Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry 16 (2008) 644–649
Bioorganic & Medicinal Chemistry

# Antiproliferative effects of 6-anilino-5-chloro-1H-benzo[*d*]imidazole-4,7-dione in vascular smooth muscle cells

Sung-Yu Hong,[a] Kyu-Won Kwak,[c] Chung-Kyu Ryu,[b] Soo-Jung Kang[a] and Kwang-Hoe Chung[c,*]

[a]*National Research Laboratory for Cardiovascular Therapy, BioBud Inc., Geumchen-gu, Seoul 153-777, Republic of Korea*
[b]*College of Pharmacy, Ewha Womans University, Seodaemun-ku, Seoul 120-750, Republic of Korea*
[c]*Thrombosis & Vascular Biochemistry Laboratory, Department of Biochemistry, College of Medicine, Pochon CHA University, Sungnam 463-836, Republic of Korea*

Received 14 August 2007; revised 15 October 2007; accepted 16 October 2007
Available online 24 October 2007

**Abstract**—It has been known that benzimidazol-4,7-diones have antiproliferative activity against various cancer cell lines. Recently, we have also reported that these compounds strongly inhibited the proliferation of vascular smooth muscle cell (SMC) and human umbilical vein endothelial cells (HUVECs). Although benzimidazol-4,7-diones have important biological activities, the molecular mechanism of the compounds in these cells remains to be elucidated. In order to investigate the anti-proliferation mechanism of the compounds in smooth muscle cell, we selected 6-anilino-6-chloro-5-chloro-1*H*-benzo{*d*}midazole-4,7-dione (BUD-0203) among 12 benzimidazol-4,7-dione derivatives and examined its antiproliferative effects. Phosphorylation of the extracellular-signal regulated kinase (ERK) reached a maximal level at 1 h after treatment with BUD-0203 and was sustained during the examined period. We also observed that phosphorylation of p38 reached a maximal level at 4 h and decreased to control levels after 8 h. These results showed that BUD-0203 sustainedly activated MAP kinase pathways in SMC. However, this compound did not induce cell cycle arrest in G1 or G2 phase in these cells. We also demonstrated that BUD-0203 not only induced apoptosis of SMC, but it also strongly inhibited SMC migration induced by platelet-derived growth factor (PDGF) or serum.Taken together, our experiments indicate that benzimidazol-4,7-diones induce apoptosis of smooth muscle cell via simultaneously prolonged activation of MAP kinase pathways including ERK, p38, and JNK/SAPK, similar with the apoptosis mechanism reported previously.


## 1. Introduction

The abnormal proliferation and migration of SMCs is known to be involved in various pathogeneses of cardiovascular diseases such as atherosclerosis and restenosis after angioplasty.[1] During restenosis after angioplasty, the accumulation of SMCs is believed to be due to the combined process of cell proliferation and directed migration of quiescent cells from arterial media into the intima.[2,3] SMC migration and proliferation is an important factor of the formation of atherosclerotic plaques.[2,3] Several growth factors, including platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF), induce the proliferation and migration of arterial SMCs.[4,5]

Extracellular signals are transmitted to intracellular molecules to coordinate appropriate responses. Among the signal transduction pathways, mitogen-activated protein kinase (MAPK) pathways play a critical role in the proliferation of eukaryotic cells.[6] These cascades consist of three kinase modules that include an MAPK, which is activated by an MAPK/ERK kinase (MEK), which, in turn, is activated by an MEK kinase (MEKK).[7] Activation of MAPK pathways is observed following the treatment of cells with growth factors, tumor necrosis factor (TNF), interleukin-1, and upon exposure to stresses (heat, UV).[8] A central function of the MAPK pathways is the activation of gene expression, which is mediated through the phosphorylation of transcription factors. ERK, p38, and JNK pathways have dual functions. The activation of the pathways leads to proliferation of SMCs,[9] or to growth arrest

*Keywords*: 6-Anilino-5-chloro-1H-benzo[*d*]imidazole-4,7-dione; Antiproliferation; Vascular smooth muscle cells; MAP kinase pathway; Cell cycle; Apoptosis; Restenosis.
* Corresponding author. Tel.: +82 31 725 8379; fax: +82 31 725 8350; e-mail: hoe@cha.ac.kr

0968-0896/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2007.10.069

and apoptosis of SMCs.[10] It has been reported that transient activation of p38 and JNK leads to cell survival, whereas persistent activation of those proteins induces apoptosis of the cells.[11,12]

Compounds containing the heterocyclic quinone group represent an important class of biologically active molecules.[13] Some benzimidazole-4,7-dione derivatives have been found to exhibit cytotoxic activities against human lymphoblastic leukemia, non-Hodgkin lymphoma, and other cancer cell lines.[14,15] The compounds also showed anti-fungal activities[16] and inhibited protozoal purin nucleoside phosphorylase.[17]

We recently reported that some benzimidazole-4,7-dione derivatives strongly inhibited the proliferation of SMCs[18] and HUVECs.[19]

In this study, we chose 6-anilino-5-chloro-1H-benzo[*d*]imidazole-4,7-dione (we mentioned this benzimidazole-4,7-dione derivative as BUD-0203) among 12 benzimidazole-4,7-dione derivatives as a model drug for elucidating antiproliferative effects in SMCs because it had potent anti-proliferation activity and showed lowest cytotoxicity in HUVEC and NIH3T3 cell. We proposed that the compounds would be candidates for drugs that inhibit vascular SMCs. We further investigated the effect of BUD-0203 on the migration of SMCs induced by PDGF or serum and apoptosis of SMCs. We also studied the effect of the compound on the MAPK pathways, including ERK1/2 and p38 of SMCs, to verify the mechanism of apoptosis induced by the compound.

## 2. Results

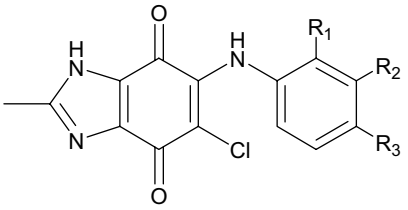
### 2.1. 6-Anilino-5-chloro-1H-benzo[*d*]imidazole-4,7-dione derivative (BUD-0203)

In the previous study, we reported that benzimidazole-4,7-dione derivatives had anti-proliferative activity against SMCs[18] and HUVECs.[19] Among the benzimidazole-4,7-dione derivatives, we chose 6-anilino-5-chloro-1H-benzo[*d*]imidazole-4,7-dione (Table 1) as a test compound to study the mechanism because it had potent and selective anti-proliferation activity as compared to the other benzimidazole-4,7-dione derivatives.

### 2.2. Activation of ERK1/2, p38, JNK/SAPK pathways by BUD-0203

To examine whether the BUD-0203 activates the MAPK pathways, we measured the phosphorylation level of ERK1/2, p38 by using Western blotting. As shown in Figure 1, BUD-0203 induced the phosphorylation of ERK1/2. The phosphorylation of ERK1/2 was dramatically increased at 5 min after the addition of the compound to culture media, while the expression level of ERK1/2 was not changed. The phosphorylation of p38 was increased at 2 h after exposure to the chemical, and it reached the maximum level at 4 h (Fig. 1). Notably, the phosphorylation of p38 was retained for more than 8 h. Together with our recent report that the compound activated the phosphorylation of JNK/SAPK,[18] the above data indicated that BUD-0203 activated all MAPK pathways, including the ERK1/2, p38, and JNK/SAPK pathways.

**Table 1.** Structures and $IC_{50}$ values of the benzimidazole-4,7-dione derivatives for inhibition of SMC, HUVEC, and NIH-3T3 cells




| Compounds | $R_1$ | $R_2$ | $R_3$ | $IC_{50}$ (μM) | | |
|---|---|---|---|---|---|---|
| | | | | SMC | HUVEC | NIH-3T3 |
| BUD-0201 | H | Br | H | 1.0 | 0.8 | 0.8 |
| BUD-0202 | H | H | CH3 | 0.8 | 1.0 | 1.6 |
| BUD-0203 | H | H | H | 0.8 | 1.2 | 5.2 |
| BUD-0204 | H | H | Cl | 2.8 | 0.8 | 1.3 |
| BUD-0205 | H | H | F | 3.0 | 1.0 | 1.2 |
| BUD-0206 | Cl | Cl | H | 0.6 | 0.3 | 2.3 |
| BUD-0207 | Cl | H | Cl | 0.8 | 0.5 | 1.2 |
| BUD-0208 | F | H | F | 1.2 | 1.0 | 0.8 |
| BUD-0209 | H | Cl | H | 0.9 | 0.6 | 1.6 |
| BUD-0210 | F | F | F | 0.8 | 0.5 | 1.6 |
| BUD-0211 | H | H | $CF_3$ | 0.6 | 0.6 | 1.1 |
| BUD-0212 | H | H | I | 1.0 | 2.0 | 0.6 |

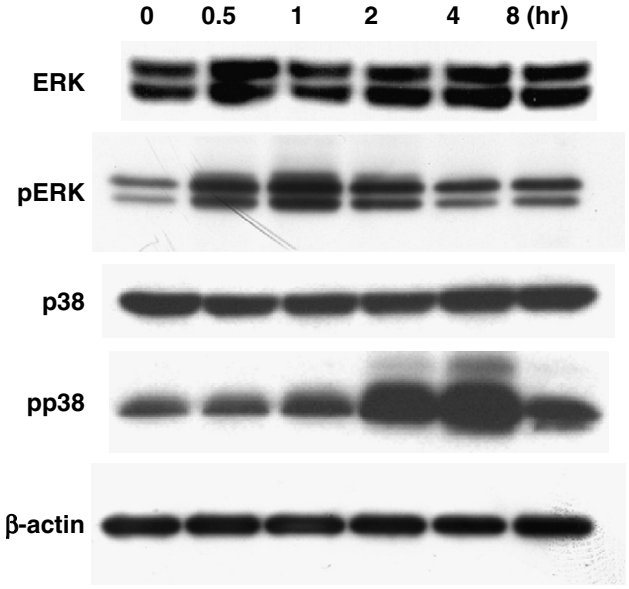



**Figure 1.** Activation of ERK and p38 kinases in BUD-0203-treated rat aortic smooth muscle cells. Cells treated with 1 μM of the compound for the indicated period of time were immunoblotted with anti-ERK1/2, anti-phospho ERK1/2, anti-p38, and anti-phospho-p38 antibody, respectively.

### 2.3. Induction of apoptosis by BUD-0203

To investigate whether BUD-0203 induces the apoptosis of smooth muscle cells, the cells were treated with BUD-

0203, and the activated form of caspase 3 was then detected by Western blotting. As shown in Figure 2, the cleaved form of caspase 3, of which the molecular weight is 17 and 12 kDa, was increased in the presence of the compound. This result revealed that the compound induced the apoptosis of smooth muscle cells via activation of caspase 3.

### 2.4. The effect of BUD-0203 on the cell cycle of SMC

To investigate the effect of BUD-0203 on the cell cycle of SMCs, we analyzed the DNA contents of the cell by flow cytometry. As shown in Figure 3, BUD-0203 decreased the percentage of the cells in G0/G1, but did not change the percentage in G2/M, which indicated that the compound did not arrest the cell cycle of SMCs at G1 or G2 phase.

Next, to further confirm the result of flow cytometric analysis, we examined the expression level of cyclin E in the presence of BUD-0203. In this experiment, the cells were pretreated with PDGF and 10% serum to fully induce cell growth. As shown in Figure 4, the increase of cyclin E expression induced by PDGF and serum was not affected by the treatment of the compound. This result indicated that the compound did not arrest the cell cycle at G0/G1 phase in SMCs.

### 2.5. Inhibition of SMC migration by BUD-0203

Together with SMC proliferation, migration of SMCs across basement membrane to the intima is an important step in restenosis. In order to investigate whether BUD-0203 affects the migration of SMCs induced by PDGF and serum, a Transwell plate migration assay was performed as described in Section 4. As expected, the migration of SMCs induced by PDGF and serum was significantly reduced by the compounds (Fig. 5).

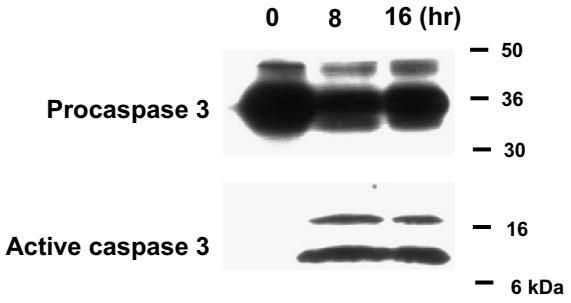



**Figure 2.** Induction of apoptosis by BUD-0203 in rat aortic smooth muscle cells. Cells treated with 1 μM of the compound for 8 and 16 h were immunoblotted with anti-caspase 3 antibody.

## 3. Discussion

Restenosis after angioplasty is a major limitation to the long-term success of the procedure. It is known that restenosis involves the migration of SMCs across the basement membrane to the intima, where the cells pro-

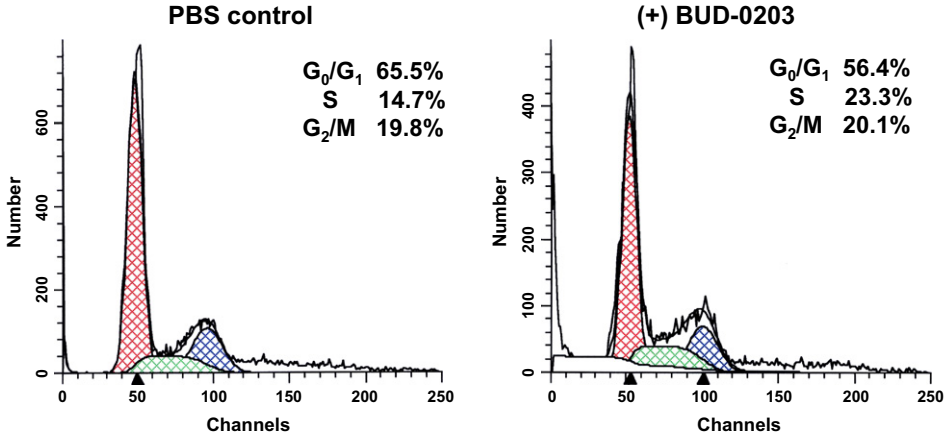



**Figure 3.** DNA content analysis in rat aortic smooth muscle cells by flow cytometry. Cells were treated with 1 μM of BUD-0203 for 16 h. Cells were then collected and stained with propidium iodide (PI). The stained cells were analyzed by flow cytometry (Becton–Dickinson, Franklin Lakes, NJ).

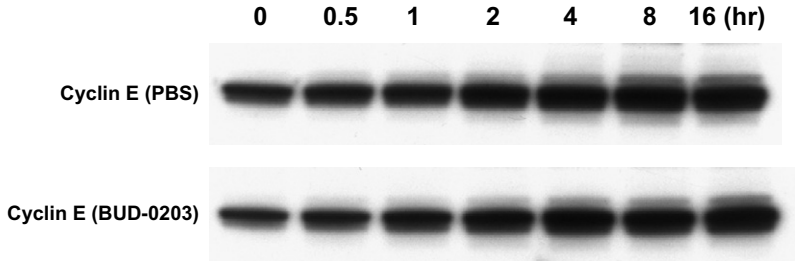



**Figure 4.** Effect of BUD-0203 on expression of cyclin E in rat aortic smooth muscle cells. SMCs treated with 1 μM of BUD-0203 for the indicated period of time were immunoblotted with anti-cyclin E antibody.

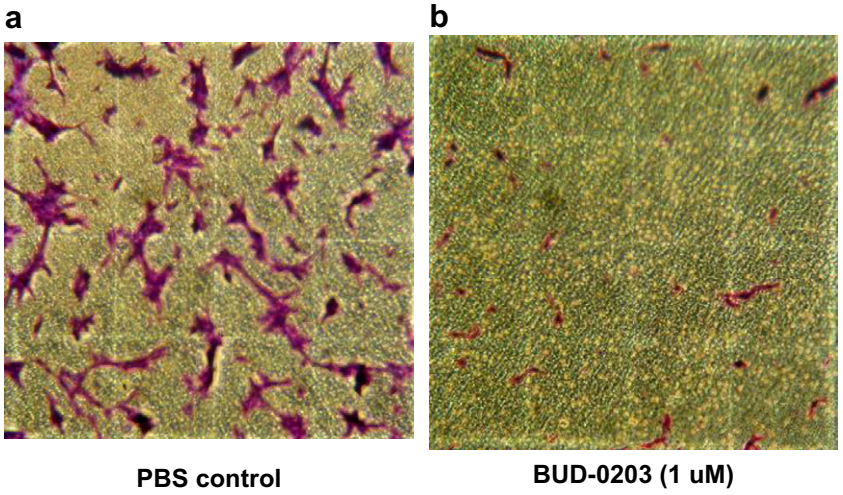



**Figure 5.** BUD-0203 inhibited the migration of rat aortic smooth muscle cells induced by bFGF and serum. The migration assay was performed by using a Transwell plate assay as described in Section 4. SMCs in serum-free DMEM with 1% FBS were added to the upper compartment of a Transwell. The lower compartment contained 600 μl of DMEM with 1% FBS and PDGF (10 ng/ml). After 12 h, cells on the upper surface of the membrane were removed by gentle wiping. The inserts were incubated with hematoxylin for 5 min at room temperature. The membranes were then mounted on glass slide and the number of migrating cells was counted.

liferate.[20] The migration process involves degradation of the extracellular matrix, cell detachment, and new cell adhesions. In the previous study, we showed that BUD-0203 inhibited the proliferation of SMCs in the presence of PDGF and serum. In this study, we examined whether the compounds also inhibited the migration of SMCs induced by PDGF and serum because the migration is an important step of restenosis. Our finding that the compound significantly inhibited migration of SMCs reveals the possibility of BUD-0203 as lead compound for the development of more potent anti-restenosis drugs.

To investigate the antiproliferative mechanism of BUD-0203 in SMCs, we examined the effect of the compound on the MAPK pathway. We found that BUD-0203 activated the ERK, p38, and JNK/SAPK pathways in SMCs. In general, ERK, p38, and JNK/SAPK play a critical role in cell proliferation. However, in many cases, the activation of the pathways leads to cell death.[11,21] It has been reported that the duration of activation of ERK, p38, and JNK/SAPK determined the fate of cells.[11,12,22] Persistent activation of ERK, p38, and JNK/SAPK mediates growth arrest or apoptosis,[11,21,22] whereas transient activation leads to proliferation.[12] BUD-0203 induced prolonged activation of ERK, p38, and JNK/SAPK. On the basis of our results, we suggest that BUD-0203 could inhibit proliferation of SMCs through the persistent activation of ERK, p38, and JNK/SAPK.

The result that BUD-0203 activated caspase 3 showed that the compound induced apoptosis of SMCs. It has been reported that several compounds such as taxol, cantharidin, and cisplatin induced apoptosis of cultured animal cells, and the apoptosis is closely related with the activation of ERK, p38, and JNK/SAPK.[21,23,24] Our results also show that BUD-0203 induced apoptosis of SMCs through the activation of ERK, p38, and JNK/SAPK.

On the other hand, a lot of compounds that inhibit cell proliferation and induce apoptosis also arrest the cell cycle at G1 or G2 phase. However, BUD-0203 failed to induce cell cycle arrest at G1 or G2 phase. Interestingly, the percentage of cells in S phase was increased by 10% after the compound was added. This result indicates that the compound might affect DNA synthesis for cell proliferation.

In conclusion, our results showed that a synthetic benzimidazole-4,7-dione derivative, BUD-0203, inhibited the migration of SMCs and induced apoptosis of the cells. ERK, p38, and JNK/SAPK play an important role in mediating apoptotic signal transduction. The benzimidazole-4,7-dione derivatives, especially BUD-0203, might be good candidates for restenosis therapy because they strongly inhibit the proliferation and migration of SMCs.

## 4. Materials and methods

### 4.1. Materials

Antibodies to rat ERK, phospho-ERK, p38, and phospho-p38 were purchased from Biosource International Inc. (Camarillo, CA). The antibody to cleaved caspase 3 was obtained from Stressgen (Victoria, Canada). The anti-cyclin E antibody was purchased from Upstate (Lake Placid, NY).

### 4.2. Cell culture and proliferation assay

Rat aortic smooth muscle cells (RAoSMCs) were isolated by a modification of the method of Chamley et al.[25] The thoracic aortas from 6- to 8-week-old Sprague–Dawley rats were removed and transferred on ice in serum-free Dulbecco's modified Eagle's medium (DMEM; Invitrogen Co., Carlsbad, CA) containing 100 U/ml of penicillin and 100 μg/ml of streptomycin. The aorta was freed from

connective tissue, transferred into a Petri dish containing 5 ml of an enzyme dissociation mixture containing DMEM with 1 mg/ml of collagenase type I (Sigma, St. Louis, MO) and 0.5 μg/ml elastase (USB Bioscience, Cleveland, OH), and incubated for 30 min at 37 °C. The aorta was then transferred into DMEM, and the adventitia was stripped off with forceps under a binocular microscope. The aorta was transferred into a plastic tube containing 5 ml of the enzyme dissociation mixture and incubated for 2 h at 37 °C. The suspension was centrifuged (1500 rpm for 10 min), and the pellet was resuspended in DMEM containing 10% fetal bovine serum (FBS). Cells were cultured over several passages (up to 10). RAoSMCs were cultured in DMEM supplemented with 10% FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin in 75-$cm^2$ flasks at 37 °C in a humidified atmosphere of 95% air and 5% $CO_2$ (Forma Scientific, Inc., Marietta, OH).

HUVECs obtained from umbilical cord veins were cultured in Medium 199 (Gibco BRL, Grand Island, NY, USA) supplemented with 20% fetal bovine serum, 25 mM HEPES, 10 U/ml heparin, 100 U/ml penicillin, 100 μg/ml streptomycin, and 20 ng/ml bFGF. HUVECs were plated at a density of $5 \times 10^3$ cells/well in 100 μl M199 containing 20% (v/v) fetal bovine serum in gelatin-coated 96-well plates (Costar, Corning, NY, USA). After 24 h incubation, the cells were treated with test compounds in 100 μl M199 containing 5% FBS and 10 ng/ml bFGF for 48 h.

Cell proliferation was determined using a colorimetric assay kit based on the uptake of WST by viable cells (Premix WST-1 cell proliferation assay system, Takara Bio Inc., Otsu, Japan). The assay kit is dependent on the reduction of tetrazolium salt WST-1, which results in formation of a dark red formazan product, by various mitochondrial dehydrogenase of viable cells.

### 4.3. SMC migration assay

SMCs were dissociated by treatment with trypsin–EDTA, washed three times in PBS, and resuspended in serum-free DMEM with 1% FBS. SMCs ($5 \times 10^4$ per 100 μl) in serum-free DMEM with 1% FBS were added to the upper compartment of a Transwell plate with 8-μm polycarbonate membrane (Corning Costar, Cambridge, MA). The lower compartment contained 600 μl of DMEM with 1% FBS and PDGF (10 ng/ml). The plates were incubated at 37 °C, 5% $CO_2$ for 12 h. The upper compartment of the chamber insert was rinsed with PBS, and the cells were fixed in 4% paraformaldehyde at room temperature for 20 min. Cells on the upper surface of the membrane were removed by gentle wiping. The inserts were incubated with hematoxylin for 5 min at room temperature. The membranes were then mounted on glass slides, and the number of migrating cells was counted.

### 4.4. Western blotting

RAoSMCs were cultured for 72 h in DMEM containing 0.2% FBS and treated with the compound (1 μM). The cells were pooled and homogenized in RIPA buffer [150 mM NaCl, 50 mM Tris (pH 7.6), 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin] at 4 °C. After incubation for 30 min on ice, insoluble materials were removed by centrifugation at 14,000 rpm for 15 min, and the protein lysate concentrations were measured by Bradford assay. The same amounts and proportions of proteins from whole cell lysates or precipitated immune complexes were resolved on SDS–PAGE and blotted onto nitrocellulose membranes. The membrane was incubated with primary antibodies overnight at 4 °C, followed by incubation with HRP-conjugated secondary antibodies for 50 min at room temperature and detection with enhanced chemiluminescence (ECL) reagent.

### 4.5. Analysis of cell cycle by flow cytometry

RAoSMCs ($1 \times 10^6$ cells) were seeded in a 100-mm culture dish and incubated in DMEM with 10% FBS and PDGF (10 ng/ml) for 24 h. The test compound was then added to the cell. After 24 h of treatment, cells were collected and fixed with 70% ice-cold ethanol, followed by washing with PBS. Cells were stained with propidium iodide (PI) in the presence of RNase at 37 °C for 30 min. The stained cells were analyzed by flow cytometry (Becton–Dickinson, Franklin Lakes, NJ).

## Acknowledgments

We thank Dr. Tae-Hee Lee for comments on this manuscript. This study was supported by a grant of National Research Laboratory Project from the Ministry of Commerce, Industry, and Energy, Republic of Korea (No. 10020214).

## References and notes

1. Schwartz, R. S. In *Text Book of Interventional Cardiology*; Topol, E. J., Ed., 2nd ed.; W.B. Saunders: Philadelphia, PA, 1994; p 365.
2. Jackson, C. L.; Reidy, M. A. *Ann. N. Y. Acad. Sci.* **1992**, *667*, 141.
3. Epstein, S. E.; Siegall, C. B.; Biro, S.; Fu, Y. M.; FitzGerald, D.; Pastan, I. *Circulation* **1991**, *84*, 778.
4. Newby, A. C.; George, S. J. *Cardiovasc. Res.* **1993**, *27*, 1173.
5. Ferns, G. A.; Raines, E. W.; Sprugel, K. H.; Motani, A. S.; Reidy, M. A.; Ross, R. *Science* **1991**, *253*, 1129.
6. Robinson, J. R.; Cobb, M. H. *Curr. Opin. Cell. Biol.* **1997**, *9*, 180.
7. Cobb, M. H.; Goldsmith, E. J. *J. Biol. Chem.* **1995**, *270*, 14843.
8. Dent, P.; Yacoub, A.; Fisher, P. B.; Hagan, M. P.; Grant, S. *Oncogene* **2003**, *22*, 5885.
9. Bornfeldt, K. E.; Campbell, J. S.; Koyama, H.; Argast, G. M.; Leslie, C. C.; Raines, E. W.; Krebs, E. G.; Ross, R. J. *J. Clin. Invest.* **1997**, *100*, 875.
10. Mayr, M.; Li, C.; Zou, Y.; Huemer, U.; Hu, Y.; Xu, Q. *FASEB J.* **2000**, *14*, 261.
11. Guo, Y. L.; Baysal, K.; Kang, B.; Yang, L. J.; Williamson, J. R. *J. Biol. Chem* **1998**, *273*, 4027.
12. Roulston, A.; Reinhard, C.; Amir, P.; Williams, L. T. *J. Biol. Chem.* **1998**, *273*, 10232.

13. Middleton, R. W.; Parrick, J. In *The Chemistry of the Quinonoid Compounds*; Patai, T., Rappoport, Z., Eds.; John Wiley & Sons: London, 1988; p 1019.
14. Antonini, I.; Claudi, F.; Cristalli, G.; Franchetti, P.; Grifantini, M.; Martelli, S. *J. Med. Chem.* **1988**, *31*, 260.
15. Craigo, W. A.; LeSueur, B. W.; Skibo, E. B. *J. Med. Chem* **1999**, *42*, 3324.
16. Ryu, C. K.; Song, E. H.; Shim, J. Y.; You, H. J.; Choi, K. U.; Choi, I. H.; Lee, E. Y.; Chae, M. J. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 17.
17. Alvarez, F.; Gherardi, A.; Nebois, P.; Sarciron, M. E.; Petavy, A. F.; Walchshofer, N. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 977.
18. Hong, S. Y.; Chung, K. H.; You, H. J.; Choi, I. H.; Chae, M. J.; Han, J. Y.; Jung, O. J.; Kang, S. J.; Ryu, C. K. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 3563.
19. Chung, K. H.; Hong, S. Y.; You, H. J.; Park, R. E.; Ryu, C. K. *Bioorg. Med. Chem.* **2006**, *14*, 5795.
20. Bauters, C.; Isner, J. M. *Prog. Cardiovasc. Dis.* **1997**, *40*, 107.
21. Huh, J. E.; Kang, K. S.; Chae, C.; Kim, H. M.; Ahn, K. S.; Kim, S. H. *Biochem. Pharmacol.* **2004**, *67*, 1811.
22. Alblas, J.; Slager-Davidov, R.; Steenbergh, P. H.; Sussenbach, J. S.; van der Burg, B. *Oncogene* **1998**, *16*, 131.
23. Bacus, S. S.; Gudkov, A. V.; Lowe, M.; Lyass, L.; Yung, Y.; Komarov, A. P.; Keyomarsi, K.; Yarden, Y.; Seger, R. *Oncogene* **2001**, *20*, 147.
24. Arany, I.; Megyesi, J. K.; Kaneto, H.; Price, P. M.; Safirstein, R. L. *Am. J. Physiol. Renal. Physiol.* **2004**, *287*, F543.
25. Chamley, J. H.; Campbell, G. R.; McConnell, J. D.; Groschel-Stewart, U. *Cell. Tissue Res.* **1977**, *177*, 503.