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# Anti-proliferation effect of 3-amino-2-imino-3,4-dihydro-2*H*-1,3-benzothiazin-4-one (BJ-601) on human vascular endothelial cells: G0/G1 p21-associated cell cycle arrest

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**Abstract**

The aim of this study was to examine the anti-proliferation effect of 3-amino-2-imino-3,4-dihydro-2*H*-1,3-benzothiazin-4-one (BJ-601) on human vascular endothelial cells and its possible molecular mechanism underlying. Our data showed that BJ-601 at a range of concentrations (0–40 μM) dose- and time-dependently decreased cell number in cultured human dermal microvascular endothelial cells (HDMVECs), but not human fibroblasts. The BJ-601-induced growth inhibition in HDMVECs was reversible. [$^3$H]thymidine incorporation demonstrated that BJ-601 arrested the HDMVECs at the G0/G1 phase of the cell cycle. Western blot analysis revealed that BJ-601 (0–40 μM) dose-dependently increased the levels of the protein p21, but not of p27, p53, cyclins A, D1, D3 and E, cyclin-dependent kinase 2 (CDK2), and CDK4 in HDMVECs. Immunoprecipitation showed that the formation of the CDK2–p21 complex, but not CDK2–p27, CDK4–p21 and CDK4–p27 complexes, was increased in the BJ-601-treated HDMVECs. Kinase assay further demonstrated that CDK2, but not CDK4, kinase activity was decreased in a dose-dependent manner in the BJ-601-treated HDMVECs. Pretreatment of HDMVECs with a p21 antisense oligonucleotide, which blocked the expression of p21 protein, reversed the BJ-601-induced inhibition of [$^3$H]thymidine incorporation into HDMVECs. Moreover, cotreatment of the endothelial cells with protein kinase C (PKC) inhibitor, staurosporine, prevented the BJ-601-induced decrease of [$^3$H]thymidine incorporation into HDMVECs. Administration of BJ-601 dose-dependently inhibited capillary-like tube formation of HDMVECs in Matrigel. In conclusion, these data suggest that BJ-601 inhibits HDMVECs proliferation by increasing the level of p21 protein, which in turn inhibits CDK2 kinase activity, and finally causes retardation of the cell cycle at the G0/G1 phase.


*Keywords:* BJ-601; Angiogenesis; p21; p53; Cyclin-dependent kinase; Endothelial cells

## 1. Introduction

Angiogenesis or neovascularization refers to formation of new capillary blood vessels as extensions of existing vessels, and is a tightly controlled process, which involves both endothelial cells and pericytes. 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; migration of endothelial cells from the existing vessel toward the angiogenic stimulus; proliferation of endothelial cells

*Abbreviations:* BJ-601, 3-amino-2-imino-3,4-dihydro-2*H*-1,3-benzothiazin-4-one; HDMVECs, human dermal microvascular endothelial cells; HUVECs, human umbilical vein endothelial cells; CDK, cyclin-dependent kinase; PKC, protein kinase C; HEPES, 4-(2-hydroxy-ethyl)-1-piperazineethanesulfonic acid; PMSF, phenylmethylsulphonyl fluoride; SDS, sodium dodecyl sulfate; NP-40, Nonident P-40; ECGS, endothelial cell growth supplement; M199, medium 199; FBS, fetal bovine serum; CKI, CDK-inhibitory protein; NBT, 4-nitro blue tetrazolium; BCIP, 5-bromo-4-chloro-3-indolyl-phosphate; DTT, dithiothreitol; FACS, fluorescence-activated cell sorter

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within the sprout; and the formation of a lumen with subsequent branching. Angiogenesis is thought to be regulated by angiogenic and anti-angiogenic factors [1]. In the physiological situation, the activity of angiogenic and anti-angiogenic factors maintains this in balance. However, persistent and unregulated angiogenesis is often found to be a critical causal factor in many pathological conditions [2].

Normally, vascular proliferation occurs only during embryonic development, in the ovary and uterus during the reproductive cycle and in tissues undergoing wound healing. In contrast, many pathological conditions (e.g., atherosclerosis, cancer, and diabetic retinopathy) are characterized by persistent, unregulated angiogenesis [2]. Control of the angiogenic process could become the primary method for treatment of these disorders. Accordingly, experimental and clinical investigators continue to search for new therapeutic strategies for inhibiting angiogenesis. Since proliferation of endothelial cells is one major event essential for angiogensis, our approach as pursued in this study is to identify medicinal agents capable of retarding the cell cycle in vascular endothelial cells.

Structurally, 3-amino-2-imino-3,4-dihydro-2*H*-1,3-benzothiazin-4-one (BJ-601) is a benzothiazinone derivative (Fig. 1). Previous studies have shown that benzothiazinone can block the voltage-dependent L-type calcium channels [3,4], and inhibit the activity of angiotension converting enzyme [5]. It has been suggested that such benzothiazinone derivatives may offer some useful medicinal actions including: analgesic, anti-inflammatory, antimicrobial, etc. [6]. Moreover, the benzothiazinone analogues have been shown to inhibit xanthine oxidase activity [7]. However, the anti-angiogenic activity of benzothiazinone and derivatives has not been addressed. While studying the cytotoxicity of benzothiazinone derivatives, we found that BJ-601 exerts significant inhibition on the growth of human vascular endothelial cells. This finding prompted us to study the anti-angiogenic effect of BJ-601 and its underlying molecular mechanisms.

Here, we demonstrate that BJ-601 dose-dependently inhibited the growth of HDMVECs by interrupting the transition of the cell cycle from the G1 into S phase and this occurred when CDK2 activity was inhibited just as the level of p21 protein increased. We show further that this p21-mediated cell cycle arrest was PKC-dependent.

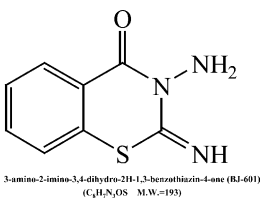


Fig. 1. Structure of 3-amino-2-imino-3,4-dihydro-2*H*-1,3-benzothiazin-4-one (BJ-601).

## 2. Methods and materials

### 2.1. Materials

BJ-601 was synthesized in our laboratory according to the method previously described [8]. The product was recrystallized with benzene to give white crystals, mp 149–152 °C (lit. 141–143 °C).

4-(2-Hydroxy-ethyl)-1-piperazineethanesulfonic acid (HEPES), glycerol, phenylmethylsulphonyl fluoride (PMSF), sodium dodecyl sulfate (SDS), Nonident P-40 (NP-40), and endothelial cell growth supplement (ECGS) were purchased from Sigma Chem. (St. Louis, MO, USA). Medium 199 (M199), trypsin-EDTA, and kanamycin were purchased from Life Technologies (Carlsbad, CA, USA). Matrigel was purchased from BD Bioscience Pharmigen (San Diego, CA, USA). Medium 200 (M200) was purchased from Cascade Biologics (Portland, OR, USA). Fetal bovine serum (FBS) was purchased from HyClone (Logan, UT, USA). Antibodies specific for cyclins, CDKs, and CDK-inhibitory proteins (CKIs) were purchased from Transduction Laboratories (Lexington, KY, USA). An antibody specific for G3PDH was purchased from Biogenesis (Kingston, NH, USA). Anti-mouse IgG conjugated alkaline phosphatase was purchased from Jackson Immuno Research Laboratories (West Grove, PA, USA). 4-Nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) were purchased from Kirkegaard & Perry Laboratories (Gaithersburg, MD, USA). Protein assay agents were purchased from Bio-Rad (Hercules, CA, USA).

### 2.2. Cell culture

HDMVECs isolated from human foreskin or human umbilical venous endothelial cells (HUVECs) isolated from human umbilical vein were grown in M199 medium 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. At confluence, the cells were disaggregated in trypsin solution, and the cells were washed with M199 containing 10% FBS, harvested at $125 \times g$ for 5 min, and then subcultured according to standard protocols. Cells from passages 5 to 9 were used.

### 2.3. [$^3$H]thymidine incorporation

The [$^3$H]thymidine incorporation was performed as previously described [9,10]. Briefly, HDMVECs or HUVECs 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 either 0.05% DMSO (control) or various concentrations of BJ-601 was added to the cells and the cultures were allowed to incubate for 21 h. During the last 2 h of the incubation (with or without BJ-601), [$^3$H]thymidine was added at 1 μCi ml$^{-1}$ (1 μCi = 37 kBq). Incorporated [$^3$H]thymidine was extracted in 0.2N NaOH and measured in a liquid scintillation counter.

### 2.4. Cell counting

To study the anti-proliferative effect of BJ-601 on the vascular endothelial cells, we examined changes in cell number in response to BJ-601 treatment in subcultured HDMVECs. 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 BJ-601 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

To confirm that the results of our studies of cellular proliferation in HDMVECs were not due to cell death caused by BJ-601 treatment, we conducted viability assay by treating the cells with BJ-601 for 3 days at the maximal dose (40 μM) used in the studies of cell growth inhibition. Cell viability was estimated by trypan blue exclusion assay as previously described [11]. Four samples were analyzed in each experiment.

### 2.6. Protein preparation and Western blotting

To determine the expression levels of cyclins, CDKs, CKIs, and G3PDH in HDMVECs, the cells were cultured in 10 cm petri dishes. After reaching 70–80% confluence, the cells were rendered quiescent and then M199 supplemented with 10% FBS and 0.05% DMSO (control) or various concentrations of BJ-601 was added to the cells and the cultures were allowed to incubate for 21 h. After incubation, the total proteins were extracted and Western blot analyses were performed as described previously [10,12].

### 2.7. Immunoprecipitation

As previously described [10], CDK2 or CDK4 was immunoprecipitated from 200 μg of protein by using anti-CDK2 or anti-CDK4 antibody (2 μg ml$^{-1}$) and protein A agarose beads (1/10 V). The precipitates were washed with washing buffer, resuspended 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

The assays for CDK2 and CDK4 kinase activities in the BJ-601-treated and control HDMVECs were measured as previously described [10].

### 2.9. Flow cytometry

As previously described [13], the cells were seeded onto 10 cm petri dishes and grown in M199 supplemented with 10% FBS and ECGS. After the cells had grown to 70–80% confluence, they were rendered quiescent and challenged with 10% FBS. Then, after release using trypsin-EDTA, they were washed twice with PBS and fixed in 70% ethanol at 4 °C. Nuclear DNA was stained with a reagent containing propidium iodine (8 μg ml$^{-1}$) and DNase-free RNase (100 μg ml$^{-1}$) and measured using a fluorescence-activated cell sorter (FACS).

### 2.10. Capillary-like tube formation assay

Capillary-like tube formation assay was performed as described previously [14] with minor modifications. The 96-well plates were coated with 50 μl Matrigel (10 mg ml$^{-1}$) by incubating at 37 °C for 1 h. HDMVECs 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 $4 \times 10^4$ cells per well without or with BJ-601 (5–40 μM). Three samples for each treatment were tested. The plates were then incubated for a further 3 h at 37 °C, and capillary-like tube formation was observed under phase contrast microscopy.

### 2.11. Statistical analysis

Values represent the means ± S.E. Three to four samples were analyzed in each experiment. Comparisons were subjected to one-way ANOVA followed by Fisher's least significant difference test. Significance was accepted at $P < 0.05$.

## 3. Results

### 3.1. Inhibition of cell proliferation in HDMVECs by BJ-601

Fig. 2a showed a time-dependent decrease in cell count in the BJ-601 (15 μM)-treated HDMVECs as compared with control DMSO-treated cells. As illustrated in Fig. 2b, treatment of HDMVECs with BJ-601 (0–40 μM) for 3 days

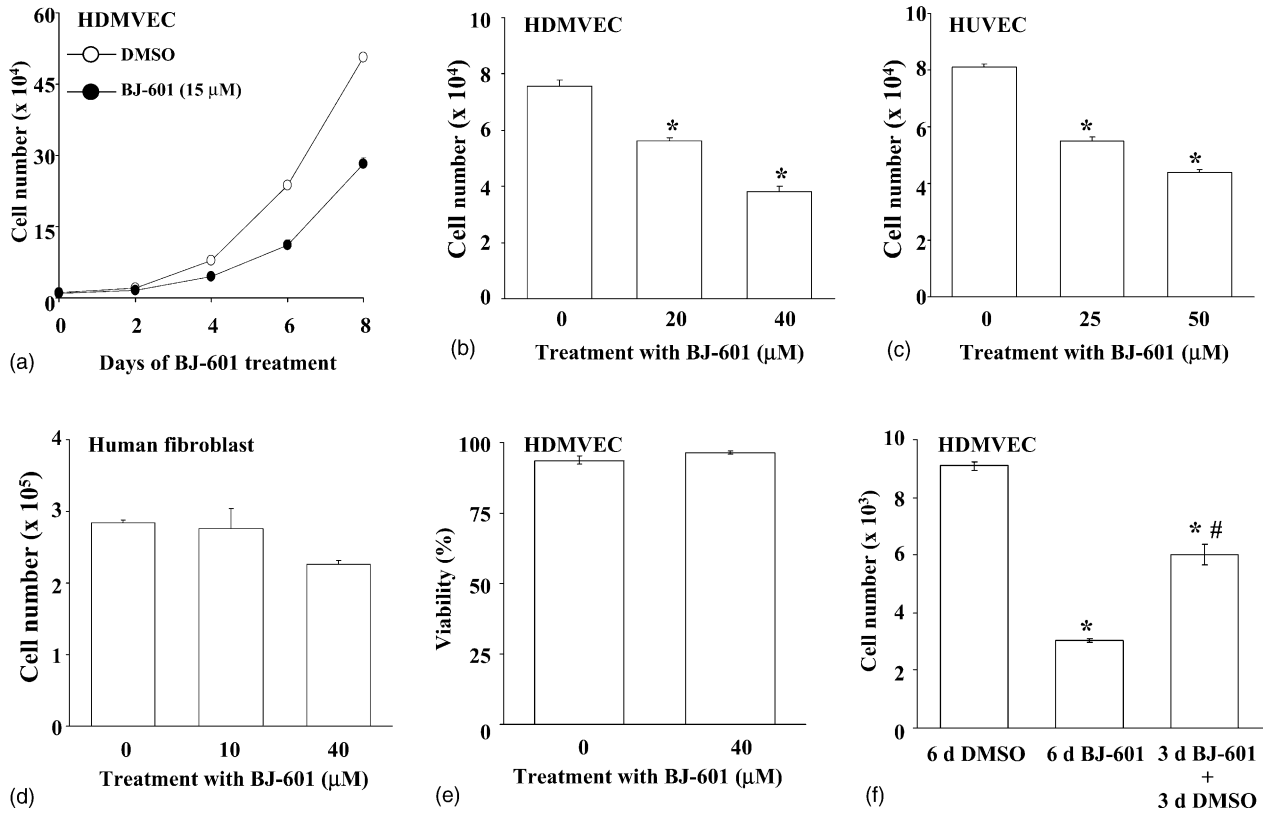


Fig. 2. Effects of BJ-601 on cell growth rate in subcultured human vascular endothelial cells. The cells were treated with DMSO (control) or BJ-601. Media without (control) or with BJ-601 were changed daily until cell counting. (a) Time-dependent inhibition of HDMVECs growth by 15 μM BJ-601 treatment. (b) Dose-dependent inhibition of HDMVECs growth by BJ-601. (c) Dose-dependent inhibition of HUVECs growth by BJ-601. (d) Treatment with BJ-601 (0–40 μM) for 3 days did not significantly affect cell growth of human fibroblasts. (e) There was no significant difference in viability between control and BJ-601-treated HDMVECs. (f) BJ-601-induced inhibition of HDMVECs proliferation was reversed by removal of BJ-601. Treatment of HDMVECs with 40 μM BJ-601 for 6 days induced a significant inhibition of cell number (67% inhibition) as compared with the cells treated with 0.05% DMSO. However, treatment of the cells with 40 μM BJ-601 for 3 days followed by 0.05% DMSO without BJ-601 for an additional 3 days reversed the inhibition (34% inhibition). Three to four samples were analyzed in each group, and values represent the means ± S.E. Significance was accepted at $P < 0.05$. The symbol (*) indicates that BJ-601-treated group is different from control group. The symbol (#) indicates that 3d BJ-601 + 3d DMSO-treated group is different from BJ-601-treated group.

induced a decrease in cell number in a dose-dependent manner. This BJ-601-induced decrease in the cell number was also observed in HUVECs (Fig. 2c). In contrast, human fibroblasts were less sensitive to BJ-601 treatment (Fig. 2d), suggesting a preferential effect of BJ-601 on vascular endothelial cell growth inhibition. The BJ-601-induced reduction in [$^3$H]thymidine incorporation in HDMVECs can be due to retardation of the cell cycle or the occurrence of cell death. Trypan blue assays indicated that there was no significant difference in cell viability between control and BJ-601-treated HDMVECs (Fig. 2e). We also examined the reversibility of the BJ-601-induced growth inhibition in HDMVECs. As illustrated in Fig. 2f, treatment of HDMVECs with 40 μM BJ-601 for 6 days induced a 67% reduction of cell number as compared with the cells treated with 0.05% DMSO for 6 days. However, treatment of the cells with 40 μM BJ-601 for 3 days followed by 0.05% DMSO without BJ-601 for an additional 3 days induced only 34% inhibition as compared with the cells treated with 0.05% DMSO for 6 days. These results suggest that the BJ-601-induced inhibition of cell growth is reversible.

### 3.2. Retardation of cell cycle in G0/G1 phase by BJ-601 treatment

In order to demonstrate more sharply the actions of BJ-601 on a specific phase of the cell cycle, the cells were switched to media with 2% FBS for 24 h to render them quiescent and to synchronize their cell cycle activities. They were then returned to culture media supplemented with 10% FBS and either 0.05% DMSO (control) or BJ-601 (15 μM) and, at various times thereafter, they were treated with [$^3$H]thymidine. As illustrated in Fig. 3, BJ-601 induced a reduction of the [$^3$H]thymidine incorporation into HDMVECs during the S phase of the cell cycle.

### 3.3. Alterations in cell cycle activity by BJ-601 treatment

As shown in Fig. 4a, treatment of HDMVECs with BJ-601 for 21 h at a range of concentrations (0–40 μM), which caused the inhibition of [$^3$H]thymidine incorporation and cell growth arrest, did not induce any significant changes of the protein expression levels of cyclins A, D1,

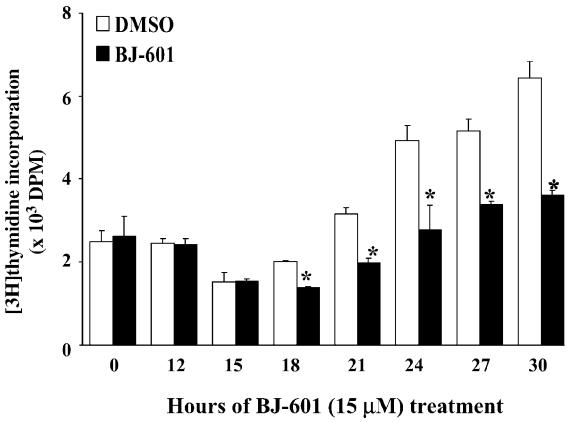


Fig. 3. Retardation of the cell cycle in HDMVECs by BJ-601. Three to four samples were analyzed in each group, and values represent the means ± S.E. Significance was accepted at $P < 0.05$. The symbol (*) indicates that BJ-601-treated group is different from control DMSO-treated group.

D3, E, CDK2 and CDK4, suggesting that BJ-601-induced cell cycle arrest in HDMVECs is not through alternations 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 BJ-601-treated HDMVECs. Fig. 4b showed that the protein levels of p21, but not p27, were dose-dependently increased in the BJ-601-treated HDMVECs 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 BJ-601-treated HDMVECs. As illustrated in the Fig. 4b, BJ-601 treatment did not cause any significant change in the level of p53 protein.

The CKI exerts its inhibitory effect on the kinase activity through formation of cyclin–CDK complexes. Accordingly, we found that the formation of the CDK2–p21, but not CDK2–p27, CDK4–p21 and CDK4–p27 complex, was increased in the BJ-610 (Fig. 5a). To demonstrate that the increased p21 protein is associated with inhibition of CDK activation, which is necessary for cell cycle progression from G1 to S phase, we examined the CDK kinase activity. Fig. 5b shows that the assayable CDK2, but not CDK4, kinase activity was significantly decreased in the HDMVECs treated with BJ-601.

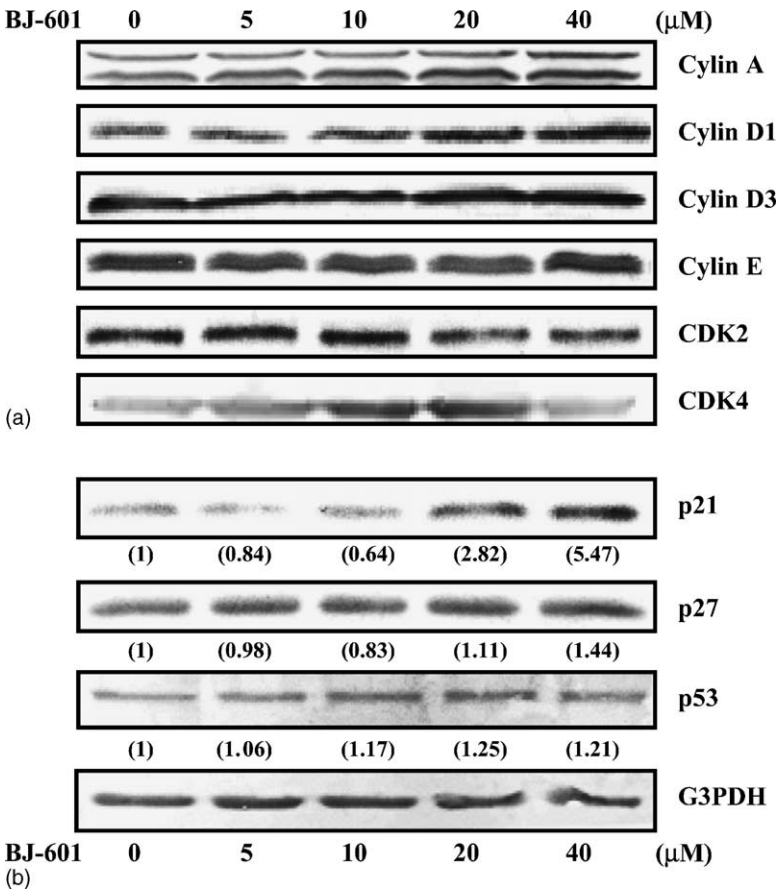


Fig. 4. Effect of BJ-601 on the protein levels of cyclins, CDKs and CKIs. Western blot analysis was performed to examine the changes of protein levels of cyclins, CDKs, and CKIs in the BJ-601-treated HDMVECs. Proteins were extracted from the cultured HDMVECs at 21 h after BJ-601 treatment and probed with proper dilutions of specific antibodies. (a) BJ-601 (0–40 μM) did not induce any significant change of the protein levels of cyclins A, D1, D3, and E, CDK2, and CDK4. Results from a representative experiment are shown. (b) Treatment of HDMVECs with BJ-601 (0–40 μM) for 21 h dose-dependently increased the protein levels of p21, but not p27 and p53. Results from a representative experiment are shown. Membrane was probed with anti-G3PHD antibody to verify equivalent loading. The protein levels in each treatment after normalization with the levels of G3PDH protein are shown in parentheses. CDK, cyclin-dependent kinase.

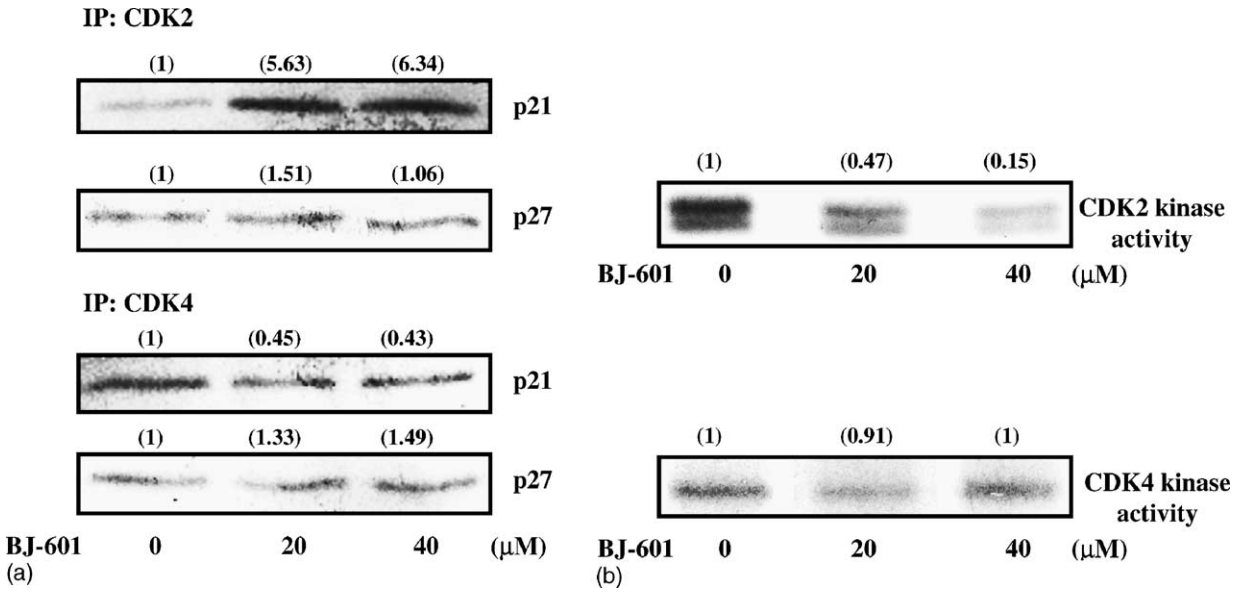


Fig. 5. Effect of BJ-601 on the formations of CKI–CDK complex and CDK kinase activity. (a) BJ-601 induced upregulation of the formations of CDK2–p21 complex in a dose-dependent manner. The formations of CDK2–p27, CDK4–p21 and CDK4–p27 complex were not affected significantly by BJ-601 treatment. Results from a representative experiment are shown. (b) Treatment of the HDMVECs with BJ-601 dose-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 Section 2. The intensity ratio of each band versus G3PDH was shown in parentheses. CDK, cyclin-dependent kinase.

In the experiments of Fig. 6, [$^3$H]thymidine uptake was employed to show how DNA synthesis (and cell cycle activity) was affected by the BJ-601 treatment, and to illustrate further the role of p21. As shown in Fig. 6a and b, treatment of the cells with 20 μM BJ-601 induced 30% decreases in [$^3$H]thymidine uptake. In Fig. 6a, the third and fourth bars showed that in the presence of 10 and 20 nM p21 AS, the expression of p21 was greatly reduced, and, as a consequence, the BJ-601 induced decreases in [$^3$H]thymidine were virtually eliminated. Incidentally, bar 5 showed that the p21 AS by itself, while decreasing p21 expression, did not affect [$^3$H]thymidine uptake. Hence, we conclude that BJ-601 induced expression of p21, which in turn, blocked CDK activation, reduced DNA synthesis and thereby arrested the cell cycle function at G0/G1. As a control study, when p27 AS was used in place of the p21 AS, so that p21 expression was unimpaired, BJ-601 induced decrease in [$^3$H]thymidine uptake occurred as expected (Fig. 6b). Evidently, p27 was not involved in this action of BJ-601.

### 3.4. Involvement of PKC-pathway in the BJ-601-induced anti-proliferation

To examine whether a PKC-mediated signaling pathway is involved in the BJ-601-induced anti-proliferation in vascular endothelial cells, the cells were pretreated with staurosporine, a PKC inhibitor, followed by BJ-601. As shown in Fig. 7, pretreatment the cells with staurosporine ($1 \times 10^{-4}$ nM) prevented the BJ-601-induced inhibition of [$^3$H]thymidine incorporation into endothelial cells.

### 3.5. Anti-angiogenic effect of BJ-601

Since endothelial cell proliferation and migration are processes integral to the formation of capillary-like tube structures, we used an in vitro Matrigel assay to examine the effect of BJ-601 on tube formation. As illustrated in Fig. 8, the number of cord-like structures formed after 3 h of incubation in the presence of 10 μM BJ-601 was visibly lower than in the control cells treated with PBS (Fig. 8c). The BJ-601-induced decrease in the capillary-like tube formation was in a dose-dependent manner. At 40 μM BJ-601, tube formation was virtually undetectable (Fig. 8e).

## 4. Discussion

Control of vascular development has been suggested to offer new therapeutic approaches to many angiogenesis-related disorders. In the present study, we demonstrated that BJ-601 at a range of concentrations (0–40 μM) dose-dependently inhibited DNA synthesis, decreased cell number, and suppressed tube formation in cultured HDMVECs (Fig. 2). These results were not due to cell death, indicating that there was an inhibitory effect of BJ-601 on the mechanisms for normal cell function and division in the subcultured HDMVECs. The BJ-601-induced growth inhibition in human vascular endothelial cells is reversible. To our knowledge, this is the first demonstration that BJ-601 inhibits the growth of human vascular endothelial cells.

By thymidine incorporation analysis, we demonstrated that BJ-601 treatment decreased DNA synthesis and arrested the cells at the G0/G1 phase of the cell cycle

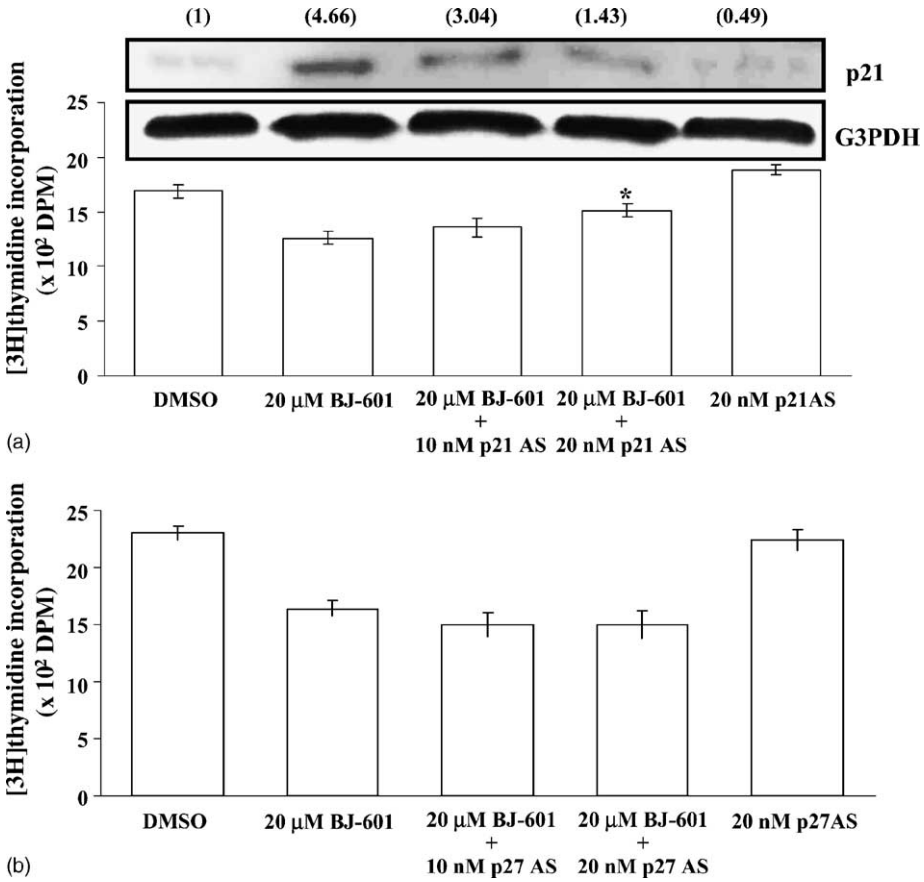


Fig. 6. Involvement of p21 in the BJ-601-induced decrease of [$^3$H]thymidine incorporation in HDMVECs. Pretreatment of HDMVECs with AS p21 (a), but not AS p27 (b), dose-dependently reversed the BJ-601-induced up-regulation of p21 protein and decrease of [$^3$H]thymidine incorporation. The levels of p21 protein in each treatment after normalization with the levels of G3PDH protein are shown in parentheses. Values represent the means ± S.E. AS p21, antisense p21 oligonucleotide; AS p27, antisense p27 oligonucleotide. Three to four samples were analyzed in each group, and values represent the means ± S.E. Significance was accepted at $P < 0.05$. The symbol (*) indicates that p21 AS + BJ-601-treated group different from BJ-601-treated group.

(Fig. 3). It has been suggested that the cell cycle is regulated by the activation of specific CDKs and coordinated successive activation of certain CDKs occurs late in the G1 phase and is instrumental in the transition from the G1 to the S phase [15,16]. 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

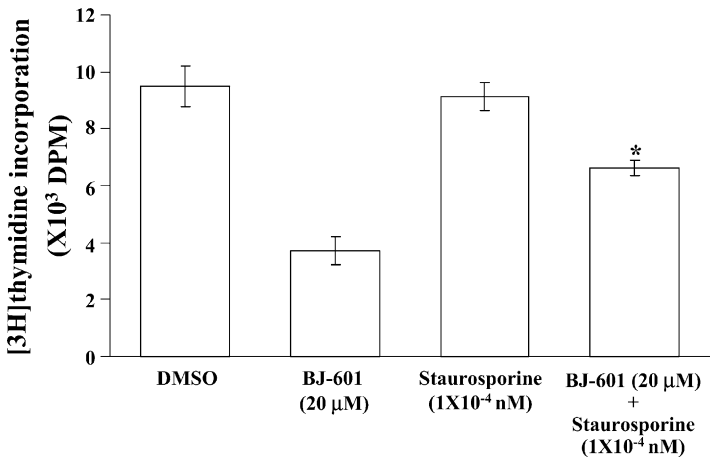


Fig. 7. Involvement of PKC activation in the BJ-601-induced decrease of [$^3$H]thymidine incorporation. HDMVECs were co-treated with staurosporine ($1 \times 10^{-4}$ nM) and BJ-601 (20 μM). In the presence of staurosporine, the BJ-601-induced inhibition of [$^3$H]thymidine incorporation was prevented. Values represent the means ± S.E. Significance was accepted at $P < 0.05$. The symbol (*) indicates that staurosporine + BJ-601 group different from BJ-601-treated group, DMSO-treated control group, and staurosporine-treated group.

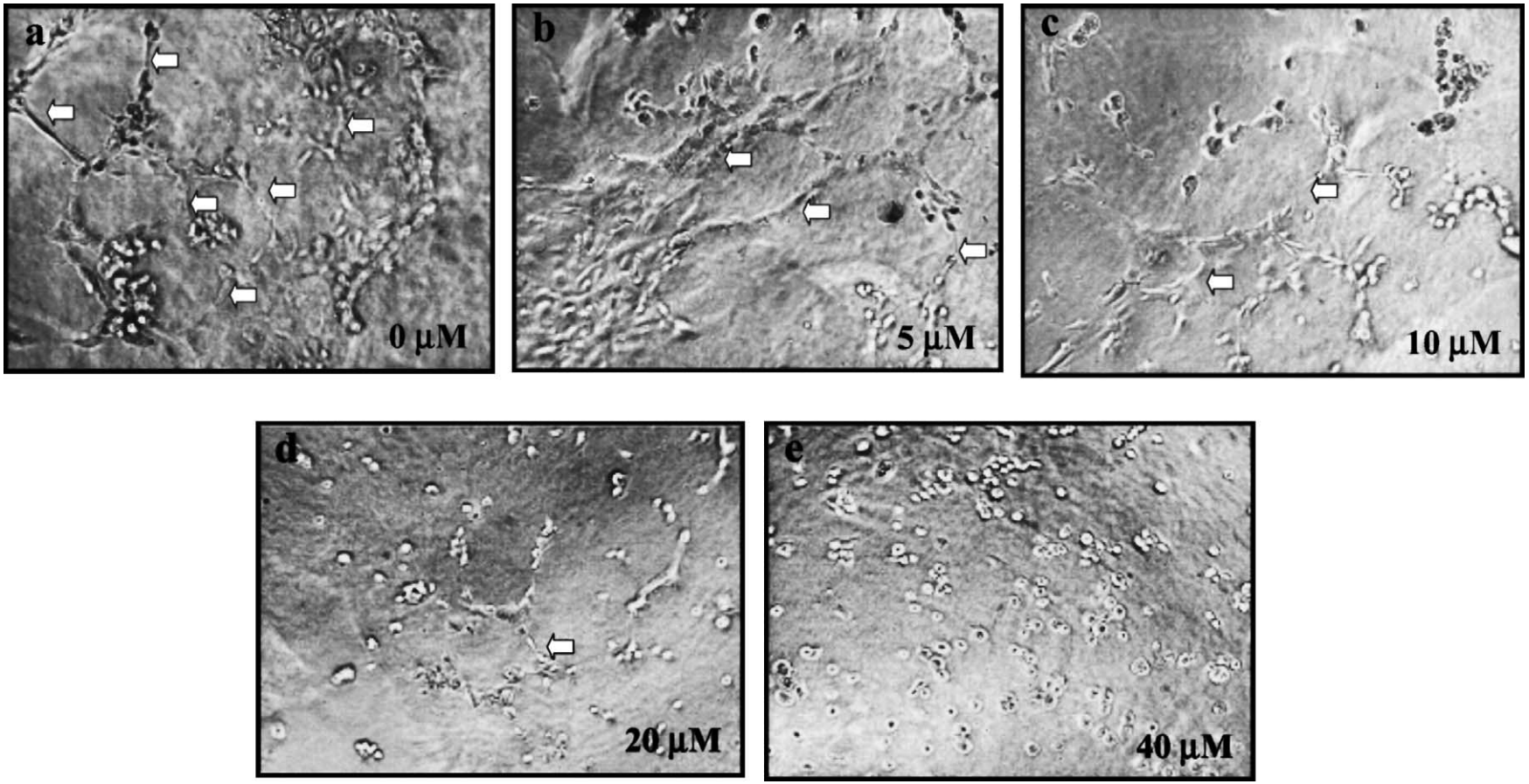


Fig. 8. BJ-601 inhibits capillary-like tube formation of HDMVECs in Matrigel. HDMVECs were suspended in M200 media supplemented with 10% FBS and endothelial cell growth supplement, and plated onto a layer of Matrigel at a density of $4 \times 10^4$ cells per well without (a) or with BJ-601 at a concentration of 5 (b), 10 (c), 20 (d) or 40 μM (e). Results from a representative experiment are shown. Arrow markers indicate representative tube formations.

CKIs [17]. Cyclins have been identified as cyclins A, D1, D3 and E, whereas the most common CDKs are designated CDK2 and CDK4. The formations of cyclin A–CDK2 and cyclin E–CDK2 complexes occur late in the G1 phase as cells prepare to synthesize DNA [18], and formation of the cyclin E complex is a rate-limiting step in the G1/S transition [19]. The basic mechanisms for cell cycle regulation appear to be universal. In the present study, we demonstrated that BJ-601 at a concentration of 40 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 HDMVECs, indicating that the action of BJ-601 on the growth of endothelial cells is not through a mechanism to reduce the cyclins or CDKs. Examining the expression of CDK inhibitory proteins, we found that treatment of HDMVECs with BJ-601 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, but not CDK2–p27, CDK4–p21 and CDK4–p27complex, was increased and the assayable CDK2, but not CDK4, kinase activity was decreased in the BJ-601-treated HDMVECs. These findings suggest that BJ-601 inhibits the CDK2 kinase activity through an increase in p21 expression. The important role of p21 in the BJ-601-induced anti-proliferation in the HDMVECs is confirmed by the antisense p21 oligonucleotide experiment showing that pretreatment with a p21 antisense oligonucleotide, but not p27 antisense oligonucleotide, reversed the BJ-601-induced inhibition in [$^3$H]thymidine incorporation. Accordingly, we concluded that BJ-601 induced an increase in p21 expression, which in turn inhibited the CDK2 enzyme activity and led to the impairment of HDMVECs in the transition from the G1 to S phase.

It has been demonstrated that p21 is a transcriptional target of the tumor suppressor gene p53 [20,21]. Expression of p53 in the cells can induce cell growth arrest through transcriptional activation of p21 [22]. However, treatment of HDMVECS with BJ-601 did not affect the expression level of p53 protein, suggesting that change of p53 protein is not involved in this process. Previously, we have demonstrated that a PKC-mediated signaling pathway has also been suggested to be involved in the regulation of p21 expression [10]. In the present study, treatment of HDMVECs with PKC inhibitor (staurosporine) prevented the BJ-601-induced inhibition in [$^3$H]thymidine incorporation (Fig. 7), suggesting that PKC-mediated signaling pathway might be involved in the BJ-601-induced anti-proliferation in vascular endothelial cells. Since staurosporine may alter other kinase besides PKC and we have not used any other specific PKC inhibitor in the present study, a definitive conclusion about the role of PKC is not yet at hand. A further study to address this issue is still ongoing in our research to identify the primary target molecules of BJ-601 in regulating p21 function.

At present, we have demonstrated the anti-proliferation and anti-capillary-like tube formation of BJ-601 in human vascular endothelial cells. Hence, we conclude that the BJ-601-induced cell cycle arrest in HDMVECs occurred when the cyclin–CDK system was inhibited just as p21 protein levels increased. The findings from the present studies suggest the potential application of BJ-601 in the treatment of angiogenesis-related disorders.

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