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Structure and anti-proliferation function of 5,5-diphenyl-2-thiohydantoin (DPTH) derivatives in vascular endothelial cells

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Abstract

Previously, we identified DPTH, an analogue of antiepileptic drug phenytoin (5,5-diphenylhydantoin, DPT), capable of retarding the cell cycle in the human vascular endothelial cells. Our data suggest that DPTH inhibits human umbilical venous endothelial cells (HUVEC) proliferation by increasing the level of p21 protein, which in turn inhibits the activities of cyclin-dependent kinase (CDK)2 and CDK4, and finally interrupts the cell cycle. To search chemicals with more potency in anti-angiogenic activity, we designed and synthesized several chemical compounds based on the structure–activity relationship consideration. We evaluated the anti-angiogenic activity of these compounds by examining their effects on DNA synthesis, cell number, p21 induction and capillary-like tube formation. Our results showed that introduction of side chain containing an aromatic ring structure with right spatial arrangement at sulfur atom of DPTH enhanced the anti-angiogenic activity in HUVEC.

Keywords: DPTH; Angiogenesis; p21; Endothelial cells; Capillary-like tube formation

1. Introduction

Angiogenesis, the formation of new capillary blood vessels as extensions of existing vessels, is a complex process regulated by multiple stimulatory and inhibitory factors. Angiogenesis is essential for many physiological processes and important in the pathogenesis of many disorders (Risau, 1997). In the physiological condition, the activity of stimulators and inhibitors of angiogenesis maintains it in balance. Normally, vascular proliferation occurs only during embryonic development, the female reproductive cycle and wound healing. In contrast, many pathological conditions (e.g., atherosclerosis, cancer, and diabetic retinopathy) are characterized by persistent, unregulated angiogenesis (Folkman, 1995).

The events that are thought to be essential for angiogenesis include: local degradation of the basement membrane of the parent vessel, allowing protrusion of endothelial cells; outward migration of endothelial cells in tandem to form a capillary sprout; proliferation of endothelial cells within the sprout; and the formation of a lumen with subsequent branching. Control of vascular development could permit new therapeutic approaches to these disorders. During the past few years, experimental and clinical investigators continue to search for new therapeutic strategies for preventing the occurrence of angiogenesis. One approach, as pursued in this study, seeks to identify medicinal agents capable of retarding the cell cycle in the vascular endothelial cells.

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Previously, we identified DPTH, an analogue of antiepileptic drug phenytoin (5,5-diphenylhydantoin, DPT), capable of retarding the cell cycle in human vascular endothelial cells (Shih et al., 2004; Fig. 1). DPTH dose-dependently inhibited DNA synthesis and decreased cell number in cultured human umbilical venous endothelial cells (HUVEC). Studies of [3H] thymidine incorporation revealed that treatment of HUVEC with DPTH decreased DNA synthesis and arrested the cells at the G0/G1 phase of the cell cycle. Moreover, DPTH inhibited HUVEC proliferation by increasing the level of p21 protein, which in turn inhibited the activities of CDK2 and CDK4, and finally interrupted the cell cycle. The findings from our studies suggest that DPTH might have the potential to inhibit the process of angiogenesis. Although DPTH exerts an anti-angiogenic activity, the concentrations used in the in vitro studies might be too high for clinical uses. Accordingly, we designed and synthesized a series of DPTH derivatives to seek chemicals with more potency in anti-angiogenic activity.

2. Materials and methods

2.1. Materials

DPTH derivatives reported in this study were synthesized as previously described (Cain and Naegele, 1954). DPTH derivatives were prepared by treating DPTH with alkyl or naphthalenyl halides in base medium at room temperature. A typical reaction is exemplified as follows: 5,5-Diphenyl-3-thiohydantoin (DPTH, 1.0 mmol) was dissolved in ethanol (5 ml) to which was added aq. NaOH (1.1 mmol) and 1-bromooctane (1.1 mmol). The whole was stirred at 25 °C until the disappearance of starting material. The crude product was then recrystallized with methanol.

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. An antibody specific for p21 was 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.

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 37 °C incubator. After the cells had grown to confluence, they were disaggregated in trypsin solution, washed with M199 containing 10% FBS, centrifuged at 125 ×*g* for 5 min, resuspended, and then subcultured according to standard protocols. Cells from passages 5–9 were used.

2.3. [3H]thymidine incorporation

The [3H]thymidine incorporation was performed as previously described (Lee et al., 1997; Hsu et al., 2003). Briefly, HUVEC were applied to 24-well plates in growth medium (M199 plus 10% FBS and 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 30 μM of DPTH derivatives was added to the cells and the cultures were allowed to incubate for 24 h. During the last 3 h of the incubation with or without DPTH, [3H]thymidine was added at 1 μCi ml^{-1} (1 μCi=37 kBq). Incorporated [3H]thymidine was extracted in 0.2 N NaOH and measured in a liquid scintillation counter.

2.4. MTT assay

Cell number was estimated by a modified MTT [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay as previously described (Yu et al., 2004). The cells were treated daily with 30 μM of DPTH derivatives for anti-angiogenic activity. Four samples were analyzed in each experiment.

2.5. Protein preparation and Western blotting

To determine the expression levels of p21 and G3PDH in HUVEC, the total proteins were extracted and Western blot analyses were performed as described previously (Lin et al., 2002; Ho et al., 2004). Briefly, HUVEC were cultured in 10 cm Petri dishes. After reaching subconfluence, the cells were rendered quiescent, treated with 30 μM of DPTH derivatives 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 12,000 ×*g* 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

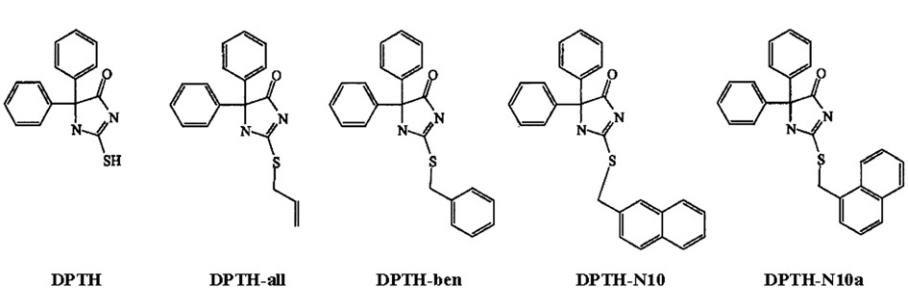

Fig. 1. Chemical structures of DPTH derivatives.

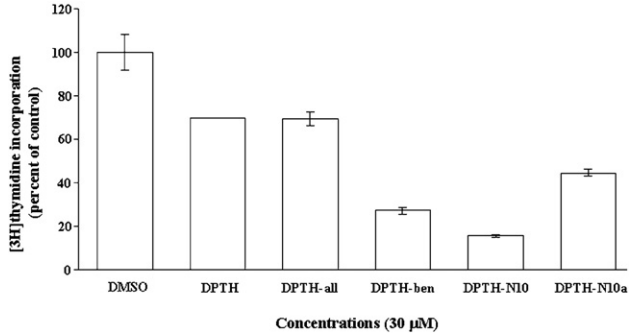

Fig. 2. Effects of DPTH derivatives on thymidine incorporation in subcultured HUVEC. To study the effect of DPTH derivatives on the cell cycle, [3H] thymidine incorporation was conducted after HUVEC release from quiescence by incubation in culture media supplemented with 10% FBS and 0.05% DMSO (control) or 30 μM DPTH derivatives in 0.05% DMSO. Three to four samples were analyzed in each group, and values represent the means±s.e. mean.

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 p21, or G3PDH. The blot was then incubated with anti-mouse or anti-rabbit IgG linked to HRP (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.

2.6. Capillary-like tube formation assay

Capillary-like tube formation assay was performed as described previously with minor modifications (Ho et al., 2004). The 96-well plates were coated with 50 μl Matrigel (10 mg/ml) (BD Bioscience Pharmigen, CA, USA) by incubating at 37 °C for 1 h. HUVEC were suspended in M200 (Cascade Biologics, Portland, OR, USA) 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 or with DPTH derivatives (30 μM). The plates were then incubated for a further 2 h at 37 °C, and capillary-like tube formation was observed under microscope.

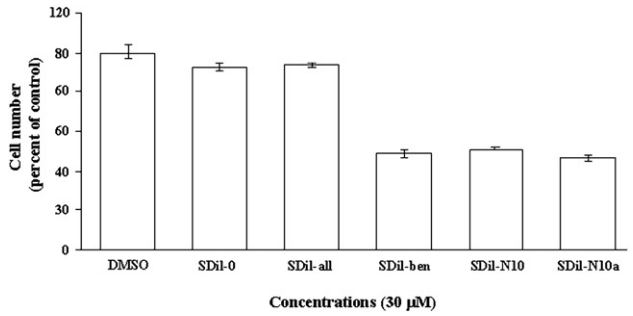

Fig. 3. Effects of DPTH derivatives on cell growth rate in subcultured HUVEC. To study the effect of DPTH derivatives on cell growth rate in HUVEC, MTT assay was conducted after the cells were daily treated without or with DPTH derivatives (30 μM) for 3 days. Three to four samples were analyzed in each group, and values represent the means±s.e. mean.

2.7. Statistical analysis

Values represent the means±s.e. mean. Three to four samples were analyzed in each experiment.

3. Results

3.1. Anti-proliferative activity of DPTH derivatives in HUVEC

To study the anti-proliferative effect of DPTH derivatives on the vascular endothelial cells, we examined changes in [3H] thymidine incorporation (a measurement of DNA synthesis) in response to treatment with DPTH derivatives in subcultured HUVEC. As illustrated in Fig. 2, treatment of HUVEC with DPTH derivatives for 24 h induced a decrease in [3H]thymidine incorporation. The inhibitory activity of these compounds is DPTH-N10 ≥ DPTH-ben > DPTH-N10a >> DPTH-all ≥ DPTH > control. We further examined the effect of DPTH derivatives on cell number of HUVEC. In the experiment of Fig. 3, HUVEC were cultured for 3 days with or without DPTH derivatives (30 μM), and then the cells were harvested and counted. The data showed that the degree of decreased cell number by these compounds was DPTH-N10 ≥ DPTH-ben ≥ DPTH-N10a >> DPTH-all ≥ DPTH > control. This result is consistent with the study of thymidine incorporation.

3.2. P21 induction by DPTH derivatives

Previously, we demonstrated the DPTH-induced cell cycle arrest in HUVEC through induction of p21 expression. The p21 protein, a CDK-inhibitory protein, represents the major signaling molecule involved in the DPTH-induced cell cycle arrest in HUVEC. Accordingly, we examined the levels of p21 protein in HUVEC treated with DPTH derivatives. As shown in

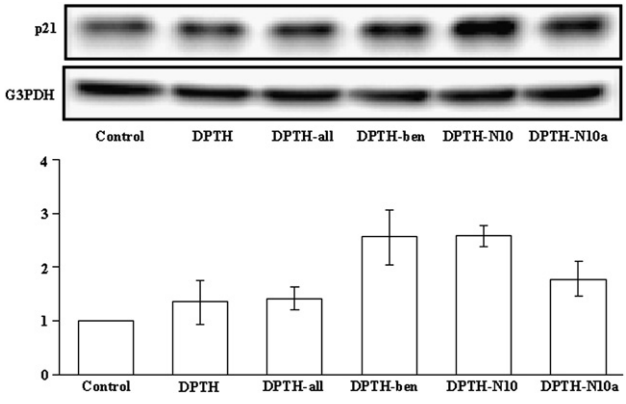

Fig. 4. Effect of DPTH derivatives on the protein level of p21. Western blot analysis was performed to examine the changes of protein level of p21 in HUVEC treated with DPTH derivatives at a concentration of 30 μM. Proteins were extracted from the cultured HUVEC at 24 h after treatment with DPTH derivatives and probed with anti-p21 antibodies. Membrane was probed with anti-G3PHD antibody to verify equivalent loading. Upper panel shows a representative experiment. Lower panel shows quantitative results ($n=3$).

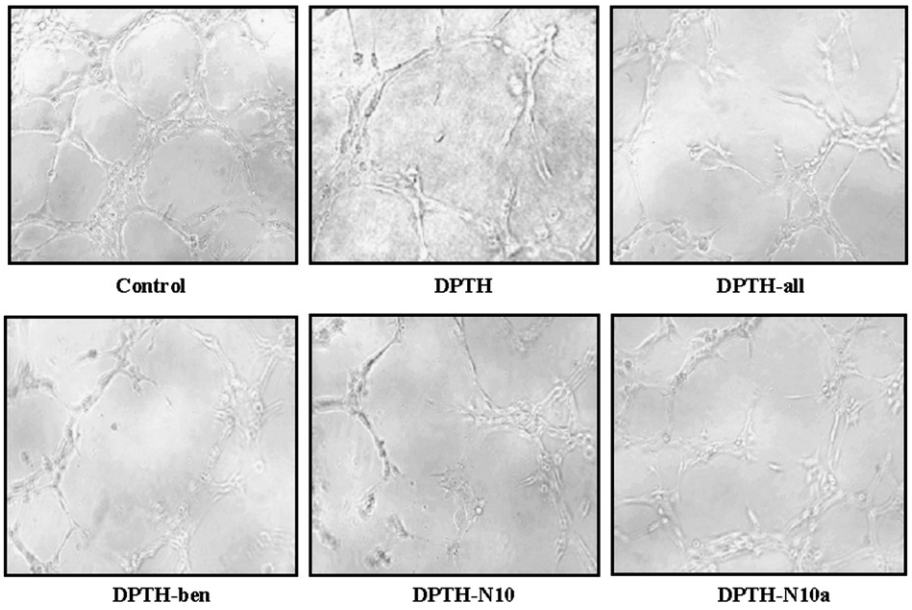

Fig. 5. Effect of DPTH derivatives on capillary-like tube formation of HUVEC in Matrigel. HUVEC were suspended in M200 media supplemented with 10% FBS and endothelial cell growth supplement, and plated onto a layer of Matrigel at a density of 4×10^4 cells/well without (control) or with DPTH derivatives at a concentration of 30 μM for 2 h.

Fig. 4, treatment of HUVEC with DPTH derivatives (30 μM) caused an increase in p21 protein level. The degree of increased p21 protein level by these compounds was DPTH-N10 ≥ DPTH-ben > DPTH-N10a >> DPTH-all ≥ DPTH > control.

3.3. Effect of DPTH derivatives on capillary-like tube formation

To examine the anti-angiogenic activity of DPTH derivatives, we conducted the capillary-like tube formation assay. As shown in Fig. 5, DPTH derivatives inhibited the capillary-like tube formation in an order of DPTH-N10 ≥ DPTH-ben > DPTH-N10a >> DPTH-all ≥ DPTH > control.

4. Discussion

Control of vascular development has been suggested to be new therapeutic approaches to many angiogenesis-related disorders. Previously, we demonstrated that DPTH at a range of concentrations (12.5–50 μM) inhibited DNA synthesis and decreased cell number in cultured HUVEC (Shih et al., 2004). DPTH inhibited the proliferation of HUVEC by increasing the level of p21 protein, which in turn inhibited the activities of CDK2 and CDK4, and finally interrupted the cell cycle. In the present study, we designed several DPTH derivatives by introduction of hydrocarbon side chain serves as an initiative derivatization. The synthesis of DPTH derivatives was targeted to get further anti-angiogenic compounds of high activity. Our results showed that introduction of aryl side chain at sulfur atom of DPTH exerted an increased anti-proliferation activity in HUVEC with increasing hydrophobicity or aromaticity of aryl side chain. It turned out that DPTH derivatized with a naphthalenyl group, which contains planar double aromatic rings, most significantly increased anti-proliferation activity in HUVEC as compared to that with a benzene group that is only a single aromatic ring. To our knowledge, this is the first demonstration that the introduction of an aromatic ring structure at sulfur atom of DPTH could enhance the anti-proliferation activity in HUVEC.

DPTH-all was introduced to an allyl side chain, a three carbon entity with a double bond at the end, at sulfur atom of DPTH. Our data showed that an allyl side chain did not cause any significant change in anti-proliferation activity in HUVEC as compared with DPTH. This indicated that the chain length of an allyl chain might not be long enough as a naphthalenyl or a benzyl group to have efficacious hydrophobic interaction with binding domain inducing anti-proliferation activity. Hypothesis of inefficacious interaction with binding domain further explained that the naphthalenyl group in DPTH-N10a was introduced to sulfur atom at the β position leading to a decrease in anti-proliferation activity compared to that of DPTH-N10, which has its naphthalenyl group introduced at the α position. Furthermore, the anti-proliferation activity was higher for DPTH-ben, which contains a benzene ring, than that for DPTH-N10a, which possesses the same naphthalenyl group as that of DPTH-N10 but at the β position. It demonstrated that a desirable interaction between substrate and binding domain determined not only by the chain length of aryl side chain, but also by the right spatial direction of aryl side chain related to the sulfur atom. Our results showed that introduction of an aromatic ring structure with right spatial arrangement at sulfur atom of DPTH enhanced the anti-proliferation activity in HUVEC.

In the present study in vitro, we found that DPTH-N10 with a 2-methylnaphthalenyl functionality at sulfur atom of the parent compound DPTH exerts the strongest inhibition activity on the growth of human vascular endothelial cells among a series of alkyl or aryl congeners tested. By using capillary-like tube

formation, aortic ring culture, and chick embryo chorioallantoic membrane (CAM) assays, we further confirmed the anti-angiogenic effect of DPTH-N10 (unpublished data).

It has been generally believed that progression of cell cycle activity is regulated by coordinated successive activation of certain CDKs (Hunter and Pines, 1994; Morgan, 1995). This CDK activation is in turn modulated by association with a number of regulatory subunits called cyclins, and with a group of CDK-inhibitory proteins designated CKIs (Sherr and Roberts, 1995). Among these CKIs are two known as p21 and p27. Cyclin A-CDK2 and cyclin E-CDK2 complexes form late in the G1 phase as cells prepare to synthesize DNA (Lees, 1995). Previously, we showed that DPTH inhibits HUVEC proliferation by increasing the level of p21 protein, which in turn inhibits the activities of CDK2 and CDK4, and finally interrupts the cell cycle (Shih et al., 2004). It seems that the p21 protein represents the major signaling molecule involved in the DPTH-induced cell cycle arrest in HUVEC. Although we could not rule out the possibility that structural modifications might interfere the cell cycle progress of HUVEC through a different signaling pathway, our study in delineation of molecular mechanism underlying DPTH-N10-induced cell cycle arrest in HUVEC showed that DPTH-N10 induced cell cycle arrest in HUVEC through a same molecular mechanism as DPTH by increasing p21 protein levels (unpublished data). These findings suggest that the different anti-proliferation activity in HUVEC among the DPTH derivatives might be due to their lipophilicity or binding activity to the binding domain of a receptor or a targeting molecule.

5. Conclusion

In conclusion, we demonstrated that a positive correlation among the decreased thymidine incorporation (Fig. 2), decreased cell number (Fig. 3), increased p21 induction (Fig. 4) and inhibition of capillary-like tube formation (Fig. 5) in the DPTH derivatives-treated HUVEC. Although the primary target molecule of DPTH action is still unclear at this point, our results from the present study suggest that introduction of an aromatic ring structure with right spatial arrangement at the sulfur atom of DPTH enhanced the anti-angiogenic activity.

Acknowledgments

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