ORIGINAL ARTICLE

Habiterpenol, a novel abrogator of bleomycin-induced G2 arrest in Jurkat cells, produced by *Phytohabitans suffuscus* 3787_5

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A small molecule named habiterpenol produced by actinomycete *Phytohabitans suffuscus* 3787_5 was found to abrogate bleomycin-induced G2 arrest in Jurkat cells. Habiterpenol showed no cytotoxic effect on Jurkat cells even at 273 μM ; however, the compound inhibited bleomycin-induced G2 arrest in Jurkat cells with an IC_{50} value of 3.55 μM , while it showed no effect on colchicine-induced M arrest even at 273 μM . These results indicated that habiterpenol selectively abrogated bleomycin-induced G2 arrest in Jurkat cells.

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INTRODUCTION

DNA damage induces signal-transduction pathways known as checkpoints, which arrest cell cycle progression and permit DNA repair.¹ Checkpoints arrest cells in the G1 phase to prevent replication of damaged DNA, and in the G2 phase to prevent the segregation of damaged chromosomes during mitosis. Normal cells can repair DNA at G1 and G2 phases, whereas most cancer cells have mutations in genes involved in the G1 checkpoint, such as *p53*, *p16^{TNK4}* or *Rb*,² and can repair DNA only in the G2 phase. Accordingly, disruption of the G2 checkpoint in cancer cells is expected to increase sensitivity to DNA-damaging reagents such as bleomycin, cisplatin and camptothecin, causing selective cell death of cancer cells by the accumulation of mutations in genes;³ therefore, the G2 checkpoint is a potential target for development of a novel therapy against intractable cancers. At the G2 checkpoint, DNA damage activates the ataxia telangiectasia-mutated (ATM) and the ataxia telangiectasia and Rad 3-related (ATR) members of the phosphoinositide kinase family, and induces the phosphorylation of histone H2A.X.⁴ A signal is then transmitted through the downstream protein kinases Chk1 and Chk2,^{4–6} which are able to phosphorylate Cdc25C at Ser216.⁷ This phosphorylation is thought to prevent directly Cdc25C from activating cyclic-dependent kinase-1.⁸ Chk1 and Chk2 can also phosphorylate and activate Wee1, a kinase that catalyzes cyclic-dependent kinase-1 inhibitory phosphorylation.^{9,10}

Jurkat, a human T-cell leukemia-derived cell line, depends on the G2 checkpoint to repair DNA damage as it loses the G1 checkpoint by lacking functional *p53*.³ We established a cell-based assay using Jurkat cells to screen microbial culture broths for G2 checkpoint abrogators.^{11–14}

Bleomycin and colchicine were used to measure whether a culture broth (or sample) shows G2 checkpoint abrogation activity. Bleomycin, a DNA-damaging agent, arrests the cell cycle at the G2 phase of Jurkat cells, whereas microtubule-disrupting colchicine arrests at the M phase. Samples were selected that showed abrogation of bleomycin-induced G2 arrest but had no effect on colchicine-induced Mphase arrest. During our continuous screening for G2 checkpoint abrogators, a new diterpenoid, designated habiterpenol (Figure 1), was isolated from the culture broth of *Phytohabitans suffuscus* 3787_5. The physicochemical properties and structure elucidation of habiterpenol will be described elsewhere.¹⁵ In this study, the fermentation, isolation and effect on the drug-induced cell cycle checkpoint of habiterpenol are described.

RESULTS

Taxonomy of the producing strain 3787_5

Strain 3787_5 was isolated from a soil sample collected on Ishigaki Island, Okinawa, Japan. The strain grew well on inorganic salts starch agar and tyrosine agar, and the colony color was pale yellow to pale orange. Mass of the aerial mycelia produced on inorganic salts starch agar, glycerol asparagine agar and others was white. From observation of the scanning electron micrograph of the strain (Figure 2), the long spore chains were formed on the aerial mycelia. The spores were cylindrical in shape, approximately 1.0 μm long $\times$ 0.8 μm wide in size and had a smooth surface. No soluble pigment was produced. The cell-wall peptidoglycan contained glutamic acid, glycine, alanine, *meso*-diaminopimelic acid and lysine. The predominant menaquinones of the strain were MK-9 (H_4), MK-9 (H_6), MK-10 (H_4) and MK-10 (H_6). The 16S rRNA gene sequence (1280 nucleotides) was

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Figure 1 Structure of habiterpenol (1).

Figure 2 Scanning electron micrograph of strain 3787_5 after incubation on inorganic salts starch agar for 3 weeks at 27 °C.

determined and analyzed using EzTaxon-e database (<http://eztaxon-e.ezbiocloud.net/>). The result indicated that the gene sequences between 3787_5 and *Phytohabitans suffuscus* K07-0523^T (AB490769) show 100.0% similarity.¹⁶ On the basis of the morphological and cultural properties, chemotaxonomy and 16S rRNA gene sequence similarity, strain 3787_5 was identified with *P. suffuscus*.

Time course of habiterpenol production by fermentation of *P. suffuscus* 3787_5

The production of habiterpenol by the strain started on day 20 after inoculation. The concentrations reached a maximal level at 2.3 mg l⁻¹ on day 40, and then gradually decreased on day 50 (data not shown).

Isolation of habiterpenol

The isolation procedure of habiterpenol from the culture broth of strain 3787_5 is summarized in Figure 3. Activity-guided isolation was carried out. The culture broth (4.5 l) was centrifuged at 3000 r.p.m. to obtain the mycelium. The mycelium was treated with EtOH (2.0 l) for 2 h, and EtOH extracts were filtered to remove the mycelium. After concentration of the extracts to remove EtOH, the aqueous solution (0.80 l) was adjusted at pH 3.0 and extracted with EtOAc (0.80 l). The organic layer was dried over Na₂SO₄ and concentrated under reduced pressure to give brown materials (2.34 g). The materials were dissolved in a small amount of MeOH and purified by ODS column chromatography (i.d. 4.0 × 30 cm, Chromatorex ODS, 100–200 mesh; Fuji Silysia Chemical, Aichi, Japan). The active principles were eluted stepwise with CH₃CN:H₂O (0, 60 and 100%, 200 ml each) and acetone (200 ml).

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Culture broth (4.5 l)
| centrifuged at 3,000 rpm for 10 min
Mycelia cake
| treated with EtOH (2.0 l)
| filtered
| concentrated to remove EtOH
| extracted with EtOAc (0.8 l, pH3)
EtOAc extract (2.34 g, yield)
| ODS column chromatography (40φ x 300 mm)
| Eluent: 60 and 100% CH3CN : H2O, acetone (1.0 l each)
Acetone fraction (81.4 mg)
| Preparative HPLC
| Column: PEGASIL C4 (20φ x 250 mm)
| Mobile phase: 85–90% CH3CN–0.05% H3PO4 (45 min linear gradient)
| Flow rate: 8 ml/min
| Detection: UV 210 nm
Active fraction (R.T.; 27 min)
| concentrated to remove CH3CN
| extracted with EtOAc (0.2 l)
Habiterpenol (16.4 mg)
  
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Figure 3 Isolation procedure of 1.

Habiterpenol was recovered from the acetone fraction, which was concentrated under reduced pressure to give brown oily materials (81.4 mg). The materials containing habiterpenol were dissolved in MeOH and finally purified by HPLC under the following conditions: column, Pegasil C4 (i.d. 20 × 250 mm; Sensyu Scientific, Tokyo, Japan); mobile phase, a linear gradient from 85 to 90% CH₃CN in 0.05% H₃PO₄ for 45 min; flow rate, 8 ml min⁻¹; detection, UV 210 nm. Habiterpenol was eluted as a peak with a retention time of 27 min. The fraction of the peak was collected and concentrated to remove CH₃CN. The aqueous solution was extracted with EtOAc and the organic layer was concentrated to dryness to give pure habiterpenol (16.4 mg) as a brown solid.

Abrogation of bleomycin-induced G2 arrest in Jurkat cells by habiterpenol

When Jurkat cells were treated with bleomycin (20 μM) for 24 h, cell cycle analysis by FACS showed distribution rates in sub-G1 and G2/M of 12% and 60%, respectively. Cell viability was about 90%, indicating that bleomycin (20 μM) arrested the cell cycle at the G2 phase (Figure 4a). Similarly, when cells were treated with colchicine (25 nM), the rates in the sub-G1 and G2/M were 24% and 51%, respectively, indicating that colchicine (25 nM) arrested the cell cycle at the M phase (Figure 4a). These data are comparative to those reported previously.¹⁴ On the other hand, when Jurkat cells were treated with habiterpenol (0.237–273 μM), the compound did not change distribution in the sub-G1, G1, S and G2/M even at 273 μM (Figure 4a).

In combination of habiterpenol (0.237–273 μM) and bleomycin (20 μM), the cell cycle pattern changed markedly (Figure 4a). Cells in the G2/M phase decreased in a dose-dependent manner with an IC₅₀ value (the concentration of habiterpenol required to reduce 50% of the number of cells in the G2/M phase) of 3.55 μM, and concomitantly cells in the sub-G1 phase increased (Figure 4b). On the other hand, in combination of habiterpenol (0.237–273 μM) and colchicine (25 nM), the distribution rates in the G2/M were unchanged up to 273 μM, indicating that habiterpenol had no effect on colchicine-induced M-phase arrest (Figures 4a and c). These data indicate that habiterpenol selectively abrogated bleomycin-induced G2 arrest in Jurkat cells.

Figure 4 Effect of **1** on the cell cycle status of Jurkat cells treated with bleomycin and colchicine. (a) Jurkat cells (5.0×10^5 cells ml^{-1}) treated with **1** only or in combination with bleomycin (20 μM) or colchicine (25 nM). The distribution in the cell cycle was analyzed by flow cytometry 24 h after incubation. (b and c) Jurkat cells (5.0×10^5 cells ml^{-1}) were incubated in a 96-well plate with (b) bleomycin (20 μM) or (c) colchicine (25 nM) at the indicated concentrations for 24 h. After treatment, the distribution in the cell cycle was analyzed by FACS (sub-G1: ■; G2/M: ▲). Each value is the mean $\pm$ s.d. of three independent experiments. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

Synergistic enhancement of bleomycin cytotoxicity to Jurkat cells in combination with habiterpenol

As fungal pycnidione, an abrogator of bleomycin-induced G2 arrest, was found to potentiate the cytotoxicity of bleomycin to Jurkat cells,¹⁴ it was also tested whether habiterpenol enhanced bleomycin cytotoxicity. By the thiazolyl blue tetrazolium bromide (MTT) assay, bleomycin alone and habiterpenol alone gave IC_{50} values of 159 and 142 μM against the growth of Jurkat cells, respectively. Next, in the presence of habiterpenol at 0.273, 2.73 or 27.3 μM , the IC_{50} values of bleomycin were measured. As shown in Figure 5, the combination of bleomycin and habiterpenol showed synergistic cytotoxicity to Jurkat cells. For example, in the presence of 2.6 μM habiterpenol, the IC_{50} value of bleomycin was 15.9 μM , over 10 times more potent than bleomycin alone.

Other biological activity

Habiterpenol (10 μg per 6 mm paper disk) showed very weak antimicrobial activity against *Bacillus subtilis* (inhibitory zone: 7 mm), but no antimicrobial activity against 13 other microorganisms.

DISCUSSION

Habiterpenol-producing actinomycete strain 3787_5 was isolated from Ishigaki Island, and 16S rRNA gene sequence analysis and taxonomic study indicated that this strain belonged to genus *Phytohabitans* in the family Micromonosporaceae, which was propounded as a new genus in 2009.¹⁶ Although taxonomic studies of this genus were carried out,¹⁷ the secondary metabolites have not

Figure 5 Effects of combined **1** and bleomycin in Jurkat cells. Isobolograms at IC₅₀ based on the results of thiazolyl blue tetrazolium bromide (MTT) assays of Jurkat cells treated with habiterpenol and bleomycin are shown (IC₅₀ of **1** or bleomycin: ■ and combination with **1** and bleomycin: ●). The data are the average of three experiments.

been reported so far; therefore, habiterpenol was first discovered as a novel small bioactive compound produced by this genus.

As demonstrated in this study, habiterpenol abrogated bleomycin-induced G2 arrest in Jurkat cells (Figure 4) and synergistically potentiated the cytotoxicity of bleomycin (Figure 5), but showed no effect on colchicine-induced M-phase arrest (Figure 4). G2 checkpoint is expected as a potential target to develop a new type of anticancer agent.¹⁸ Several target molecules are involved in the G2 checkpoint, namely Chk1, Chk2, ATM, ATR, Wee1 and Cdc25C. In fact, a number of small-molecule inhibitors have been reported. Among them, CBP501 is being developed as an anticancer drug, and is currently in randomized phase II clinical trials for patients with non-small-cell lung cancer and malignant pleural mesothelioma.¹⁹

In response to DNA damage, the cell activates a complex integrated network of signaling pathways and checkpoints to repair the damaged DNA. DNA damage is sensed by either the MRN/ATM/CHK2 pathway or the ATRIP/ATR/CHK1 pathway.²⁰ Although these two pathways functionally overlap in response to DNA damage and share most downstream effectors, the DNA double-strand break primarily activates the MRN/ATM/CHK2 pathway, whereas the ATRIP/ATR/CHK1 pathway mainly responds to DNA replication fork collapse caused by bulky DNA lesions.^{21–23} Adriamycin is also known to cause G2 arrest in Jurkat cells, and habiterpenol was found to show only a slight effect on adriamycin-induced G2 arrest in Jurkat cells (the rate in the sub-G1 was 25% at 273 μM), which was different from bleomycin. Researchers reported that bleomycin- and adriamycin-induced DNA damage activates MRN/ATM/CHK2 and both the ATRIP/ATR/CHK1 and MRN/ATM/CHK2 pathways, respectively.^{24,25} Therefore, habiterpenol might mainly affect the MRN/ATM/CHK2 pathway. The target molecule of habiterpenol remains to be defined.

METHODS

Materials

RPMI1640, bovine serum albumin, ATP, MTT, deuteriochloroform, ellipticine, MG132, propidium iodide, RNase, colchicine, Triton X-100 and IGEPAL CA-630 were purchased from Sigma-Aldrich (St Louis, MO, USA). Penicillin (100 U ml⁻¹) and streptomycin (100 μg ml⁻¹) solution were obtained from

GIBCO (Grand Island, NY, USA). Fetal bovine serum was from Hyclone (Logan, UT, USA). Bleomycin (Nippon Kayaku, Tokyo, Japan) and Block Ace (a blocking agent made from milk; Yukijirushi Nyugyo, Tokyo, Japan) were also used.

Taxonomic studies

The International *Streptomyces* Project media recommended by Shiring and Gottlieb²⁶ and media recommended by Waksman²⁷ were used to investigate the cultural characteristics. Cultures were observed after incubation for 2 weeks at 27 °C. The morphologic characteristics were observed by scanning electron microscope (JSM-5600; JEOL, Tokyo, Japan) after incubation on inorganic salts starch agar for 3 weeks at 27 °C and fixation with 4% osmium tetroxide vapor. Purified cell wall was obtained as described by Kawamoto *et al.*²⁸ and 1 mg purified cell wall was hydrolyzed at 100 °C with 1 ml of 6 N HCl for 15 h. The hydrolysate was dissolved in 100 μl water and used for amino-acid analysis. The amino-acid composition was determined by HPLC using the Pico-Tag method (Waters, Milford, MA, USA). Menaquinones were extracted and purified by the method of Collins *et al.*²⁹ and then analyzed by LC/MS (JSM-T 100LP; JEOL) with a CAPCELL PAK C18 column (Shiseido, Tokyo, Japan) eluted with methanol/2-propanol (7:3). 16S rRNA gene sequence was amplified by polymerase chain reaction and sequenced on a DNA sequencer (3130 Genetic Analyzer; Applied Biosystems, Carlsbad, CA, USA).

Fermentation

A slant culture of strain 3787_5 was grown and maintained on an agar medium consisting of 1.0% starch, 0.3% NZ amine, 0.1% yeast extract, 0.3% meat extract, 1.2% agar and 0.3% CaCO₃ (adjusted to pH 7.0 before sterilization). A loopful of spores of *P. suffusus* 3787_5 was inoculated into 10 ml seed medium consisting of 2.4% starch, 0.1% glucose, 0.3% peptone, 0.3% meat extract, 0.5% yeast extract and 0.4% CaCO₃ (adjusted to pH 7.0 before sterilization) in a large test tube. The inoculated test tube was incubated in a reciprocal shaker (210 r.p.m.) at 27 °C for 12 days to obtain the first seed culture (5 ml), which was transferred to 100 ml seed medium in a 500-ml Erlenmeyer flask and incubated in a rotary shaker (210 r.p.m.) at 27 °C to obtain the second seed culture. Further, the second seed culture (5 ml) was transferred to 100 ml seed medium in five 500-ml Erlenmeyer flasks and incubated in a rotary shaker (210 r.p.m.) at 27 °C to obtain the third seed culture.

For the production of habiterpenol, the culture was initiated by transferring 10 ml third seed culture into forty-five 500-ml Erlenmeyer flasks containing production medium (2.0% soluble starch, 0.5% glycerol, 1.0% defatted wheat germ (Nissin Pharma, Tokyo, Japan), 0.3% meat extract (Kyokuto Pharmaceutical Industrial, Tokyo, Japan), 0.3% dry yeast (Oriental Yeast, Tokyo, Japan), 0.3% CaCO₃, non-adjusted pH) and fermentation was carried out in a rotary shaker (210 r.p.m.) at 27 °C for 40 days.

Cell culture

Jurkat cells, a human T-cell leukemia-derived cell line, were cultured in RPMI 1640 containing 10% fetal bovine serum and penicillin (1 U ml⁻¹)/streptomycin (1 μg ml⁻¹) at 37 °C in a humidified atmosphere of 5% CO₂.

Cell cycle analysis

The cell cycle status was analyzed according to the method described previously.¹⁴ Briefly, Jurkat cells (5.0 × 10⁴ cells per 100 μl) were treated with habiterpenol (0–273 μM) in the presence or absence of bleomycin (20 μM), adriamycin (1.8 nM) or colchicine (25 nM) for 24 h at 37 °C. The cells were centrifuged at 700 g for 5 min, the supernatant was removed by an aspirator and the pellet was resuspended in 100 μl RPMI medium. It was then stained with Krishan solution¹³ (0.20% sodium citrate, 100 mg ml⁻¹ propidium iodide, 40 mg ml⁻¹ RNase, 0.60% IGEPAL CA-630; 100 μl) for 1 h at 37 °C. The cell cycle status was determined by analyzing the DNA content using cytometry (FACSCalibur; Becton Dickinson, San Jose, CA, USA) with CELL Quest software. Cells (1.0 × 10⁴ cells) were measured and the distribution ratios in each phase (sub-G1, G1, S and G2/M) were calculated by the program ModFit LT ver. 2.0 according to the manufacturer's protocol.

MTT assay

Jurkat cells (5.0×10^5 cells ml⁻¹) were treated with habiterpenol in the presence or absence of bleomycin for 24 h. MTT ($5.0 \mu\text{g ml}^{-1}$) was added and cells were incubated for approximately 3 h.¹⁴ After the cells were lysed with 40% *N,N*-dimethylformamide, 20% SDS, 2.0% acetic acid and 0.030% hydrochloric acid, the absorbance of the solution was read at 600 nm using POWER WAVE X340 (Bio-Tek Instruments, Winooski, VT, USA).

Effects of combined habiterpenol and bleomycin on Jurkat cells

Antagonism or synergism was examined by an isobologram,³⁰ where the X and Y intercepts indicate the concentrations of each compound alone, resulting in an IC₅₀ value. The data point that falls between the axes indicates the concentration of the drug combination that inhibits cell proliferation. Data points above or below the straight line joining the intercepts (IC₅₀) indicate antagonistic or synergistic effects, respectively, whereas data points that fall on or close to the line joining the intercepts indicate additive effects.

Antimicrobial activity

Antimicrobial activity of a sample against 14 species of microorganisms was measured by the agar diffusion method using paper disks.³¹

Microorganism strains

The microorganisms used for the paper disk method were as follows: *B. subtilis* PCI 219, *Staphylococcus aureus* FDA 209P, *Micrococcus luteus* PCI 1001, *Mycobacterium smegmatis* ATCC 607, *Escherichia coli* NIHJ, *Pseudomonas aeruginosa* P-3, *Xanthomonas campestris* pv. *oryzae* KB 88, *Bacteroides fragilis* ATCC 23745, *Acholeplasma laidlawii* PG 8, *Pyricularia oryzae* KF 180, *Aspergillus niger* ATCC 6275, *Mucor racemosus* IFO 4581, *Candida albicans* ATCC 64548 and *Saccharomyces cerevisiae*.

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