inhibition of [3H]thymidine incorporation, respectively. Fig. 2b illustrated that treatment of HUVEC with DPTH-N10 (0–20 μM) concentration-dependently decreased [3H]thymidine incorporation. We further examined the effect of DPTH-N10 on cell number of HUVEC. In the experiment of Fig. 2c (left panel), HUVEC were cultured with

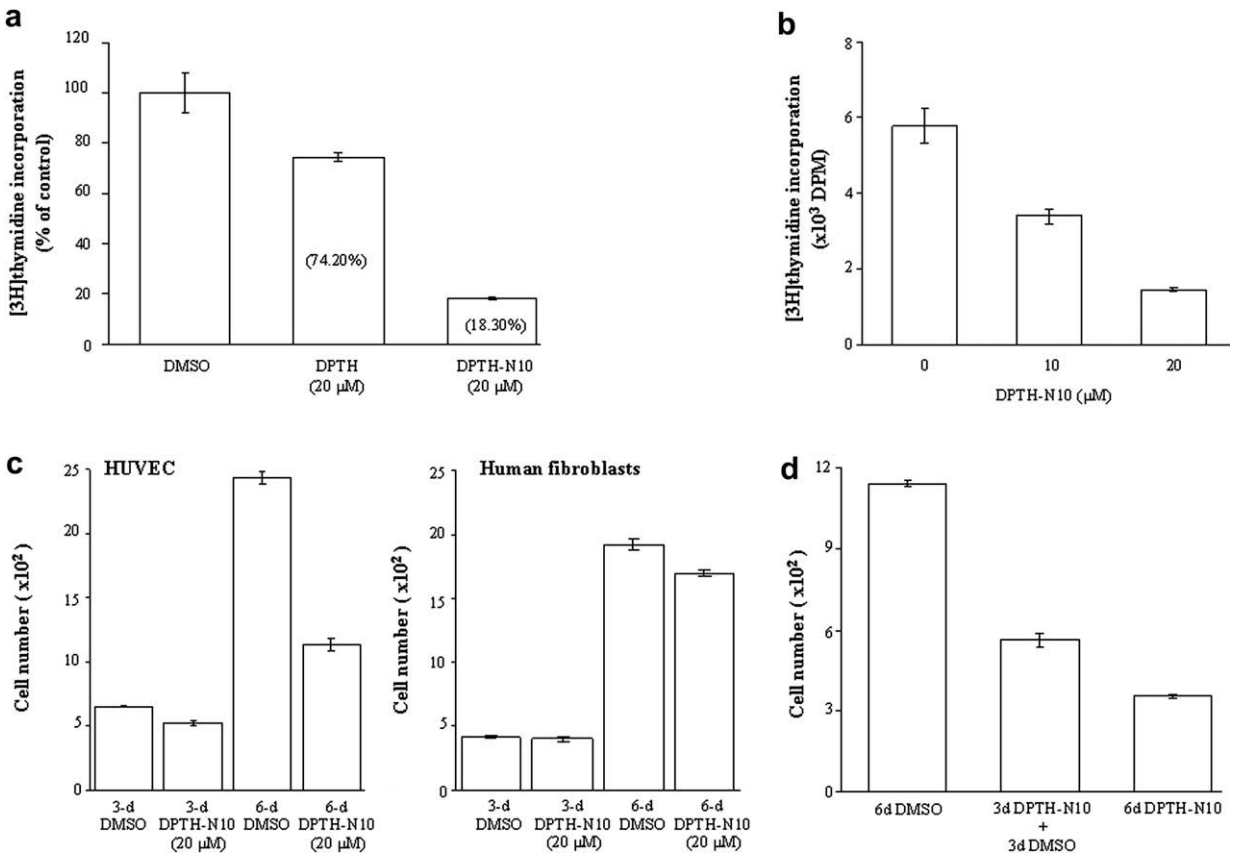

Fig. 2. Effects of DPTH-N10 on [3H]thymidine incorporation and cell growth rate in subcultured HUVEC. (a) DPTH-N10 (20 μM) showed a stronger inhibition of DNA synthesis in HUVEC as compared with DPTH (20 μM) treatment (71.7% vs. 15.8%) (b) Concentration-dependent inhibition of [3H]thymidine incorporation in HUVEC by DPTH-N10. (c) Time-dependent inhibition of cell growth was observed in HUVEC, but not human fibroblasts. Media without (control) or with DPTH-N10 were changed daily until cell counting. (d) DPTH-N10-induced inhibition of HUVEC proliferation was reversed by removal of DPTH-N10. Three to four samples were analyzed in each group, and values represent means ± SE mean. Significance was accepted at $p < 0.05$. *DPTH-N10-treated group different from control group. §DPTH-treated group different from control group. #DPTH-N10-treated group different from DPTH-treated group.

media containing DMSO (control) or DPTH-N10 (20 μM). Media without or with DPTH-10 (20 μM) were changed daily until cell counting. These data showed that a reduced cell count was observed in the DPTH-N10-treated HUVEC as compared with DMSO-treated cells. This DPTH-N10-induced decrease in the cell growth rate was in a time-dependent manner and consistent with the inhibitory effect of DPTH-N10 on [3H]thymidine incorporation. In contrast, treatment of human fibroblasts with DPTH-N10 (20 μM) for 6 days did not cause any significant growth inhibition (Fig. 2c, right panel), suggesting the specific effect of DPTH-N10 on growth inhibition of HUVEC. We also examined the reversibility of the DPTH-N10-induced inhibition of cell proliferation. As illustrated in Fig. 2d, treatment of HUVEC with 20 μM DPTH-N10 for 6 days induced a 70% reduction of cell number as compared with the cells treated with 0.05% DMSO for 6 days. However, treatment of the cells with 20 μM DPTH-N10 for 3 days followed by 0.05% DMSO without DPTH-N10 for an additional 3 days induced only 50% inhibition as compared with the cells treated 0.05% DMSO for 6 days. These results suggest that the DPTH-N10-induced inhibition of cell growth is reversible. Fig. 3a shows that DPTH-N10 reduced both FBS- and VEGF-induced increases in [3H]thymidine incorporation in HUVEC.

The DPTH-N10-induced reduction in [3H]thymidine incorporation in HUVEC can be due to retardation of cell cycle or cell death. To confirm that the results of our studies of DNA synthesis and cellular proliferation in HUVEC were not due to cell death caused by DPTH-N10 treatment, we conducted viability assays by treating the cells with DPTH-N10 for 24 h at the concentrations (0–20 μM) used in the studies of cell growth inhibition. MTT assays indicated that DPTH (0, 10 and 20 μM) did not cause any significant cell death (Fig. 3b).

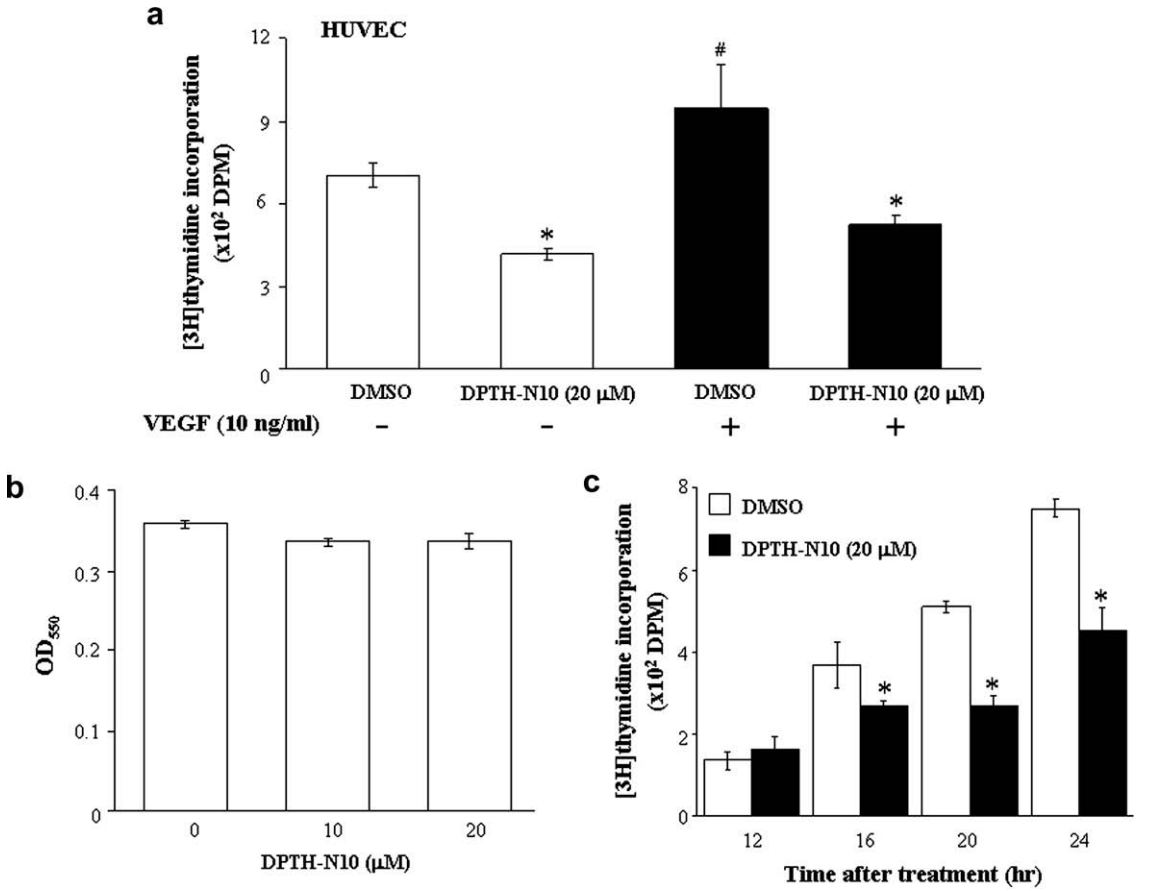

Fig. 3. (a) DPTH-N10 inhibited the VEGF (10 ng/ml)-induced increase in [3H]thymidine incorporation. (b) DPTH-N10 (0, 10 and 20 μM) did not cause cell death in HUVEC. (c). Time-dependent inhibition of cell cycle in HUVEC by DPTH-N10. Three to four samples were analyzed in each group, and values represent means ± SE mean. Significance was accepted at $p < 0.05$. *DPTH-N10-treated group different from control group. #VEGF/DMSO-treated group different from DMSO-treated group.

3.2. Arrest of cell cycle in G0/G1phase by DPTH-N10 treatment

In order to minimize data scatter and to optimize reproducibility, HUVEC were switched to media with 2% FBS for 24 h to render them quiescent and to synchronize their cell cycle activities. Then they were returned to media with 10% FBS and, at various times thereafter, they were treated with [3H]thymidine. Fig. 3c shows a reduction of the thymidine incorporation into HUVEC during the S phase of the cell cycle. The DPTH-N10-induced accumulation of HUVEC at the G0/G1 phase of the cell cycle was confirmed by flow cytometry analysis (75.28% vs. 63.60% as compared with vehicle-treated HUVEC).

3.3. Alterations in cell cycle activity by DPTH-N10 treatment

To delineate the molecular mechanism underlying DPTH-N10-induced inhibition of HUVEC cell proliferation, we further examined the levels of cell cycle regulatory proteins in the DPTH-N10-treated HUVEC. As shown in Fig. 4, treatment of HUVEC with DPTH-N10 for 24 h at concentrations (0, 10 and 20 μM), which caused the inhibition of [3H]thymidine incorporation and cell growth, did not induce any significant changes

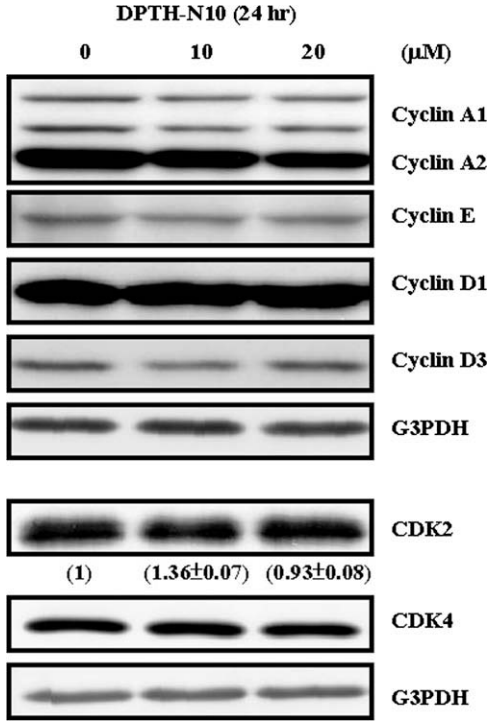

Fig. 4. Effect of DPTH-N10 on the cyclin and CDK protein levels. Western blot analysis was performed to examine the changes of protein in cyclins, CDKs, and CKIs in the DPTH-N10-treated HUVEC. Proteins were extracted from the cultured HUVEC at 24 h after DPTH-N10 treatment and probed with proper dilutions of specific antibodies. Results from a representative experiment are shown. The CDK2 protein levels were performed quantification by densitometry analysis. Values shown in parentheses represent means ± SE mean ($n = 4$). Membrane was probed with anti-G3PHD antibody to verify equivalent loading onto columns. CDK, cyclin-dependent kinase.

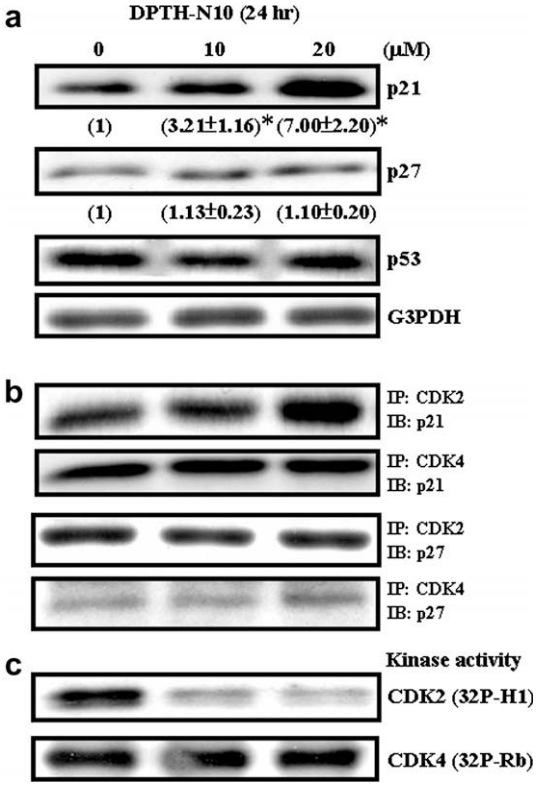

Fig. 5. Effect of DPTH-N10 on the protein levels of CKI, the formation of CKI–CDK complex and CDK kinase activity. (a) DPTH-N10 concentration-dependently increased the protein levels of p21, but not p27 and p53. Results from a representative experiment are shown. The p21 and p27 protein levels were performed quantification by densitometry analysis. Four samples were analyzed in each group, and values shown in parentheses represent means ± SE mean. Significance was accepted at $p < 0.05$. *DPTH-N10-treated group different from control group. (b) DPTH-N10 induced upregulation of CDK2–p21 complex in a concentration-dependent manner. CDK2 was immunoprecipitated by anti-CDK2 antibody, and CDK2–p21 association was detected by anti-p21 antibody, whereas CDK2–p27 association was detected by anti-p27 antibody. CDK4 was immunoprecipitated by anti-CDK4 antibody, and CDK4–p21 association was detected by anti-p21 antibody, whereas CDK4–p27 association was detected by anti-p27 antibody. Results from a representative experiment are shown. (c) Treatment of the HUVEC with DPTH-N10 concentration-dependently decreased the CDK2, but not CDK4, kinase activity. Results from a representative experiment are shown. The CDK2 and CDK4 kinase activities were determined as described in the Methods and Materials. CDK, cyclin-dependent kinase.

of the protein levels of cyclin A, cyclin D1, cyclin D3, cyclin E, CDK2 and CDK4, suggesting that DPTH-N10-induced cell cycle arrest in HUVEC is not through alterations of the protein levels of cyclins and CDKs. Since the CDK activity can be also controlled by a group of CKIs, we further examined the changes of protein levels of p21 and p27, two known CKIs, in the DPTH-N10-treated HUVEC. Fig. 5a shows that the protein levels of p21, but not p27, were concentration-dependently increased in the DPTH-N10-treated HUVEC as compared with the DMSO-treated cells (control). We also examined the change of the protein level of p53, which has been suggested to be involved in the regulation of p21 expression, in the DPTH-N10-treated HUVEC. As illustrated in the Fig. 5a, the level of p53 protein was not changed significantly in the DPTH-N10-treated HUVEC as compared with control cells. The CKI exerts its inhibitory effect on the kinase activity through binding to the cyclin-CDK complex. Accordingly, we further conducted immunoprecipitation assay to examine the effect of DPTH-N10 on the formation of the CDK–CKI complex. In the DPTH-N10-treated cells, the formation of the CDK2–p21 complex, but not CDK2–p27, CDK4–p21 and CDK4–p27 complex, was increased (Fig. 5b). To demonstrate that the increased p21 protein is associated with inhibition of CDK activation, we examined the CDK kinase activity. Fig. 5c shows that the assayable CDK2, but not CDK4, kinase activity was significantly decreased in the HUVEC treated with DPTH-N10. To further demonstrate that the increased p21 expression observed in the DPTH-N10-treated HUVEC correlated with G0/G1 arrest, the experiment illustrated in Fig. 6 was conducted. Thus, in the sample labeled DPTH-N10 (for 20 μM DPTH-N10-treated alone), the [3H]thymidine incorporation was decreased. Sample DPTH−N10+AS p21 was pretreated with a p21 antisense oligonucleotide (AS), which blocked the expression of p21 protein. Treatment of HUVEC with AS p21 or AS p27 alone did not cause any significant change in [3H]thymidine incorporation into HUVEC. Consequently, pretreatment of the HUVEC with AS p21 dose-dependently reversed the DPTH-N10-induced decrease in [3H]thymidine incorporation (Fig. 6). In contrast, pretreatment of HUVEC with AS p27 failed to preventing the DPTH-N10-induced decrease in [3H]thymidine incorporation.

3.4. Anti-angiogenic effect of DPTH-N10

To confirm the anti-angiogenic effect of DPTH-N10, we conducted the capillary-like tube formation, aorta ring culture and CAM assays. In the presence of DPTH-N10 (5–10 μM), the capillary-like tube formation (Fig. 7a), and the growth of capillary like tubes in the rat aorta ring (Fig. 7b) and in the chick embryo chorioallantoic membrane (Fig. 7c) were decreased. Our results showed that DPTH-N10 at concentration as low as 5 μM inhibited the growth of capillary like structures (Fig. 7b).

4. Discussion

Previously, we demonstrated that DPTH, an analogue of the anti-epileptic drug phenytoin (5,5-diphenylhydantoin, DPH), exerts an anti-proliferation effect on cultured HUVEC, suggesting the potential applications as an anti-angiogenic agent of DPTH [5]. The present study was undertaken to examine the anti-angiogenic effect of DPTH-

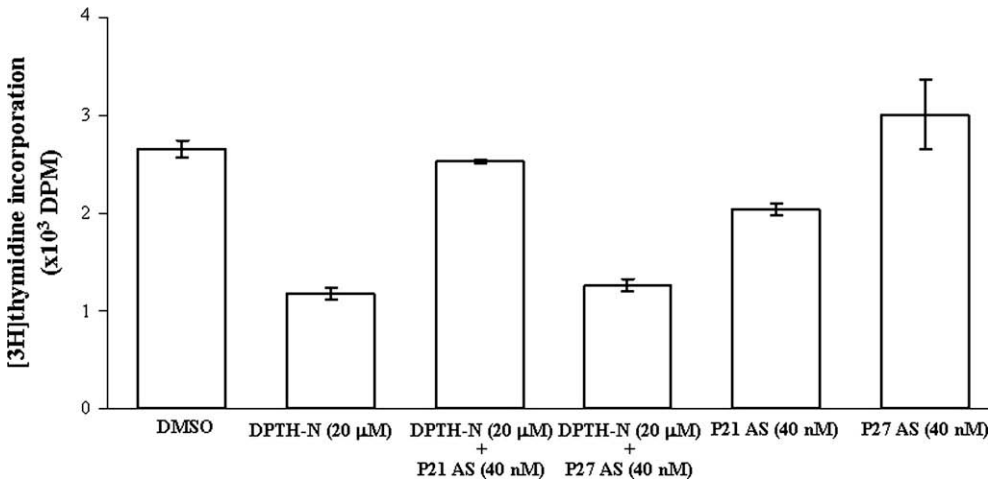

Fig. 6. Involvement of p21 in the DPTH-N10-induced decrease of [3H]thymidine incorporation in HUVEC. Antisense p21 or p27 oligonucleotide was added to HUVEC at a final concentration up to 40 nM at 16 h before the cell was challenged with 10% FBS and 20 μM DPTH-N10 for additional 21 h. Values shown in parentheses represent means ± SE mean. ($n = 4$). Significance was accepted at $p < 0.05$. *As compared with DMSO-treated group. #As compared with DPTH-N10-treated group. AS, antisense oligonucleotide.

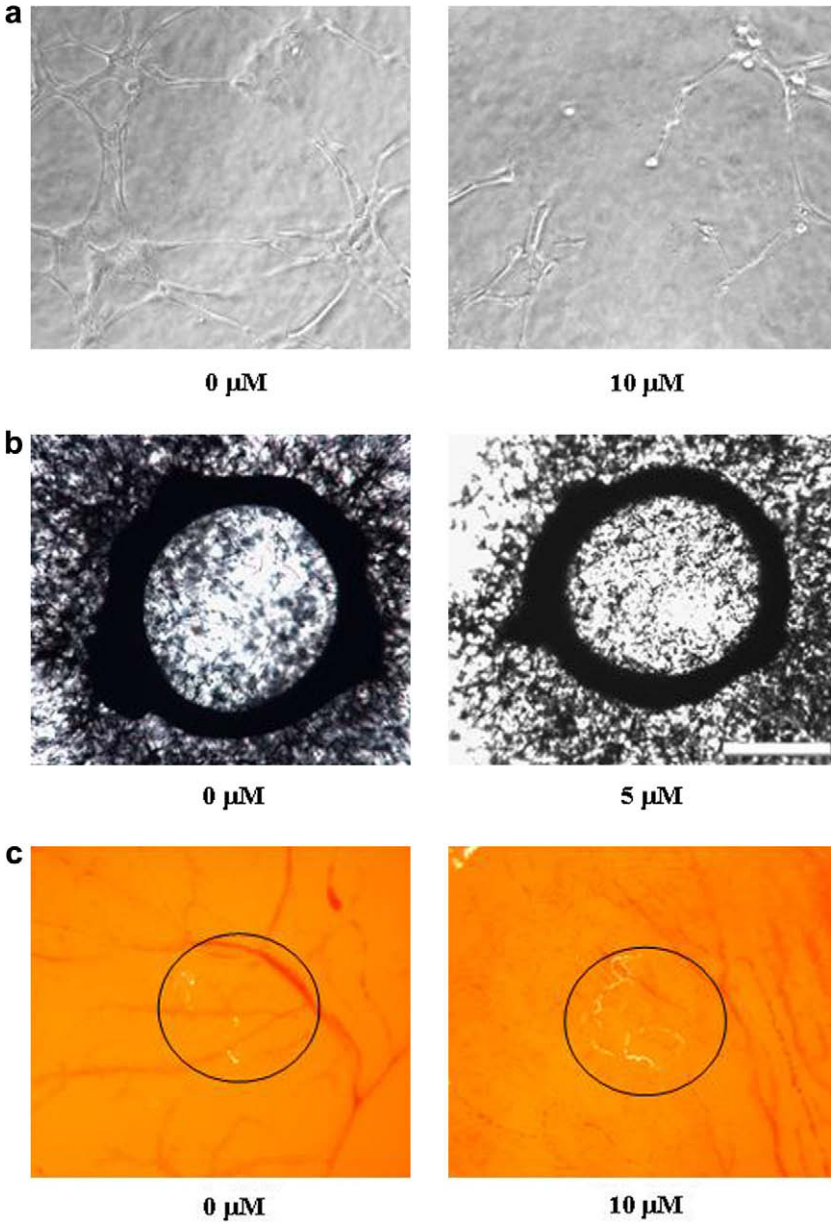

Fig. 7. Anti-angiogenic effect of DPTH-N10. (a) DPTH-N10 inhibits capillary-like tube formation of HUVEC in Matrigel. HUVEC were suspended in M200 media supplemented with 10% FBS and endothelial cell growth supplement, and plated onto a layer of Matrigel at a density of 4×10^4 cells/well without (left panel) or with DPTH-N10 at a concentration of 10 μM (right panel). (Bar = 1 mm). (b) DPTH-N10 inhibits the growth of capillary in the rat aorta ring. Gross appearance of the rat aorta ring treated with DMSO (left panel) or 5 μM DPTH-N10 (right panel). (c) DPTH-N10 inhibits sprouting angiogensis. Angiogenesis was assessed by using the CAM assay. Gross appearance of the chick embryo chorioallantoic membrane treated with methylcellulose disks containing DMSO (left panel) or 10 μM DPTH-N10 (right panel). Sprouting angiogenesis was observed after 72 h addition of DMSO (control) or DPTH-N10. Images were photographed digitally at 10×.

N10, a derivative of DPTH. Our *in vitro* studies demonstrated that DPTH-N10 at concentrations of 0–20 μM inhibited DNA synthesis and decreased cell number in cultured HUVEC in a dose- and time-dependent manner (Fig. 2b and c), but not in human fibroblasts (Fig. 2c), suggesting the specific effect of DPTH-N10 on HUVEC growth inhibition. A similar phenomenon was observed in other anti-

proliferation chemicals, such as DPTH [3], and BJ-601 [10], suggesting that vascular endothelial cells are more sensitive to anti-proliferation chemicals as compared with human fibroblasts. The reason is still unclear and deserves further investigation. The DPTH-N10-induced growth inhibition was reversible (Fig. 2d). As compared with DPTH, DPTH-N10 exerts a much stronger inhibitory activity on HUVEC growth (Fig. 2a). In the present study, we conducted the capillary-like tube formation, aortic ring culture, and CAM assays to further demonstrate the anti-angiogenic effect of DPTH-N10. To our knowledge, this is the first demonstration that DPTH-N10 caused cell cycle retardation, which in turn inhibited angiogenesis.

VEGF is a potent and specific mitogen for vascular endothelial cells that is capable of stimulating angiogenesis during embryonic development and tumor formation [14–16]. By [3H]thymidine incorporation assay, we demonstrated that treatment of HUVEC with DPTH-N10 at a concentration of 20 μM caused a decrease of DNA synthesis in both serum and VEGF-induced cell growth (Fig. 3a). DPTH-N10 decreased DNA synthesis and interrupted the transition of the cell cycle from the G1 into S phase (Fig. 3c). It has been generally accepted that the passage of a cell through the cell cycle is regulated by activation of specific CDKs in the cytoplasm [17,18]. This CDK activation is in turn modulated positively by their assembly with a series of regulatory subunits called cyclins, and negatively by association with a group of CDK-inhibitory proteins designated CKIs [19]. Cyclins have been identified as cyclins A, D1, D3 and E, whereas the most common CDKs are designated CDK2 and CDK4. In the present study, we demonstrated that DPTH-N10 at concentrations of 0–20 μM, which inhibited cell cycle arrest, did not induce any significant changes of the protein levels of cyclins A, D1, D3 and E, CDK2, and CDK4 in the HUVEC, indicating that the action of DPTH-N10 on the growth of endothelial cells is not through a mechanism to reduce the cyclins or CDKs. By examination of the expression levels of CDK-inhibitory proteins, we found that treatment of HUVEC with DPTH-N10 resulted in an increase in the protein level of p21, but not p27. In accord with the established notion that p21 is one known CDK-inhibitor, we found that the formation of the CDK2–p21 complex, but not CDK2–p27, CDK4–p21 and CDK4–p27 complex, was increased and the assayable CDK2 kinase activity was decreased in the DPTH-N10-treated HUVEC. These findings suggest that DPTH-N10 inhibits the CDK2 kinase activity through an increase in p21 expression. The role of p21 in the regulation of cell cycle progress has been well documented. P21 arrests the cell cycle through binding and inactivating the CDK system [20,21]. A p21 mutation, which specifically abrogates its binding to CDKs, was identified in a primary breast tumor [21], suggesting that p21 exerts tumor suppressor properties. The important role of p21 in the DPTH-N10-induced anti-proliferation in the HUVEC is confirmed by the antisense p21 oligonucleotide experiment showing that pretreatment with a p21 antisense oligonucleotide, but not p27 antisense oligonucleotide, reversed the DPTH-N10-induced inhibition in [3H]thymidine incorporation. Accordingly, we concluded that DPTH-N10 induced an increase in p21 expression, which in turn inhibited the CDK2 enzyme activity and led to the impairment of HUVEC in the transition from the G1 to S phase.

Although DPTH-N10 exerts a stronger growth inhibition in HUVEC as compared with DPTH (Fig. 2a), it seems that DPTH-N10 and DPTH share some common signaling pathways in regulating cell cycle progress in HUVEC, suggesting that DPTH molecule is the main structure for anti-proliferation effect in HUVEC and a stronger growth inhibition of DPTH-N10 might be due to the greater lipophilicity of DPTH-N10 by introduction of the naphthylmethyl moiety or a better binding activity of DPTH-N10 to the binding domain of its receptor or targeting molecule. Although our search for the target molecule of DPTH-N10 is still ongoing, we found from a study of structure and anti-angiogenic function that the degree of growth inhibition of DPTH derivatives in HUVEC is correlated with their lipophilicity and the introduction of an aromatic ring structure with right spatial arrangement at the sulfur atom might contribute to the enhanced anti-angiogenic activity of DPTH-N10 [6].

How DPTH-N10 acts to regulate the levels of p21 protein remains unsolved in this study. It has been suggested that the p21 gene is under the transcriptional control of p53 and might promote p53-dependent cell cycle arrest or apoptosis [20]. In this study, however, our results demonstrated that DPTH-N10 treatment did not change the levels of p53 protein in HUVEC, suggesting that the DPTH-N10-induced upregulation of the p21 protein was not mediated through the p53-dependent pathway.

Although proliferation of vascular endothelial cells has been recognized to be one of the major events essential for angiogenesis, it is important to demonstrate that DPTH-N10 did exert an anti-angiogenic activity. In this present study, we conducted capillary-like tube formation, aortic ring culture, and CAM assays to examine further the anti-angiogenic activity of DPTH-N10. In deed, DPTH-N10 at a concentration as low as 10 μM inhibited tube formation, growth of capillary, and sprouting angiogenesis (Fig. 7).

The dependence of angiogenesis in the growth and metastasis of solid tumors is well recognized [3,4]. Lately, application of anti-angiogenic therapy has been suggested to be a potential therapeutic strategy against cancer development and metastasis. In this study, we evaluated the anti-angiogenic activity of DPTH-N10 and delineated the molecular mechanisms underlying. Noticeably, our preliminary data showed that DPTH-N10 also dose-dependently inhibited the proliferation of colon cancer cell line, COLO-205 (Lee et al., unpublished data). Although animal studies of DPTH-N10-mediated anti-angiogenic and anti-cancer actions are still ongoing, the anti-proliferation effect of DPTH-N10 on cultured HUVEC and cancer cell lines, but not on human primary untransformed cells (such as human fibroblasts), strongly suggest the potential applications of DPTH-N10 in the treatment of angiogenesis-related disease conditions, including cancers.

The effect of anti-epileptic drugs on the growth of tumor cells (such as lymphoid tumor cells, human breast cancer cells, etc.) has been documented [22–24]. It seems that the mechanism underlying of anti-proliferation action of these anti-epileptic drugs are different from the anti-epileptic action. As demonstrated in our previous study for DPTH [3] and in this study for DPTH-N10, the anti-proliferation action of these anti-epileptic drugs is through upregulation of p21 protein, whereas their anti-epileptic action is through blocking sustained repetitive firing in individual neurons, enhancing inhibitory events mediated by γ-aminobutyric acid (GABA), blocking T-type calcium cannels, or reducing events mediated by excitatory amino acids [25].

In conclusion, the results from the present studies indicate that DPTH-N10-induced cell cycle arrest in HUVEC occurred when the cyclin-CDK system was inhibited just as p21 protein levels increased. The findings from our previous study in DPTH and the present study in DPTH-N10 strongly suggest that DPTH might be the principal compound to develop an anti-angiogenic drug.

Conflict of interest statement

The authors have nothing to disclose.

Acknowledgments

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