Cell Cycle Inhibitory Activity of 4-Hydroxy-3-(3′-methyl-2′-butenyl)-benzoic Acid from *Curvularia* sp. KF119

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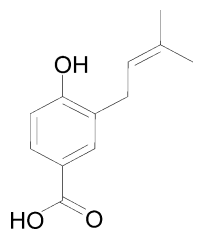
The cell cycle of eukaryotes is orchestrated partly by the products of oncogenes as well as tumor suppressor genes[1,2]. Accumulated evidences have shown that disorder of the cell cycle regulation induces or enhances tumorigenesis[3~7]. Therefore, low-molecular weight compounds or genes which can correct the aberrant progress of cell cycle have been attracted as good candidates for antitumor agents[8~10]. Based on this idea, we have screened for new cell cycle inhibitors from microbial metabolites since they have been proven as good sources of bioactive chemicals[11]. As a result, we found the known compound, 4-hydroxy-3-(3′-methyl-2′-butenyl)-benzoic acid (HMBA, Fig. 1), in a culture extract of *Curvularia* sp. KF119, which arrested the cell cycle of HeLa cell at G_1 phase.

Fig. 1. Chemical structure of 4-hydroxy-3-(3′-methyl-2′-butenyl)-benzoic acid ($C_{12}H_{14}O_3$).



HMBA (Fig. 1) was originally isolated from *Curvularia fallax* and *Discular* sp. as a phytotoxin[12,13]. In addition, the compound was reported as one of the anti-fungal and anti-bacterial agents but no study has been made on its cell cycle inhibitory activity of cultured cells. Herein, we report the isolation of HMBA from *Curvularia* sp. KF119 and its new biological activity of cell cycle inhibition.

HMBA was obtained from the culture extract of *Curvularia* sp. KF119 as described by Abraham and Arfmann[12]. Briefly, for seed cultivation, an agar piece of the stock plate was cut under sterile conditions and inoculated into a 100-ml baffle flask containing 20 ml of culture medium: 1% glucose, 1% universal peptone (Merck, Whitehouse, NJ), 2% Malt extract and 0.3% yeast extract. The flask was cultivated for 48 hours at 27°C with shaking at 140 rpm and then transferred to 1 liter baffle flasks containing the same medium (100 ml/flask) for large-scale cultivation. All flasks were cultured for 7 days at 27°C. The mycelium was separated from the medium by filtration and extracted three times with ethylacetate (EtOAc). The solvent was evaporated and the crude extract was separated on Si-60 columns with a *n*-hexane - EtOAc gradient (from 9 : 1 to 1 : 1). The collected fractions were purified further by preparative thin layer chromatography (TLC). The purified compound displayed a molecular ion of $C_{12}H_{14}O_3$ in the mass spectra analysis. ^{1}H-NMR revealed that the compound contains a 1,3,4-trisubstituted benzene with an isopentenyl side chain, and ^{13}C-NMR data were in good agreement with the known 4-hydroxy-3-(3′-methyl-2′-butenyl)-benzoic acid[12,13]

Using the purified HMBA, we first investigated the effect of compound on the growth of various cultured cells including HeLa (human cervical carcinoma), HT1080 (human fibrosarcoma), B16/BL6 (murine melanoma) and BAECs (bovine aortic endothelial cells). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay monitoring cell viability via mitochondrial activity of the cells was used. As shown in Fig. 2A, HMBA inhibited the proliferation of each cell line at the almost same dose that gave an IC_{50} of 150 μg/ml. These data demonstrated that HMBA may exhibit the growth inhibitory activity by acting on common cellular machinery of both normal and cancer cell types. Next, the effect of HMBA on the viability of HeLa cells was investigated using trypan blue staining assay up to 72 hours. Interestingly, the viability of HeLa cells was not affected up to 300 μg/ml of HBMA treatment (Fig. 2B), implying that the

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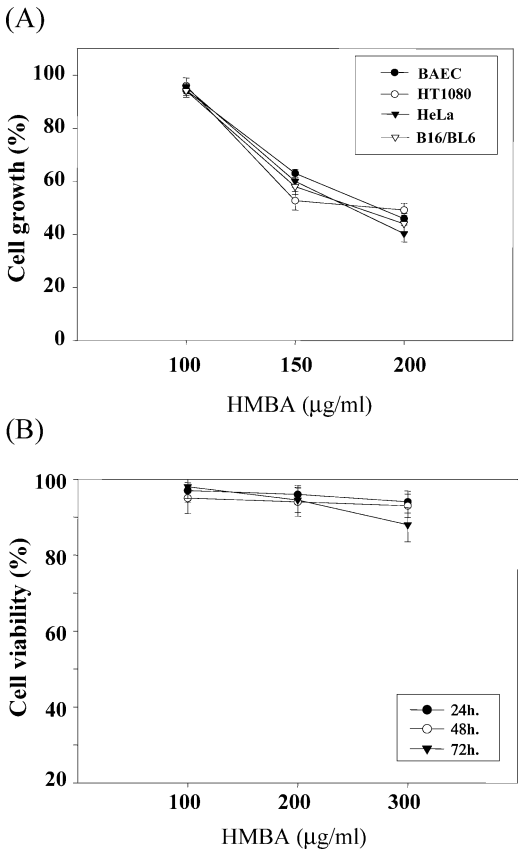
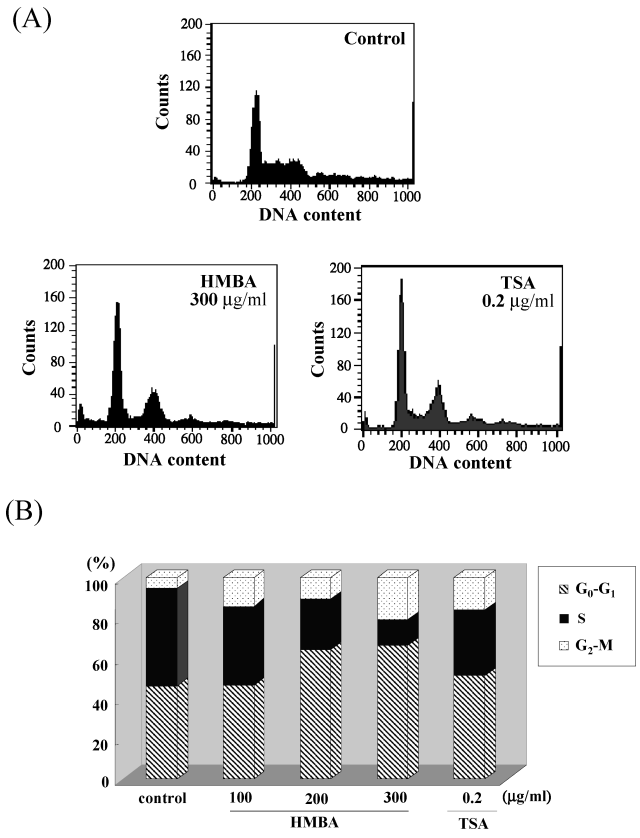
Fig. 2. Effect of HMBA on the proliferation of cultured cell lines.



The proliferation of several cultured cell lines was measured by MTT assay. (A) Effect of HMBA on the growth of various cell lines. HMBA inhibited the growth both normal and cancer cells BAEC; (○), HT1080; (●), HeLa (▼), B16/BL6 (▽). (B) Effect of HMBA on the viability of HeLa cells. The cells were stained with trypan blue and counted by hemocytometer. 100 μg/ml; (●), 200 μg/ml; (○), 300 μg/ml; (▼). Data shown are representative of at least three independent experiments.

Fig. 3. Effect of HMBA on the cell cycle progression of HeLa cells.



HeLa cells were treated with DMSO (0.05%), HMBA (300 μg/ml) or TSA (0.2 μg/ml). The stained cells were analyzed using a FACS Vantage flow cytometer. The experiment was conducted three times independently.

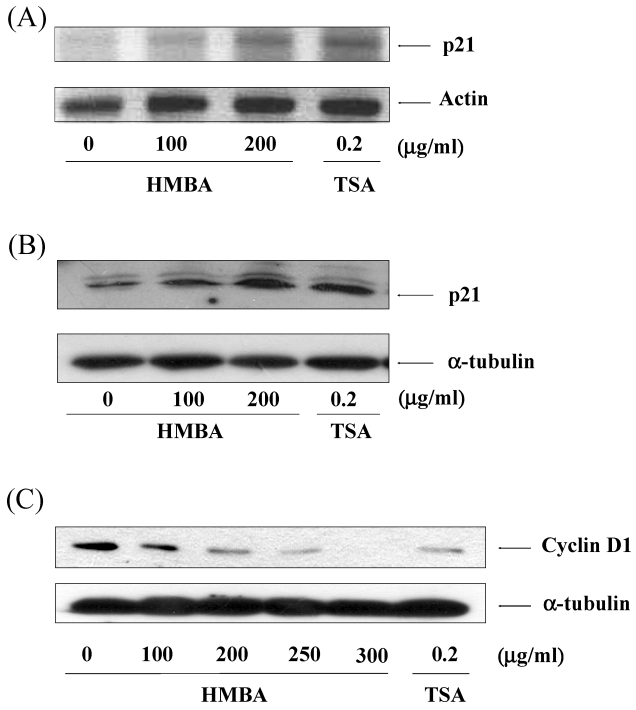
growth inhibitory activity of HMBA on the cell is not due to mere cytotoxicity of the compound.

We further investigated the effect of HMBA on the cell cycle progression using flow cytometry analysis. HeLa cells were seeded in a 6-well plate (1×10^5 cells/well) and incubated for 24 hours. After being starved for 12 hours with serum-free DMEM for synchronization, the cells were treated with HMBA and incubated for 24 hours. The cells were harvested with trypsinization, followed by fixation and permeabilized in the presence of 70% ethanol. The cells were centrifuged and suspended in phosphate-buffered saline (PBS, pH 7.4). The DNA histograms were determined using a Beckton-Dickenson FACS Vantage (San jose, CA) and the cell cycle distribution was analyzed using a Cell Quest software version 3.2 (Beckton-Dickinson). As shown in Fig. 3, control cells with vehicle treated only exhibited 46.23% in G_0/G_1, 48.52% in DNA synthesis and 5.25% in G_2-M phase; In contrast, HMBA showed a dose dependent increase of the percentage of cells at G_0/G_1 phase whereas that of S phase significantly decreased, as like the case of trichostatin A (TSA), a well known cell cycle inhibitor targeting histone deacetylase (HDAC). These data demonstrated that HMBA arrests cell cycle at G1 phase resulted in the inhibition of cell proliferation.

To decipher the molecular mechanisms of HMBA for its cell cycle inhibitory activity, the effect of HMBA on the cell cycle regulating factors was investigated. Total cellular

RNA was isolated from cultured cells treated with or without HMBA using RNeasy mini kit (Qiagen, Valencia, CA) and reverse transcribed by Molony murine leukemia virus reverse transcriptase (Life Technology, Grand Island, NY) using Oligo-d$(T)_{15}$ primer (Life Technology). For the determination of p21 mRNA content in the cells, a standard PCR was performed using 5′-GACACCACTGGAGGGTG-ACT-3′ (sense) and 5′-CTCTTGGAGAAGATCAGCCG-3′ (anti-sense) as primers. β-actin mRNA amounts were determined using the RT primer pair commercially available from Stratagene (Heidelberg, Germany) and used to normalize the cDNA content. The PCR products were resolved in a 1% agarose gel electrophoresis and visualized by ethidium bromide staining. As shown in Figure 4A, HMBA showed a dose-dependent increase in a cyclin-dependent kinase (CDK) inhibitor $p21^{WAF1}$ mRNA whereas the expression of β-actin was not affected by the compound. This result suggests that HMBA inhibits cell cycle progression through the specific regulation in the expression of $p21^{WAF1}$ gene. We next examined changes in the protein level of $p21^{WAF1}$ and other cell cycle related gene such as cyclin D1 in response to HMBA. Western blot analysis using total cellular proteins (20~25 μg) from HMBA treated cells showed that HMBA induces the expression of $p21^{WAF1}$ protein in a dose-dependent manner (Fig. 4B). Interestingly, however, the expression of cyclin D1 protein was suppressed by HMBA treatment (Fig. 4C). Together, HMBA exhibits the cell cycle inhibitory activity through cooperative expressional regulation of cell cycle inhibitor and activator. The molecular mechanism of HMBA on the cell cycle inhibition appears to be similar to that caused by the inhibition of HDAC as shown in parallel experiments of TSA in Fig. 4. Nonetheless, HMBA does not inhibit the activity of HDAC *in vitro* and *in vivo*, indicating the different mode of action for HMBA from that of TSA (data not shown).

Fig. 4. Effect of HMBA on expression of $p21^{WAF1}$ and cyclin D1 in HeLa cells.



(A) Effect of HMBA on expression of $p21^{WAF1}$ mRNA in HeLa cells. HeLa cells treated with HMBA (100~200 μg/ml) for 24 hours. Then total RNA was isolated, reverse-transcribed into cDNA and amplified with p21 specific oligonucleotide primers.

(B) Effect of HMBA on protein expression of $p21^{WAF1}$ in HeLa cells. Cells were treated with various concentrations of HMBA for 24 hours. Cell lysis and western blotting were performed as described.

(C) Cells were treated with 100, 200, 250, 300 μg/ml of HMBA for 24 hours. Cell lysis and western blotting were performed as described. Data shown are representative of at least two independent experiments.

Many studies have shown an association between cell cycle regulation and cancer, and in recent years, inhibition of the cell cycle has been highlighted as a useful phenotype for the control of cancer[9]. Anticancer agents may alter regulation of the cell cycle machinery, resulted in an arrest of the cells in different phases of the cell cycle and thereby reducing the growth and proliferation of the cancerous cells. Notably, HMBA is known as a part of the antibiotic novobiocin, a heat shock protein (HSP) 90 inhibitor[14]. Given the crucial role of Hsp90 in cell proliferation and other cellular functions such as cell differentiation and inflammation[15,16], HMBA may in part involve in regulation of molecular chaperon function. In addition, HMBA was also reported as one of the anti-fungal and anti-bacterial agents. Accordingly, HMBA may have great potential as a valuable probe for controlling and investigating the cellular function.

How can HMBA alter the expression of cell cycle regulators for its cell cycle inhibitory activity remains to be uncovered. Detailed studies whether HMBA may directly inhibit the CDK kinase activities or regulate the transcription of p21 and cyclin D1 are under investigation. It would be also of great interest to modify the functional groups of HMBA for the generation of a diverse chemical library having HMBA core structure. In summary, we report HMBA from the fungal metabolites as a new cell cycle inhibitor and expect that this unique compound can

be a useful tool for chemical genetics study of cell cycle regulation.

Acknowledgments

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