Growth Inhibition and G₂/M Phase Cell Cycle Arrest by 3,4,5-Trimethoxy-4'-bromo-*cis*-stilbene in Human Colon Cancer Cells

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Abstract – Resveratrol (3,5,4'-trihydroxy-*trans*-stilbene), a naturally occurring phytoalexin abundant in grapes and several plants, has been shown to be active in inhibiting proliferation and inducing apoptosis in several human cancer cell lines. On the line of the biological activity of resveratrol, a variety of resveratrol analogs were synthesized and evaluated for their growth inhibitory effects against several human cancer cell lines. In the present study, we found that one of the resveratrol analogs, 3,4,5-trimethoxy-4'-bromo-*cis*-stilbene, markedly suppressed human colon cancer cell proliferation (EC₅₀ = 0.01 µg/ml), and the inhibitory activity was superior to its corresponding *trans*-isomer (EC₅₀ = 1.6 µg/ml) and resveratrol (EC₅₀ = 18.7 µg/ml). Prompted by the strong growth inhibitory activity in cultured human colon cancer cells (Col2), we investigated its mechanism of action. 3,4,5-Trimethoxy-4'-bromo-*cis*-stilbene induced arrest of cell cycle progression at G₂/M phase and increased at sub-G₁ phase DNA contents of the cell cycle in a time- and dose-dependent manner. Colony formation was also inhibited in a dose-dependent manner, indicating the inhibitory activity of the compound on cell proliferation. Moreover, the morphological changes and condensation of the cellular DNA by the treatment of the compound were well correlated with the induction of apoptosis. These data suggest the potential of 3,4,5-trimethoxy-4'-bromo-*cis*-stilbene might serve as a cancer chemotherapeutic or chemopreventive agent by virtue of arresting the cell cycle and inducing apoptosis for the human colon cancer cells.

Key words □ 3,4,5-Trimethoxy-4'-bromo-*cis*-stilbene, Growth inhibition of cancer cells, G₂/M cell cycle arrest, Col2 human colon cells

INTRODUCTION

Resveratrol (3,5,4'-trihydroxy-*trans*-stilbene), a naturally occurring phytoalexin that is present in grapes, peanuts, pines and other Leguminosae family plants, has been reported to exhibit a variety of important biological effects including alleviation of atherosclerosis and coronary heart diseases (Frankel *et al.*, 1993; Pace-Asciak *et al.*, 1995). Additional biological activities of resveratrol include the antioxidant, anti-mutagenic, anti-inflammatory, or potent cancer chemopreventive effects in carcinogenesis (Jang *et al.*, 1997; Uenobe *et al.*, 1997). Moreover, resveratrol suppressed the growth of several human can-

cer cell lines, including human breast, prostate, oral squamous carcinoma, promyelocytic leukemia, and colon cancer cells (Elattar and Virji, 1999; Hsieh *et al.*, 1999; Surh *et al.*, 1999; Schneider *et al.*, 2000; Lu *et al.*, 2001). On the line of the growth inhibitory potential of resveratrol against various cancer cells, we have synthesized various resveratrol analogs to develop more potent novel chemical entities with the stilbene moiety. Here, we report that one of these resveratrol analogs, 3,4,5-trimethoxy-4'-bromo-*cis*-stilbene (BRC), strongly inhibited the growth of human cancer cells and remarkably induced arrest at G₂/M phase of the cell cycle in human colon cancer cells.

MATERIALS AND METHODS

Chemicals

Trichloroacetic acid (TCA), sulforhodamine B, propidium

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iodide, bisbenzimidazole (Hoechst 33258), RNase A, and mouse anti- β -actin monoclonal antibody were purchased from Sigma (St. Louis, MO, USA). Minimal essential medium with Earle's salt (MEME), fetal bovine serum (FBS), non-essential amino acid solution (10 mM), trypsin-EDTA solution, and antibiotic-antimycotic solution (PSF) were from Invitrogen (Grand Island, NY, USA). Mouse anti-p53 monoclonal antibody and horseradish peroxidase (HRP)-conjugated anti-mouse IgG were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Resveratrol (RES) was synthesized as described by Ali *et al.* (Ali *et al.*, 1992). Wittig reaction of triphenyl(3,4,5-trimethoxybenzyl)phosphonium bromide with 4-bromobenzaldehyde in THF in the presence of *n*-BuLi followed by silica gel column chromatographic separation afforded the known 3,4,5-trimethoxy-4'-bromo-*cis*-stilbene (BRC) and 3,4,5-trimethoxy-4'-bromo-*trans*-stilbene (BRT) (Figure 1) (Cushman *et al.*, 1991).

Evaluation of growth inhibitory potential

Human colon carcinoma (Col2, established at Department of Surgical Oncology, University of Illinois at Chicago) cells (5×10^4 cells/ml) were treated with various concentrations of test compounds for 3 days. Cells were fixed with 10% TCA solution, and cell viability was determined with sulforhodamine B (SRB) protein staining method (Lee *et al.*, 1998). The result was expressed as a percentage, relative to solvent-treated control incubations, and the IC_{50} values were calculated using non-linear regression analysis (percent survival versus concentration).

Colony-forming assay

Col2 cells (500 cells/dish in a 100 mm culture dishes) were

treated with various concentrations of test agents for 7, 24, or 48 h. The cells were washed with PBS and cultured in a drug-free medium for 7 days. Colonies were then fixed with methanol, stained with Giemsa, enumerated, and expressed as a percentage, relative to solvent-treated controls (Lee *et al.*, 1998).

Nuclear staining

Col2 cells (2×10^6 cells/dish in 100 mm culture dishes) were treated with test agents for up to 48 h. Floating cells were collected by centrifugation at $2000 \times g$ for 5 min and attached cells were trypsinized and then harvested by centrifugation. The cells combined two parts were washed with cold PBS and fixed with 90% ethanol. The fixed cells were stained with Hoechst 33258 for 30 min. The cells were then washed twice with PBS and placed on slides. Morphological change was observed under confocal microscope (Zeiss, LSM 510) (Nam *et al.*, 2001).

Analysis of cell cycle dynamics by flow cytometry

Cell cycle analysis by flow cytometry was performed as described previously (Lee *et al.*, 2002). Briefly, Col2 cells were plated at a density of 2×10^6 cells per 100 mm culture dish and incubated for 24 h. Fresh media containing various concentrations of test samples were added to culture dishes. At various time intervals (10, 24, and 30 h), the cells were harvested (trypsinization and centrifugation), fixed with 90% ethanol, and incubated with a staining solution containing 0.2% NP-40, RNase A (30 μ g/ml), and propidium iodide (50 μ g/ml) in phosphate-citrate buffer (pH 7.2). Cellular DNA content was analyzed by flow cytometry using a Becton Dickinson laser-based flow cytometer. At least 20,000 cells were used for each analysis, and results were displayed as histograms.

Evaluation of test agents on the p53 expression

Cells were exposed with various concentrations of test agents for up to 48 h. After incubation, cells were lysed and protein concentrations were determined by BCA method. Each protein (30 μ g) was subjected to 10% SDS-PAGE. Proteins were transferred onto PVDF membranes by electroblotting, and membranes were treated for 1 h with blocking buffer [5% non-fat dry milk in phosphate-buffered saline-0.1% Tween 20 (PBST)]. Membranes were then incubated with mouse anti-p53 primary antibody diluted 1: 1,000 in PBS overnight at 4°C, washed for 1 h with PBST. After washing, membranes were incubated with HRP-conjugated anti-mouse IgG diluted 1: 1,000 in PBS for 1 h at room temperature, washed for 1 h with

Fig. 1. Chemical structures of 3,4,5-trimethoxy-4-bromo-*cis*-stilbene (BRC), 3,4,5-trimethoxy-4-bromo-*trans*-stilbene (BRT) and resveratrol.

PBST, and detected using ECL reagent (Amersham Corp., Arlington Heights, IL, USA) (Lee *et al.*, 2002).

RESULTS

Effects of test compounds on Col2 cell growth and colony formation

The growth inhibitory potential of resveratrol analogs was determined in cultured Col2 cells by a colorimetric SRB protein dye staining method. The results were expressed as a percentage, relative to control cell number, indicating the extent of growth inhibitory effect of the test compound. One of these resveratrol analogs, 3,4,5-trimethoxy-4'-bromo-*cis*-stilbene (BRC) exhibited a remarkable growth inhibitory effect against Col2 colon cancer cells (Figure 2 (A)). The IC_{50} value of BRC was 0.01 μ g/ml (0.03 μ M), which was approximately over hundred to thousand times potent than 3,4,5-trimethoxy-4'-bromo-

trans-stilbene (BRT; IC_{50} = 1.6 μ g/ml (4.5 μ M)) and resveratrol (IC_{50} = 18.7 μ g/ml (81.9 μ M)), respectively. To further characterize the growth inhibitory action of BRC, Col2 cells were treated for various periods of time and dose, and then analyzed the potential to generate colonies (recover) in a drug-free medium. As shown in Figure 2 (B), the formation of colonies was significantly reduced by the treatment with BRC in a dose-dependent manner. These data suggest that BRC retards the growth of colon cancer cells.

Morphological changes by treatment with BRC

Following the potential growth inhibition of BRC against Col2 cells, we evaluated whether the growth inhibition by BRC was related to apoptosis. Primarily, the morphological changes were examined by treatment with BRC (0.1 or 0.4 μ M) for 24 h. As illustrated in Figure 3, BRC-treated cells showed the distinct round-up compared to polygonal shapes of vehicle-treated

Fig. 2. The growth inhibitory effect on human colon (Col2) cells. (A) Human colon (Col2) cells were plated at 10,000 cells in 96-well plates in MEME supplemented with 10% FBS, and incubated with the test compounds as the indicated concentrations for 3 days as described in the Materials and Methods. The data are represented as the means $\pm$ S.E. (n=3). (B) Alternatively, the cells were treated with indicated concentration of BRC for 2 days, washed, and incubated for an additional 7 days with a drug-free medium. Colony formation was enumerated and compared with solvent-treated controls. Values indicate the mean $\pm$ S.E. in duplicate tests.

Fig. 3. Morphological change of Col2 cells treated with BRC. Morphological changes of Col2 cells treated with DMSO alone or with 0.1 μ M and 0.4 μ M BRC for 24 h were observed under the phase-contrast microscope and photographed.

Fig. 4. Nuclear staining examination of Col2 cells treated with BRC. Col2 cells were treated with DMSO, or BRC (0.1 μ M) for 24 h, then stained with the Hoechst 33258 fluorescence dye, and finally observed under confocal microscope.

control cells. In addition, nuclear staining with the Hoechst 33258 dye also exhibited the nuclear condensation in the BRC-treated cells, indicative of one possible apoptotic phenomenon (Figure 4).

Effect of BRC on the regulation of cell cycle progression

To further clarify the cell cycle progression the distribution of DNA contents in cells was analyzed by flow cytometry with

the treatment of BRC for various time periods (10, 24 and 30 h). As a result, BRC (0.4 μ M) significantly accumulated in the G2/M phase of cell cycle in a time-dependent manner (41.2% at 10 h; 88.2% at 24 and 30 h), and also slightly increased in the sub-G1 phase (6.3% at 30 h), indicative of inducing apoptotic cell death (Figure 5).

Effect of BRC on p53 expression

In order to investigate whether the induction of apoptosis mediated by BRC was related to p53 expression, western blot analysis was performed. As indicated in Figure 6, there was no significant effect on p53 protein expression levels. These data suggest that apoptosis mediated by BRC is not directly related to the p53 expression.

DISCUSSION

Cancer chemoprevention is considered one promising strategy in controlling cancer progression through inhibiting, delaying, or reversing the carcinogenic process using dietary or synthetic non-toxic chemicals (Sporn and Newton, 1979; Hong and Sporn, 1997). A diverse class of compounds has been

Fig. 5. Effect of BRC on cell cycle distribution in cultured Col2 cells. Flow cytometric analysis of the DNA content treated with vehicle-control, or BRC (0.4 μ M) for 10, 24, or 30 h. The percentage of distribution in each phase of cell cycle was determined using Modfit LT V 2.0 software.

Fig. 6. Effect of BRC on the expression of p53 in cultured Col2 cells. Cells were treated with BRC (0.1, 0.2, or 0.4 μ M) and subjected to SDS-PAGE for detection of p53 expression as described in the Materials and Methods.

reported to protect against chemical carcinogenesis and thus are considered to be cancer chemopreventive agents (Kelloff *et al.*, 1992; Wattenberg, 1997). On this line, one promising phytochemical compound resveratrol has recently been attracted with its cancer chemopreventive activity in multistage carcinogenesis. In addition, resveratrol has been demonstrated to have a variety of biological activities including antioxidant, anti-proliferative and cancer chemopreventive activity in *in vivo* animal experiments (Jang *et al.*, 1997; Carbo *et al.*, 1999).

Using the prototype of resveratrol with the potential biological activity, we have synthesized and evaluated the effects of resveratrol analogs on the growth of human cancer cells for developing more potent chemical entities than resveratrol. As a result, one of these analogs, 3,4,5-trimethoxy-4-bromo-*cis*-stilbene (BRC) revealed a remarkable growth inhibitory activity against human colon cancer cells (Col2) (Figure 2). In comparison with resveratrol (3,5,4-trihydroxy-*trans*-stilbene), BRC was approximately 2,000 times more potent than resveratrol in the growth inhibition of colon cancer cells (IC_{50} : 0.01 μ g/ml (0.03 μ M) for BRC; 18.7 μ g/ml (81.9 μ M) for resveratrol). These results were well correlated with the previous our results in human lung cancer cells (Lee *et al.*, 2004) and other reported data by Lu *et al.* (Lu and Serrero, 1999), showing that polymethoxy-stilbenes increased the growth inhibition of cancer cells. In addition, in terms of geometrical structures, *cis*-configuration of BRC exhibited approximately 150 times more potent compared to the corresponding its *trans*-isomer, 3,4,5-trimethoxy-4-bromo-*trans*-stilbene (BRT; IC_{50} : 1.6 μ g/ml (4.5 μ M)). These spatial relationships for the growth inhibitory activity against human cancer cells were well exemplified by the combretastatin A-4 compound, a natural stilbene, which showed the *cis*-configuration of the compound was approximately 60 times more potent than the *trans*-isomer of the compound against human P388 murine leukemia cell (Lawrence *et al.*, 2001). Consistent with the results, the earlier study done by Cushman *et al.* (Cushman *et al.*, 1991) showed that BRC exhibited more potent cytotoxic activities against five human cancer cells A549, MCF-7, HT-29, SKMEL-5 and MLM than

BRT. However, the mechanism of action of BRC has not been fully investigated yet. In the present study, therefore, the strong growth inhibitory activity of BRC compared to BRT and resveratrol in cultured human colon cancer cells (Col2) prompted us to study its mechanism of action. As demonstrated with a colony-forming assay performed by treating Col2 cells with BRC, colony formation was also inhibited in a time- and dose-dependent manner, indicating an inhibitory activity of BRC on cancer cell proliferation. In addition, when observing morphological changes, BRC-treated cells were well discerned with round-up shapes as compared to polygonal shapes of untreated control cells under light microscope (Figure 3), and also nuclear condensation was appeared in the BRC-treated cells under confocal microscope (Figure 4). These data assumed the possible growth-arresting and apoptosis-inducing effects of BRC. Further characterization of growth inhibition by BRC was determined to evaluate whether BRC induces the growth arrest or apoptosis in greater details using flow cytometric analysis. Col2 cells were treated with 0.4 μ M BRC for up to 30 h and then subjected to an analysis of cell cycle distribution. As shown in Figure 5, cells treated with BRC remarkably accumulated in G2/M phase of the cell cycle in a time-dependent manner up to 90% by 24 h, indicated a possible growth arrest at the M/G1 phase transition period, and also the accumulation of the cells in sub-G1 phase of cell cycle was slightly increased in a time-dependent manner up to 30 h, assuming following apoptotic phenomena. Therefore, the accumulation of G2/M phase of the cell cycle and thus the arresting of the cell growth at the M/G1 phase transition appear to be probably the major mechanism of action of BRC in growth inhibition of Col2 cells. Following study to investigate whether BRC modulates p53 expression related to apoptosis was performed. As a result, western blot analysis exhibited that a slight increase of apoptotic peaks in sub-G1 cell cycle by BRC was not dependent on the expression of p53 level (Figure 6). However, employing human lung cancer cells A549 in our previous report, BRC increased p53 protein expression in a dose-dependent manner, indicating apoptosis mechanism evoked by BRC might be dif-

ferential in types of cells (Lee *et al.*, 2004). Additional studies for evaluation of the mechanism by which BRC induces apoptosis should be required in Col2 cells.

Our main findings in this study will be an additional mechanism of action in retardation of colon cancer cell growth by BRC via G2/M phase cell cycle arrest. Since the anticancer activity of many clinically relevant anticancer agents are highly related with the modulation of cell cycle regulation, the G2/M phase arrest by BRC might be valuable to control the growth of colon cancer cells.

In summary, this study suggests that BRC is a potent inhibitor of the growth of colon cancer cells. The growth inhibition of the compound is highly related to cell cycle arrest in G2/M phase and induction of apoptosis. Further studies are needed to evaluate the effects of BRC on the biomarkers or signal transduction pathways related to cell cycle regulation or apoptosis in diverse cancer cell lines. As currently reported with human colon cells, the potential growth inhibitory activity of the synthetic BRC should be taken into account when considering the further development and prioritization as a cancer chemotherapeutic agent.

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REFERENCES

- Ali, M. A., Kondo, K. and Tsuda, Y. (1992). Studies on crude drugs effective on visceral larva migrans. XV. Synthesis and nematocidal activity of hydroxystilbenes. *Chem. Pharm. Bull.* **40**, 1130-1136.
- Carbo, N., Costelli, P., Baccino, F. M., Lopez-Soriano, F. J. and Argiles, J. M. (1999). Resveratrol, a natural product present in wine, decreases tumor growth in a rat tumour model. *Biochem. Biophys. Res. Commun.* **254**, 739-743.
- Cushman, M., Nagarathnam, D., Gopal, D., Chakraborti, A. K., Lin, C. M. and Hamel, E. (1991). Synthesis and evaluation of stilbene and dihydrostilbene derivatives as potential anticancer agents that inhibit tubulin polymerization. *J. Med. Chem.* **34**, 2579-2588.
- Elattar, T. M. and Virji, A. S. (1999). The effect of red wine and its components on growth and proliferation of human oral squamous carcinoma cells. *Anticancer Res.* **19**, 5407-5414.
- Frankel, E. N., Waterhouse, A. C. and Kinsella, J. E. (1993). Inhibition of human LDL oxidation by resveratrol. *Lancet* **341**, 1103-1104.
- Himes, R. H., Kersey, R. H., Heller-Bettinger, I. and Samson, F. E. (1976). Action of the *Vinca* alkaloids vincristine, vinblastine, and desacetyl vinblastine amide on microtubules *in vitro*. *Cancer Res.* **36**, 3798-3802.
- Hong, W. K. and Sporn, M. B. (1997). Recent advances in chemoprevention of cancer. *Science* **278**, 1073-1077.
- Hsieh, T. C. and Wu, J. M. (1999). Differential effects on growth, cell cycle arrest and induction of apoptosis by resveratrol in human prostate cancer cell lines. *Exp. Cell. Res.* **249**, 109-115.
- Jang, M., Cai, L., Udeani, G. O., Slowing, K. V., Thomas, C. F., Beecher, C. W. W., Fong, H. S. S., Farnsworth, N. R., Kinghorn, A. D., Mehta, R. G., Moon, R. C. and Pezzuto, J. M. (1997). Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science* **275**, 218-220.
- Kelloff, G. J., Boone, C. W., Malone, W. F. and Steele V. E. (1992). Recent results in preclinical and clinical drug development of chemopreventive agents at the National Cancer Institute. In *Cancer chemoprevention* (L. Wattenberg, M. Lipkin, C. W. Boone, and G. J. Kelloff, Eds.), pp. 41-56. CRC Press, Boca Raton.
- Lawrence, N. J., Rennison, D., Woo, M., McGown, A. T. and Hadfield, J. A. (2001). Antimitotic and cell growth inhibitory properties of combretastatin A-4 like ethers. *Bioorg. Med. Chem. Lett.* **11**, 51-54.
- Lee, E. J., Min, H. Y., Park, H. J., Chung, H. J., Kim, S., Han, Y. N. and Lee, S. K. (2004). G2/M cell cycle arrest and induction of apoptosis by a stilbenoid, 3,4,5-trimethoxy-4-bromo-*cis*-stilbene, in human lung cancer cells. *Life Sci.* **75**, 2829-2839.
- Lee, S. K., Cui, B., Mehta, R. R., Kinghorn, A. D. and Pezzuto, J. M. (1998). Cytostatic mechanism and antitumor potential of novel 1H-cyclopenta[b]benzofuran lignans isolated from *Aglaia elliptica*. *Chemico-Biol. Interact.* **115**, 215-228.
- Lee, S. K., Heo, Y. H., Steele, V. E. and Pezzuto, J. M. (2002). Induction of apoptosis by 1,4-phenylenebis(methylene)selenocyanate in cultured human colon cancer cells. *Anticancer Res.* **22**, 97-102.
- Lu, J., Ho, C. T., Ghai, G. and Chen, K. Y. (2001). Resveratrol analog, 3,4,5,4'-tetrahydroxystilbene, differentially induces pro-apoptotic p53/Bax gene expression and inhibits the growth of transformed cells but not their normal counterparts. *Carcinogenesis* **22**, 321-328.
- Lu, R. and Serrero, G. J. (1999). Resveratrol, a natural product derived from grape, exhibits antiestrogenic activity and inhibits the growth of human breast cancer cells. *J. Cell Physiol.* **179**, 297-304.
- Nam, K. A., Kim, S., Heo, Y. H. and Lee, S. K. (2001). Resveratrol analog, 3,5,2',4'-tetramethoxy-trans-stilbene, potentiates the inhibition of cell growth and induces apoptosis in human cancer cells. *Arch. Pharm. Res.* **24**, 441-445.
- Pace-Asciak, C. R., Hahn, S. E., Diamandis, E. P., Soleas, G. and Goldberg, D. M. (1995). The red wine phenolics trans-resveratrol and quercetin block human platelet aggregation and eicosanoid synthesis: implications for protection against coronary heart disease. *Clin. Chim. Acta* **235**, 207-219.
- Schiff, P. B., Fant, J. and Horwitz, S. B. (1979). Promotion of microtubule assembly *in vitro* by taxol. *Nature* **277**, 665-667.
- Schneider, Y., Vincent, F., Duranton, B., Badolo, L., Gossé, F., Bergmann, C., Seiler, N. and Faul, F. (2000). Anti-proliferative effect of resveratrol, a natural component of grapes and wine, on human colonic cancer cells. *Cancer Lett.* **158**, 85-91.
- Sporn, M. B. and Newton, D. L. (1979). Chemoprevention of

- cancer with retinoids. *Fed. Proc.* **38**, 2528-2534.
- Surh, Y. J., Hurh, Y., Kang, J., Lee, E., Kong, G. and Lee, S. J. (1999). Resveratrol, an antioxidant present in red wine, induces apoptosis in human promyelocytic leukemia (HL-60) cells. *Cancer Lett.* **140**, 1-10.
- Uenobe, F., Nakamura, S. and Miyazawa, M. (1997). Antimutagenic effect of resveratrol against Trp-P-1. *Mutat. Res.* **373**, 197-200.
- Wattenberg, L. W. (1997). An overview of chemoprevention: current status and future prospects. *Proc. Soc. Exp. Biol. Med.* **216**, 133-141.
- Zhang, X. W., Jiang, J. F. and Xu, B. (2000). Differentiation-inducing action of 10-hydroxycamptothecin on human hepatoma Hep G2 cells. *Acta Pharmacol. Sin.* **21**, 364-368.