The Journal of Applied Pharmacology, 11, 85-90(2003)

Effects of Tubulyzines, Novel Microtubule-Binding Triazine Molecules, on Endothelial Progenitor Cell Differentiation

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(Received Mar. 17, 2003; Accepted Mar. 31, 2003)

Abstract–Microtubule-binding molecules have been developed as anti-cancer agents to overcome the toxicities of current chemotherapeutics and also have potential for use as anti-angiogenic agents. In this work, we examined the effect of novel triazine compounds, Tubulyzines (microTUBUle LYsing triaZINE), derived from the orthogonal synthesis of a triazine library, on endothelial progenitor cell differentiation. When mononuclear cells isolated from human cord blood were cultured on fibronectin-coated plates for 7 days, all the Tubulyzine compounds A, B, and C (TA, TB, and TC) tested decreased the number of adherent cells in a dose-dependent manner in a concentration ranges of 2.5 to 80 μM. TA (IC_{50} = 20 μM) showed slightly more potent activity than TB and TC. Adherent cells treated with TA also exhibited a lower level of ability to ac-LDL uptake, with low ratios of positive cells out of total adherent cells, in a dose-dependent manner and weak expression of endothelial lineage markers, KDR, CD31, and vWF at 20 μM. Therefore, these results suggest that tubulyzine A (TA) can be effectively used for the inhibition of new vessel growth by inhibiting differentiation of endothelial progenitor cells.

Keywords[ ] endothelial progenitor cell, triazine, differentiation, microtubule-binding molecules, angiogenesis

Since several recent studies show that tumor growth and metastasis are dependent on new vessel growth, preventing excessive neovascularization in cancer has been a promising strategy (OReilly *et al.*, 1994; OReilly *et al.*, 1996; Folkman, 1995; Brooks *et al.*, 1994; Good *et al.*, 1990). Neovascularization involves the recruitment of endothelial cells to the tumor vascular bed. Two possible sources of endothelialization are (1) endothelial cell migration and sprouting from preexisting mature endothelial cells, a process called angiogenesis, and (2) recruitment and differentiation of endothelial progenitor cells from circulation. The first process has been well established (Bussolino *et al.*, 1997). However, the existence of endothelial progenitor cells (EPC) in adult humans has recently been suggested and is intensively being studied. Since the first report by Asahara *et al.* (1997), the ample evidence for the existence of EPC in adult blood has been reported over the past 5 years (Kalka *et al.*, 2000; Shintani *et al.*, 2001; Asahara *et al.*, 1999; Murohara *et al.*, 2000; Nieda *et al.*, 1997; Boyer *et al.*, 2000). In a previous report, we also confirmed the existence of EPC in human cord blood (Joe *et al.*, 2002).

Recently, a new microtubule binding molecule, myoseverin, was discovered by screening a library of 2,6,9-trisubstitued purines in a morphological myocyte differentiation screen (Rosania *et al.*, 2000). This purine based molecule acts reversibly without the cytotoxic effects of non-purine-based microtubule-disrupting molecules, like taxol, vinblastine, nocodazole, and the colchicines (Perez *et al.*, 2002). In an effort to develop of an optimized myoseverin-like anti-tubulin drug, a triazine library was screened, and three triazine compounds, tubulyzines A, B and C (TA, TB, and TC) were identified (Moon *et al.*, 2002)(Fig. 1).

Among angiogenesis inhibitors being tested in a clinical trial, combretastatin A-4 is a tubulin-binding agent that resembles colchicine in structure (Pettit *et al.*, 1989). It has been shown to act as effective antivascular agent, causing the rapid and selective shutdown of animal tumor blood vessels (Dark *et al.*, 1997; Tozer *et al.*, 1999; and Chaplin *et al.*, 1999). Therefore, we are interested in determining if the new triazine com-

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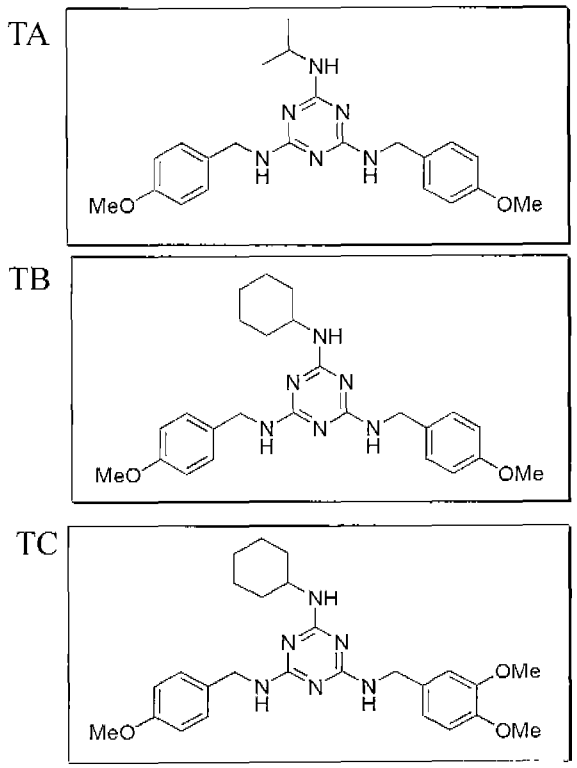

Fig. 1. Structure of triazine compounds, TA , TB, and TC.

pounds are effective in the inhibition of new vessel formation. In the paper, we addressed question of if these compounds can inhibit the differentiation of endothelial progenitor cells, which is required for neovascularization.

MATERIALS AND METHODS

Isolation of mononuclear cells from cord blood

Umbilical cord blood was placed into a disposable pyrogen-free blood bag (Green cross corp., Yongin, Korea) and was used within 1 h. Mononuclear cells were isolated from the blood using the Ficoll-Hypaque density centrifugation method. The blood was diluted with RPMI 1640 (Invitrogen, Grand Island, NY) and centrifuged at 3,000 rpm for 10 min. The cell layer was removed and diluted with phosphate-buffered saline with 20 mM EDTA. The sample was added to Ficoll-Hypaque (Amersham Pharmacia Biotech, Piscataway, NJ) and centrifuged at 400 × g for 35 min. The buffy layer was resuspended in PBS with 20 mM EDTA and centrifuged again 300 × g for 10 min. The cell pellet was washed with Medium 199 (M199, Invitrogen) and resuspended in the same medium. Clear cells were stained with trypan blue and subjected to cell counting using a hemocytometer.

Cell culture and treatment of triazine compounds

The medium used for the cell culture experiments was M199 supplemented with 10% FBS and heparin (90 μg/ml, Invitrogen). Mononuclear cells were plated on culture dishes coated with 0.1 μg/ml human fibronectin (Sigma, St. Louis, MO), at a density of 2,000 cell/mm^2, and a triazine compound was added to each well. Cells were cultured in a humidified 5% CO_2 incubator at 37°C After 3 days of culture, RBC and non-adherent cells were removed by washing with PBS, and new media and triazine compounds were added to each well. The culture was maintained through day 7 and examined for cell number and shape. The numbers of adherent cells were counted under phase-contrast microscopy at day 7. Five randomly selected microscopic fields per sample were evaluated and mean number of adherent cells per mm^2 was calculated.

Human umbilical endothelial cells (HUVECs) were isolated from fresh cords by an adaptation of the method described by Jaffe *et al.* (1984), and maintained in M199 supplemented with 20% FBS, 30 μg/ml ECGS (Endothelial cell growth supplement, Sigma) and 90 μg/ml heparin. Cells at passage between 3 and 6 were used.

Immunocytochemistry

Adherent cells cultured for 7 day were subjected to immunocytochemistry to analyze the expression of endothelial cell lineage marks von Willebrand factor (vWF, Dako, Glostrup, CA), CD31 (Dako), and KDR/ Flk-1 (Santa Cruz Biotech., Santa Cruz, CA). In this procedure, cells were grown on chamber slides and fixed with 4% paraformaldehyde. Endogenous peroxidase was inactivated with 0.6% hydrogen peroxide for 15 min in R.T. Non-specific mAb binding was blocked by incubation with 1.5% normal horse serum for 20 min. After washes, primary mAbs directed against vWF, KDR/Flk-1 or CD31 were then applied and incubated for 1 h (1:40 dilution). Negative control slides were incubated with 1.5% normal horse serum containing non-immune mouse IgG. After two washes with PBS, a biotinylated horse anti-mouse IgG Ab was applied, which was followed by the avidin-biotin immunoperoxidase treatment (Vector Laboratories, Burlingame, CA). In order to visualize the final immunoreaction products, Nova RED (Vector Laboratories) was used.

Cellular uptake of DiI-labeled acetylated-LDL

Incorporation of ac-LDL by adherent cells, one of the characteristic features of cells in endothelial lineage was examined as described previously (Murohara *et al.*, 2000). Cells cultured

on a fibronectin coated chamber slide for 7 days were incubated in medium containing 15 μg/ml DiI-labeled acetylated-Low Density lopoprotein (DiI-ac-LDL, Molecular Probes, Eugene, Oregon) for 1 h at 37°C After washes, samples were viewed with an inverted fluorescent microscope and a phase-contrast microscope, and three fields were randomly selected for photography.

Cell proliferation assay

Total MNC from cord blood were seeded on a 0.1 μg/ml fibronectin coated 96 well at a density of 2,000 cells/mm^2, and a triazine compound was added at a concentration range of 0~80 μM. After incubation for 3 days, cell viability was measured using a MTS [3-(4,5-dimethylthiazol-2-*yl*)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay kit (Promega, Madison, WI). MTS/PMS (phenazine methosulfate) solution was added to each well and incubated at 37°C in CO_2 incubator for 4 h, and light absorbance at 490 nm was detected by an ELISA reader. HUVEC was plated at a density of 1,500 cells/well, and the identical experiment was performed after attachment.

Statistical analysis

All values were presented as the means plus or minus SE. A t-test for paired samples were applied. Probabilities less than 0.01 were considered to be statistically significant.

RESULTS AND DISCUSSION

Triazine compounds, TA, TB and TC decrease adherent EPCs *in vitro*

In a previous report, we confirmed the existence of EPC in human cord blood (Joe *et al.*, 2002). In this study, we found the mononuclear cells isolated from human cord blood gave rise to differentiated cells of endothelial cell lineage after 7 days of incubation on a fibronectin matrix. Using this system, we examined if these new triazine compounds affect differentiation of endothelial progenitor cells, which exist in a fraction of mononuclear cells of human adult blood.

As shown in Fig. 2, treatment of isolated human mononuclear cells with the triazine compounds, TA, TB, and TC at 2.5~80 μM, dose dependently decreased the number of differentiated, adherent EPCs. The concentration of TA to allow 50% of total adherent cells, maximally grown under no-treatment, to grow (IC_{50}) was about 20 μM. TA was slightly more potent than TB and TC. There was no significant difference in the inhibition levels of three compounds.

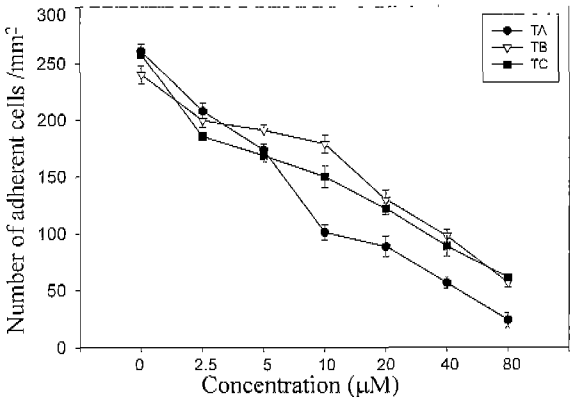

Fig. 2. Effects of Triazine compounds on EPC differentiation. Mononuclear cells were cultured on a fibronectin-coated plate under treatment with TA, TB, and TC at the indicated concentrations. After 7 days, adherent cells were examined under a phase-contrast microscopy. The number of adherent, differentiated EPCs was counted. The solvent DMSO had no effect on EPC differentiation. Data are mean ± SEM, n = 3.

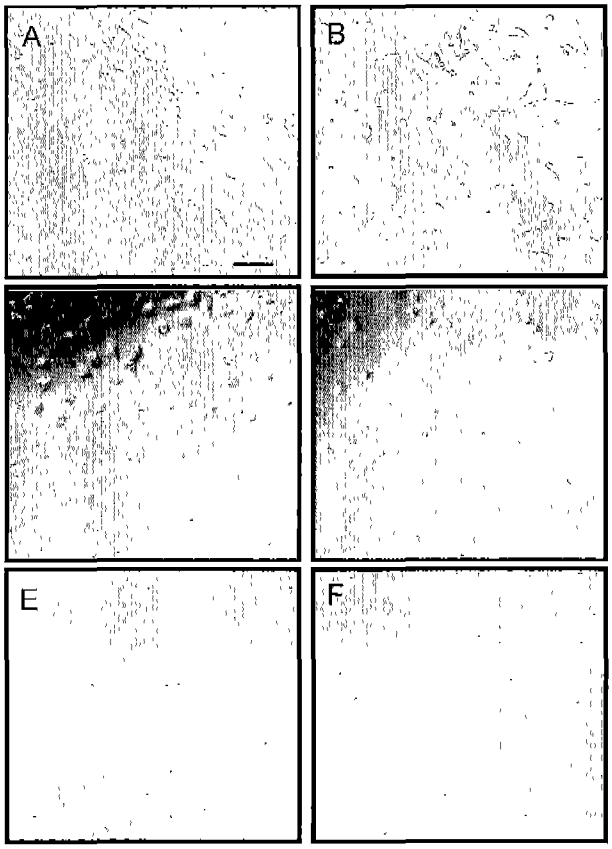

Fig. 3. The phase-contrast photomicrographs of adherent EPCs treated with TA at various concentrations for 7 days. A, 0 μM ; B, 2.5 μM ; C, 5 μM ; D, 10 μM ; E, 20 μM ; and F, 40 μM . Bar indicates 50 μm.

Most of the adherent cells of the untreated group are spindle-shaped, whereas the adherent cells treated with triazine compounds changed cell morphology from spindle shape to a spherical shape (Fig. 3). Due to the anti-tubulin effect, adherent cells may appear spherical in shape rather than spindle shaped, when treated with triazine compounds. This result is also supported by another groups report that the tumor vascular targeting agent combretastatin A-4 induces reorganization of the actin cytoskeleton (Kanthou and Tozer, 2002).

Analysis of expression of endothelial lineage markers

In order to examine whether adherent cells treated with triazine compounds carry endothelial lineage phenotypes, we analyzed the expression of endothelial lineage markers by immunocytochemistry. Expression of KDR (one of vascular endothelial growth factor (VEGF) receptors), CD31, and vWF were examined. Both KDR and CD31 exist in hematopoietic stem cells and angioblasts (non-differentiated EPCs), whereas vWF is expressed only in mature endothelial cells (Asahara *et al.*, 1997; Rafii, 2000).

As shown in Fig. 4, all three endothelial markers, KDR, CD31, and vWF were expressed in most of the adherent cells after incubation for 7 days in a control group, as reported previously (Joe *et al.*, 2002). When treated with the triazine compound TA at concentrations lower than 10 μM for 7 days, the adherent cells were stained with KDR, CD31, and vWF, without a significant difference of staining intensity. At 20 μM, weaker staining of these markers was observed. The numbers of cells stained with KDR, CD31 and vWF were decreased dose-dependently, in proportion to the number of total adherent cells. In the case of vWF, the number of stained cells reduced approximately from 60.75 ± 6.1% to about 25.7 ± 2.9% of the total adherent cell number upon treatment of TA at 20 μM. However, from the immunocytochemical analysis, inhibition of TA on EPC differentiation could not be determined quantitatively in a precise manner.

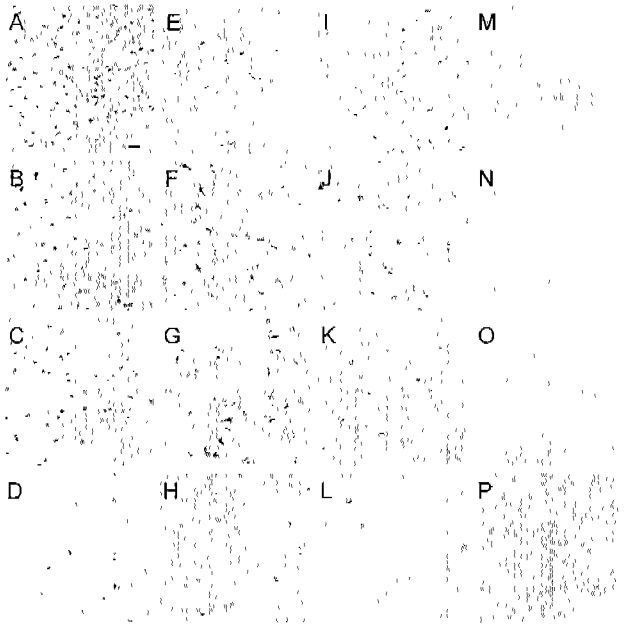

Fig. 4. Immunocytochemistry of triazine compound TA -treated EPCs Adherent cells grown for 7 days under treatment of TA at various concentrations were subjected to immunocytochemistry to analyze the expression of KDR (A, B, C and D), CD31(E, F, G and H) and vWF (I, J, K, and L). IgG was used as a negative control (M, N, O, and P). TA was treated at concentrations of 0 μM (A, E, I, and M), 5 μM (B, F, J, and N), 10 μM (C, G, K, and O) and 20 μM (D, H, L, and P). Magnification x 100. Bar indicates 50 μm.

Evaluation of the effect of TA on EPC differentiation by assessing ac-LDL uptake

Acetylated-low density lipoprotein (ac-LDL) is taken up by macrophages and endothelial cells via the "scavenger cell pathway" of low density lipoprotein (Voyta *et al.*, 1984). To evaluate whether TA inhibits EPC differentiation, we tested the ability of fluorescence-labeled ac-LDL for the cultured EPCs after treatment of TA for 7 days. When the cells were treated with triazine compound TA (5 μM, 10 μM, or 20 μM), the number of adherent cells that took up DiI-ac-LDL decreased in a dose-dependent manner (Fig. 5 A &B). At a concentration of 20 μM, the number of adherent cells that took up ac-LDL decreased to the level of 14.4 ± 2.4% of that of the cells without treatment. In addition, the intensity of fluorescence decreased also in proportion to the concentration of TA added. From these data, we could also evaluate the percentage of differentiated adherent cells with ac-LDL uptake phenotype out of the total adherent cells. As shown Fig. 5C, differentiation level of EPC was decreased in a dose-dependent manner. Without treatment, more than 88.7 ± 0.9% cells of the adherent cells took up DiI-ac-LDL at day 7 (Joe *et al.*, 2002). At a concentration of 20 μM, the proportion of the adherent cells that take up ac-LDL was drastically decreased (24.7 ± 1.1%). Therefore, we could conclude that triazine compounds also can inhibit the differentiation of EPCs to endothelial-like cells.

Effect of TA on cell proliferation

To evaluate the effect of TA on cell proliferation, a MTS assay was carried out for total mononuclear cells cultured for first 3 days. Each well contains adherent and non-adherent cells. In this assay, TA inhibited cell proliferation in a dose-

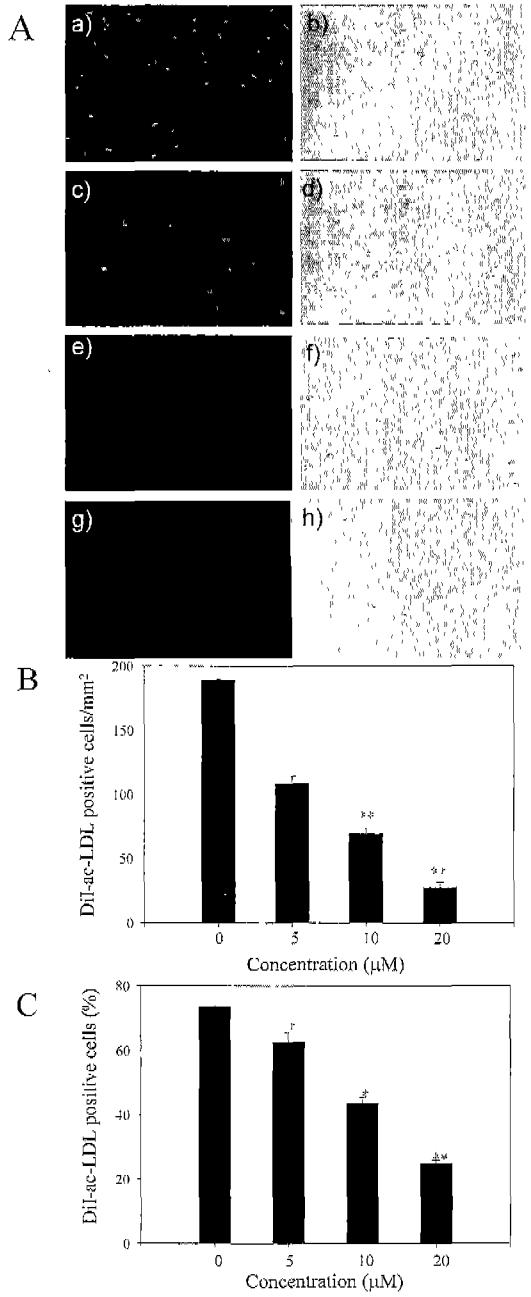

Fig. 5. Analysis of ac-LDL uptake on adherent EPCs teated with TA A. The ability to take up ac-LDL was examined for the adherent cells treated with TA at concentrations of 0 μM (a, b), 5 μM (c, d), 10 μM (e, f), and 20 μM (g, h). Fluorescence photomicrograph (a, c, e, g) and Phase contrast photomicrograph (b, d, f, h) were captured at the same time, Magnification × 100. B. The number of adherent cells that took up ac-LDL was counted per mm^2 for each treatment. C. The percentage of the number of adherent cells that took up ac-LDL to the total number of adherent cells was represented. $^{*}p < 0.01$, $^{**}p < 0.001$.

dependent manner with IC_{50} of 10-20 μM (Fig. 6A). These anti-proliferative effects of TA correspond well with the inhibi-

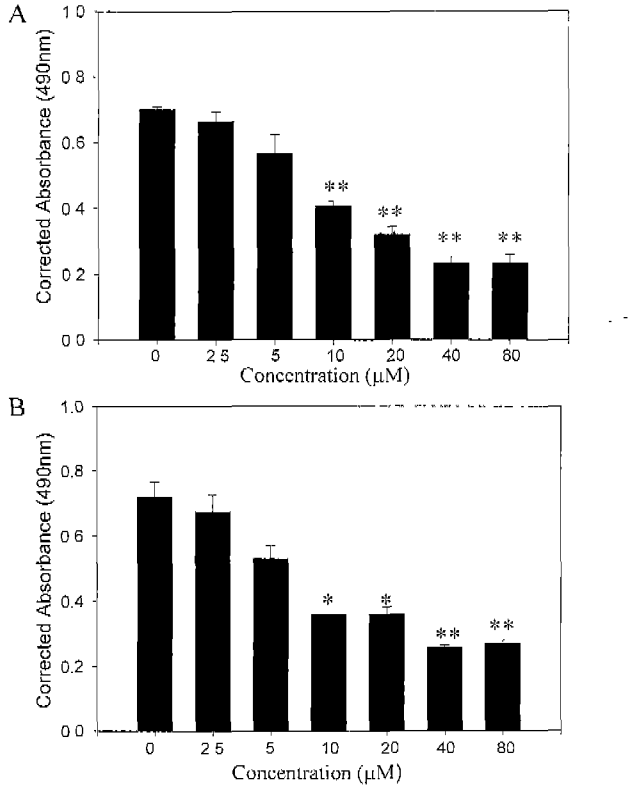

Fig. 6. Cell proliferation assay. Mononuclear cells (A) and HUVEC (B) were treated with TA at the indicated concentrations. After 72 h, both adherent and non-adherent cells were subjected to MTS assay. $^{*}p < 0.001$, $^{**}p < 0.005$.

tion of mature endothelial cell, HUVEC proliferation (Fig 6B). These results suggest that the inhibition of cell proliferation by microtubule-binding triazine compound might contribute in part in inhibition of EPC differentiation, since stem cell differentiation is generally accompanied by cell proliferation.

From these results, we can conclude that microtubule-binding triazine compounds can inhibit not only cell proliferation but also EPC differentiation. A purine-based anti-tubulin molecule, myoseverin, was also found to elicit similar effects on EPC differentiation (unpublished results). Therefore, it will be interesting to address whether other anti-tubulin drugs, being used clinically, has identical effects on EPC differentiation, although they are non-purine-based, non-triazine microtubule-disrupting molecules. Finally, it may be suggested that the triazine compound TA can be developed for application in the inhibition of neovascularization and in the treatment of cancer, in part, by targeting EPC differentiation.

ACKNOWLEGEMENT

This work was supported by the Korea science and Engi-

neering Foundation through the Vascular System Research Center at Kangwon National University. H.E Park is a recipient of fellowship of the joint program in Medical and Natural Sciences of the Catholic University of Korea.

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