Potential of Bleomycin in Jurkat Cells by Fungal Pycnidione

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Most cancer cells have mutations in genes at the G1 checkpoint and repair DNA only in the G2 phase; therefore, the G2 checkpoint is a potential target to develop novel therapy. In the course of screening, a known compound, pycnidione, was isolated from the fungal culture broth of *Gloeotinia* sp. FKI-3416. Pycnidione irreversibly abrogated bleomycin-induced G2 arrest in Jurkat cells and synergistically potentiated the cytotoxicity of bleomycin. To elucidate the mechanism of action, the effect of pycnidione on the signal transduction of the G2 checkpoint was analyzed, showing that the increased phospho-cyclin dependent kinase-1 (CDK1) level caused by bleomycin was abrogated in the presence of pycnidione, indicating that cells did not arrest at the G2 phase. Moreover, under these conditions, Chk1 and Chk2 levels were markedly down-regulated. Thus, we concluded that pycnidione abrogated bleomycin-induced G2 arrest by decreasing Chk1 and Chk2.

Key words pycnidione; G2 arrest; bleomycin

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 genes³⁾; 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 A-T and rad-3-related (ATR) members of the phosphoinositide kinase family, and induced 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 directly prevent Cdc25C from activating cyclic dependent kinase-1 (CDK1) kinase.⁸⁾ Chk1 and Chk2 can also phosphorylate and activate Wee1, a kinase that catalyzes CDK1 inhibitory phosphorylation.^{9,10)} Several G2 checkpoint abrogators have been reported to date; for example, UCN-01, a derivative of staurosporin, abrogated DNA damage-induced S and G2 arrest due to inhibition of Chk1 and perhaps Chk2.¹¹⁾ Although Phase I clinical trials of UCN-01 have been completed, UCN-01 was found to bind readily to human plasma proteins and to inhibit kinases other than Chk1 and Chk2, which may result in unexpected side effects in patients.^{12,13)} SB218078¹⁴⁾ and Gö6979¹⁵⁾ were reported recently to specifically inhibit Chk1, and their binding activity to human plasma proteins was stronger than that of UCN-01. Caffeine and PD0166285 inhibit the checkpoint kinases ATM and ATR,¹⁶⁾ and Wee1 kinase,¹⁷⁾ respectively. Suganuma *et*

al. previously reported that the short peptide corresponding to amino acids 211–221 of human Cdc25C, which is an important phosphatase in the G2 checkpoint, fused with part of human immunodeficiency virus (HIV)-TAT, can efficiently disrupt the cell cycle G2 checkpoint, which is activated by DNA damage, and can thereby sensitize cancer cells but not normal cells to anticancer reagents.³⁾ On the basis of these findings, CBP501,¹⁸⁾ a superior analog, and AZD7762¹⁹⁾ have been developed as G2 checkpoint abrogators in clinical trials.

Jurkat, a human T-cell leukemia-derived cell line, depends on the G2 checkpoint to repair DNA damage since 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.^{20–22)} 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, while 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 M phase arrest. During this screening program, pycnidione (Fig. 1A), a fungal metabolite previously reported to inhibit stromelysin,²³⁾ cell growth,²⁴⁾ and plasmodial activity,²⁵⁾ was found to abrogate G2 arrest. In this study, we describe the activity and the mechanism of action of pycnidione as a G2 arrest abrogator.

MATERIALS AND METHODS

Materials RPMI1640, bovine serum albumin (BSA), ATP, thiazolyl blue tetrazolium bromide (MTT), deuteriochloroform, ellipticine, MG132, propidium iodide, ribonuclease A, colchicine, Triton X-100, and IGEPAL CA-630 were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Penicillin (1.0×10⁴ units/mL) and streptomycin (1.0×10⁴ mg/mL) solution were obtained from GIBCO (Grand Island, NY, U.S.A.). Fetal bovine serum (FBS) was from Hyclone (Logan, UT, U.S.A.).

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Fig. 1. Effect of Pycnidione on the Cell Cycle Status of Jurkat Cells Treated with Bleomycin and Colchicine

(A) Structure of pycnidione. (B,C) Jurkat cells (5.0×10^5 cells/mL) were incubated in a 96-well plate with (B) bleomycin (a) and caffeine (b) or (C) colchicine at the indicated concentrations for 24h. After treatment, cell viability was evaluated by MTT assay ($\blacktriangle$) and the distribution in the cell cycle was analyzed by FACS (Sub G1: $\blacklozenge$, G2: $\blacksquare$ and M: $\blacktriangle$). Each value represents the mean $\pm$ S.D. of 4 independent experiments. (D) Jurkat cells (5.0×10^5 cells/mL) treated with pycnidione only or in combination with bleomycin or colchicine. The distribution in the cell cycle was analyzed by flow cytometry 24h after incubation. (E) Jurkat cells (5.0×10^5 cells/mL) were incubated with pycnidione (255 nM, middle and right panels) or methanol (left panel) for 3h. After removal of pycnidione (right panel), cells were incubated for 24h in the presence of 30 μ g/mL bleomycin (all panels). Then, DNA was stained with propidium iodide and the distribution in the cell cycle was analyzed by flow cytometry.

Bleomycin (Nippon Kayaku, Tokyo, Japan) and Block Ace (a blocking agent made from milk; Yukijirushi Nyugyo, Tokyo, Japan) were also used.

¹H-NMR (400MHz) spectra were measured with a spectrometer (XL-400; Varian, Palo Alto, CA, U.S.A.) in deuteriochloroform. The low-resolution mass spectra (LR-MS) were measured with LC/MS. LC/MS experiments were performed on the JMS-T100LP (JEOL, Tokyo, Japan) coupled with HPLC (column; Symmetry C18 (Waters, Milford, MA, U.S.A.), 2.1×150mm; solvent, 50% acetonitrile in 0.050% formic acid; detection, UV at 210nm; flow rate, 0.20 mL/min).

Antibodies Mouse monoclonal anti-CDK1 p34 (17), anti-Chk2 (A-12), anti-Cdc25C (H-6), and anti-cyclin B1 (D-11) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Mouse monoclonal anti-Chk1 (clone 2G11D5), anti-CDK2 (clone AN4.3) and anti-cyclin A (clone BF863), and rabbit polyclonal anti-Wee1 were obtained from Millipore (Billerica, MA, U.S.A.). Mouse monoclonal anti-cyclin D3 (DCS22) and anti-CDK6 (DCS83), and rabbit polyclonal anti-phospho-CDK1 (Tyr15) and anti-phospho-Histone H2A.X (Ser139) were from Cell Signaling Technology (Beverly, MA, U.S.A.). Anti-actin (clone C4; CHEMICON International, Temecula, CA, U.S.A.) was also used. Rabbit polyclonal anti-mouse immunoglobulin (Ig) and anti-rabbit Ig, horseradish peroxidase (HRP) linked whole antibody (GE Healthcare U.K. Ltd., Buckinghamshire, England) and anti-rabbit Ig (whole molecule) fluorescein isothiocyanate (FITC) conjugate (Sigma-Aldrich) were used.

Production of Pycnidione Pycnidione was purified from the culture broth of *Gloeotinia* sp. FKI-3416, isolated from a soil sample collected at Amamiooshima, Kagoshima, Japan. Briefly, the whole culture broth (2.0L) was extracted with ethanol (2.0L), concentrated by evaporation to remove the organic solvent, and re-extracted with ethyl acetate. The extract (540mg) was purified by HPLC using an octadecyl silica column, giving pure pycnidione (67.8mg).

Cell Culture Jurkat, a human T-cell leukemia-derived cell line was kindly provided by Dr. Kawabe. Jurkat cells were cultured in RPMI1640 containing 10% FBS and penicillin (100units/mL)/streptomycin (100mg/mL). The 39 human cancer cell lines for the panel are listed as follows²⁶: 7 lung cancers, NCI-H23, NCI-H226, NCI-H522, NCI-H460, A549, DMS273, and DMS114; 5 colorectal cancers, HCC-2998, KM-12, HT-29, HCT-15, and HCT-116; 6 gastric cancers, MKN-1, MKN-7, MKN-28, MKN-45, MKN-74, and St-4; 5 ovarian cancers, OVCAR-3, OVCAR-4, OVCAR-5, OVCAR-8, and SK-OV-3; 5 breast cancers, BSY-1, HBC-4, HBC-5, MDA-MB-231, and MCF-7; 2 renal cancers, RXF-631L and ACHN; 1 melanoma, LOX-IMVI; 6 gliomas, U251, SF-295, SF-539, SF-268, SNB-75, and SNB-78; and 2 prostate cancers, DU-145 and PC-3. These cell lines were cultured in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan) with 5% fetal bovine serum, penicillin (100units/mL), and streptomycin (100μg/mL) at 37°C.

MTT Assay Jurkat cells (5.0×10⁵ cells/mL) were treated with pycnidione in the presence or absence of bleomycin for 24h. MTT (5.0μg/mL) was added and cells were incubated for approximately 3h.²² After the cells were lysed with 40% *N,N*-dimethylformamide, 20% sodium dodecyl sulfate (SDS), 2.0% acetic acid, and 0.030% hydrochloric acid, the absorbance of the solution was read at 600nm using POWER WAVE X340

(Bio-Tek Instruments, Winooski, VT, U.S.A.).

Determination of Cell Growth Inhibition Profiles

Growth inhibition experiments were performed to assess the sensitivity of the cells to pycnidione as described before.²⁶ Growth inhibition was measured by determining changes in the amounts of total cellular proteins after 48h of chemical treatment using a sulforhodamine B assay. This GI₅₀ calculation method is well-established and reliable for anticancer drug screening using NCI60 as well as JFCR39.²⁶ At each test concentration, the percentage growth was calculated using the following seven absorbance measurements: growth at time 0 (*T*₀), growth of control cells (*C*), and test growth in the presence of five different concentrations (*T*) of a drug. Percentage growth inhibition was calculated as: % growth=100×[(*T*−*T*₀)/(*C*−*T*₀)] when *T*≥*T*₀, and % growth=100×[(*T*−*T*₀)/*T*] when *T*<*T*₀. GI₅₀ values, which represent 50% growth inhibition concentration, were calculated as 100×[(*T*−*T*₀)/(*C*−*T*₀)] = 50. Absolute values of GI₅₀ were then log transformed for further analysis. We confirmed the accuracy of the measured GI₅₀ data using reference control chemicals, such as mitomycin C, paclitaxel, and SN-38, in each experiment and by checking the dose–response curves.

Hierarchical Clustering Hierarchical clustering analysis was carried out using the average linkage method and “GeneSpring” software (Silicon Genetics, Inc., Redwood, CA, U.S.A.). Pearson correlation coefficients were used to determine the degree of similarity.

Cell Cycle Analysis Jurkat cells were stained with Krishan’s solution²² (0.20% sodium citrate, 100mg/mL propidium iodide, 40mg/mL ribonuclease A, 0.60% IGEPAL CA-630). The cell cycle status was determined by analyzing the DNA content using flow cytometry (FACSCalibur; Becton Dickinson, San Jose, CA, U.S.A.) with CELL Quest software. Ten thousand cells were analyzed and the distribution ratios in each phase (Sub G1, G1, S and G2/M) were calculated.

Phospho-Histone H2A.X Analysis The assay to detect phospho-histone H2A.X was performed according to the instructions for the phospho-Histone H2A.X (Ser139) antibody. Briefly, Jurkat cells were fixed with 2.5% formaldehyde for 10min at room temperature, chilled on ice for 1.0min, permeabilized with 90% ice-cold MeOH, and kept at 4°C for 30min. The cells were stained with phospho-Histone H2A.X (Ser139) primary antibody for 1.0h, further stained with anti-rabbit FITC conjugated antibody for 1.0h, and counterstained with propidium iodide (Krishan’s solution) for 20min.

Western Blotting Jurkat cell lysates were prepared with Triton lysis buffer (50mM Tris-HCl, pH7.4, 10% glycerol, 1.0% Triton X-100, 1.0mM ethylenediaminetetraacetic acid (EDTA), and 150mM sodium chloride) containing protease inhibitors (5.0mg/mL aprotinin, 5.0mg/mL leupeptin, 5.0mg/mL pepstatin, 5.0mg/mL sodium orthovanadate, and 0.50mM phenylmethanesulfonyl fluoride).²⁷ Total cell protein (20μL) was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gels and electrotransferred onto polyvinylidene difluoride membranes (Millipore). The secondary antibody was visualized using ECL Western Blotting Detection Reagents (GE Healthcare) and detected by LAS-4000 mini using Science Lab 2005 software (Fujifilm Co., Tokyo, Japan). Sequential reprobing of membranes with a variety of antibodies was performed after inactivation of HRP with 0.10% sodium azide or after the

complete removal of primary and secondary antibodies from the membrane in stripping buffer, according to the manufacturer's instructions.

Synthesis of *O*-Dimethylpyncnidione A solution of pyncnidione (5.3 mg, 9.7 nmol) in benzene/MeOH (1:1, 0.50 mL) was treated with (trimethylsilyl)diazomethane (2.0 M in hexane, 14 μ L, 28 nmol) at 0°C, and the mixture was stirred at 0°C for 30 min. Acetic acid was added, and the reaction mixture was concentrated *in vacuo*. The residue (6.2 mg) was finally purified by preparative HPLC (column; PEGASIL ODS (Senshu Scientific Co., Ltd., Tokyo, Japan), 4.6 $\times$ 250 mm; solvent, 40% acetonitrile; detection, UV at 210 nm; flow rate, 1.0 mL/min). Under these conditions, *O*-dimethylated pyncnidiones D1 to D4 were eluted as peaks with retention times of 13.2, 16.2, 19.0 and 24.0 min, respectively. The fractions were concentrated *in vacuo* to dryness to give pure *O*-dimethylpyncnidiones D1 (0.60 mg), D2 (0.60 mg), D3 (0.60 mg) and D4 (0.71 mg) as white crystals. D1: ¹H-NMR (deuteriochloroform, 400 MHz) δ : 7.00 (s, 2H), 6.52 (s, 2H), 5.36–5.35 (m, 2H), 3.92 (s, 3H), 3.91 (s, 3H), 3.57–3.54 (m, 1H), 3.05 (dd, 1H, J =4.4, 16.0 Hz), 2.70 (dd, 1H, J =5.2, 17.6 Hz), 2.68–2.65 (m, 1H), 2.42 (s, 3H), 2.39 (s, 3H), 2.45–2.26 (m, 3H), 2.18 (d, 1H, J =4.0 Hz), 1.87–1.84 (m, 1H), 1.75–1.50 (m, 3H), 1.26 (s, 3H), 1.23 (s, 3H), 1.09 (s, 3H), 1.05 (s, 3H), 1.22–1.03 (s, 2H); low resolution mass spectrum (LR-MS), m/z 577 (M+H⁺). D2: ¹H-NMR (deuteriochloroform, 400 MHz) δ : 7.03 (s, 1H), 6.89 (s, 1H), 6.50 (s, 1H), 6.46 (s, 1H), 5.41–5.37 (m, 2H), 3.92 (s, 3H), 3.91 (s, 3H), 3.70–3.50 (m, 1H), 3.20–3.28 (m, 1H), 2.69–2.61 (m, 2H), 2.41 (s, 3H), 2.38 (s, 3H), 2.40–2.32 (m, 3H), 2.18–2.16 (m, 1H), 1.83–1.75 (m, 1H), 1.75–1.50 (m, 3H), 1.35 (s, 3H), 1.23 (s, 3H), 1.18 (s, 3H), 1.09 (s, 3H), 1.17–1.03 (s, 2H); LR-MS, m/z 577 (M+H⁺). D3: ¹H-NMR (deuteriochloroform, 400 MHz) δ : 7.04 (s, 1H), 6.91 (s, 1H), 6.51 (s, 1H), 6.45 (s, 1H), 5.42–5.30 (m, 2H), 3.92 (s, 3H), 3.89 (s, 3H), 3.60–3.55 (m, 1H), 2.85 (dd, 1H, J =5.2, 17.2 Hz), 2.73–2.62 (m, 2H), 2.39 (s, 3H), 2.34 (s, 3H), 2.42–2.17 (m, 3H), 2.03–2.01 (m, 1H), 1.93–1.91 (m, 1H), 1.90–1.80 (m, 3H), 1.30 (s, 3H), 1.29 (s, 3H), 1.20 (s, 3H), 1.07 (s, 3H), 1.32–1.06 (s, 2H); LR-MS, m/z 577 (M+H⁺). D4: ¹H-NMR (deuteriochloroform, 400 MHz) δ : 7.03 (s, 1H), 7.02 (s, 1H), 6.48 (s, 1H), 6.46 (s, 1H), 5.51–5.40 (m, 2H), 3.93 (s, 3H), 3.91 (s, 3H), 3.74–3.68 (m, 1H), 2.98–2.91 (m, 1H), 2.72 (dd, 1H, J =3.6, 13.2 Hz), 2.64 (dd, 1H, J =5.6, 17.6 Hz), 2.37 (s, 3H), 2.37 (s, 3H), 2.47–2.09 (m, 4H), 1.89–1.80 (m, 1H), 1.78–1.43 (m, 3H), 1.25 (s, 3H), 1.23 (s, 3H), 1.12 (s, 3H), 1.08 (s, 3H), 1.17–1.01 (s, 2H); LR-MS, m/z 577 (M+H⁺).

RESULTS

Abrogation of Bleomycin-Induced G2 Arrest in Jurkat Cells by Pyncnidione First, we prepared the concentrations of bleomycin and colchicines for cell cycle analysis using Jurkat cells. In cells treated with bleomycin (30 μ g/mL) for 24 h, the distribution ratios in the Sub G1 and G2/M were 12.4% and 72.2%, respectively, and cell viability was 88.9%, indicating that 30 μ g/mL bleomycin arrested the cell cycle at the G2 phase (Fig. 1B). Furthermore, in combination with caffeine (3.0 mM), a G2 checkpoint abrogator,¹⁶⁾ bleomycin-induced G2 arrest was abrogated. Similarly, when cells were treated with colchicine (10 ng/mL), the ratios in the Sub G1 and G2/M were 18.6% and 60.0%, respectively, indicating that 10 ng/mL col-

chicine arrested the cell cycle at the M phase (Fig. 1C). Thus, we used 30 μ g/mL bleomycin and 10 ng/mL colchicine in our cell cycle experiments.

The effect of pyncnidione on bleomycin-induced G2 arrest or colchicine-induced M phase arrest in Jurkat cells was tested (Fig. 1D). Pyncnidione alone did not change the distribution in the Sub G1, G1, S and G2/M up to 255 nM. With a combination of pyncnidione (27–255 nM) and bleomycin, the cell cycle pattern changed markedly. Cells in the Sub G1 phase increased, and conversely, cells in the G2 phase decreased in a dose-dependent manner. On the other hand, pyncnidione had no effect on colchicine-induced M phase arrest in Jurkat cells. These data indicate that pyncnidione selectively abrogated bleomycin-induced G2 arrest.

Next, we tried to remove the pyncnidione by washing the cells. Jurkat cells were pretreated with pyncnidione (255 nM) for 3 h, and then cells were washed with PBS to remove pyncnidione in the medium. Pyncnidione-pretreated cells were incubated in the presence of bleomycin (30 μ g/mL) for 24 h, and the cell cycle status was analyzed. As shown in Fig. 1E, pretreatment still caused the abrogation of bleomycin-induced G2 arrest, suggesting that pyncnidione strongly bound to target(s) in Jurkat cells.

Effect of Pyncnidione on Cyclin Levels in Bleomycin-Treated Jurkat Cells When cells were arrested at the G2 phase, it was reported that cyclin D3 levels were moderately increased, and the levels of cyclins A and B1 were markedly increased^{28,29)}; therefore, the protein levels of CDK/cyclin complex were analyzed (Fig. 2). In bleomycin-treated cells, the protein levels of cyclins D3, A and B1 were increased (Fig. 2, lane 3), indicating that Jurkat cells were arrested at the G2 phase, while in combination with pyncnidione (lane 4), the levels of cyclins D3, A and B1 decreased to similar levels to untreated control cells (lane 1), indicating that cells were not arrested at the G2 phase.

Synergistic Potentiation of Bleomycin Cytotoxicity to Jurkat Cells in Combination with Pyncnidione It is well known that G2 checkpoint abrogators increase the cytotoxicity of DNA-damaging anticancer agents.^{16,18–20)} Pyncnidione alone (Fig. 3A) or bleomycin alone (Fig. 3B) inhibited cell proliferation in a dose-dependent manner with an IC₅₀ value of 510 nM or 100 μ g/mL, respectively. Note that 100 nM pyncnidione did not show marked cytotoxicity (Figs. 1B, 3A). In the presence of 100 nM pyncnidione, bleomycin more strongly inhibited cell proliferation with an IC₅₀ value of 11 μ g/mL (Fig. 3B), indicating that pyncnidione potentiated the cytotoxicity of bleomycin to Jurkat cells 9.2-fold. Interestingly, the potentiating effect of pyncnidione was attenuated in the presence of MG132 (Fig. 3B), a specific proteasome inhibitor,³⁰⁾ indicating that the proteasome pathway was involved in the pyncnidione-dependent potentiation of bleomycin activity.

To investigate the existence of a possible synergistic effect, isobologram analysis of the two-drug interaction was performed.³¹⁾ As shown in Fig. 3C, data points of bleomycin (30 μ g/mL) and pyncnidione (0–255 nM) were plotted below the solid line, indicating that the interaction between pyncnidione and bleomycin is synergistic. Next, we investigated the mechanism of action of this synergistic effect.

Effect of Pyncnidione on Phosphorylation Levels of Histone H2A.X DNA damage caused by bleomycin, ionizing radiation or radiomimetic agents results in rapid phosphoryla-

Fig. 4. Effect of Pycnidione on the Signal Transducers of DNA Damage-Induced G2 Arrest

(A) Jurkat cells ($1-2 \times 10^5$ cells/mL) were incubated with pycnidione alone (a) or in combination with bleomycin (b) for 24h. After incubation, the cells were fixed and stained with anti-phospho-Histone H2A.X (Ser139) antibody and analyzed by flow cytometer. DNA content (propidium iodide) was plotted on the x-axis and phosphorylated H2A.X (FITC) was plotted on the y-axis. (B) Cell lysates were prepared from Jurkat cells ($2.5-5.0 \times 10^5$ cells/mL) cultured in the absence or presence of pycnidione and/or with bleomycin for 24h. The relative phosphorylation levels of CDK1 in bleomycin-treated cells were calculated, normalized to the levels of total CDK1, and only bleomycin-treated cells were set as a control (1.00). (C) Cell lysates were prepared from Jurkat cells ($2.5-5.0 \times 10^5$ cells/mL) cultured in the absence (lanes 1—4) or presence (lanes 5—8) of pycnidione and/or with bleomycin for 24h.

thermore, in combination with pycnidione, the levels further increased in a pycnidione-dependent manner. These results indicated that the DNA damage signal driven by bleomycin was not disrupted by pycnidione.

Decrease in Phosphorylation Levels of CDK1 by Pycnidione The entry of eukaryotic cells into mitosis is regulated by CDK1 kinase activation, a process controlled at several steps, including cyclin binding and phosphorylation of CDK1 at Tyr15.⁷⁾ Therefore, the phosphorylation levels of CDK1 were examined (Fig. 4B). The levels were very low in the absence or presence of pycnidione without bleomycin, indicating that pycnidione itself had no effect on phosphorylation. When the cells were treated with bleomycin, the phosphorylation levels significantly increased to arrest cells at the G2 phase.⁹⁾ Under these conditions, the combination with pycnidione led to a decrease in phosphorylated CDK1 levels driven by bleomycin. When the ratio (pCDK1 vs. CDK1) in bleomycin-treated cells was defined as 1.00 (lane 5), the ratio in cells in combination with pycnidione (lane 8) was estimated to be 0.10, the same as in untreated cells. These results indicate that cells abrogated G2 arrest to progress the cell cycle. Taken together, pycnidione blocks signal transduction between ATM and CDK1, leading to the abrogation of bleomycin-induced G2 arrest.

Effect of Pycnidione on the Levels of Proteins Involved in G2 Arrest Signal Transduction Induced by Bleomycin At the G2 checkpoint, a DNA damage signal from ATM is transmitted through the downstream proteins, Chk1, Chk2, Cdc25C, Wee1 and CDK1^{4–6,8–10)}; therefore, the levels of these proteins were examined (Fig. 4C). Compared with those in bleomycin-treated cells (lane 5), the levels of Chk1 and Chk2 in cells treated with bleomycin in combination with pycnidione (216 nM) markedly fell to 16% and 13% (lane 8), respectively. Thus, the down-regulation of Chk1 and Chk2 levels reduced the phosphorylation of CDK1 (Fig. 4B), leading to abrogation of bleomycin-induced G2 arrest by pycnidione. As described above (Fig. 2), the levels of proteins involved in other phases of the cell cycle, such as CDK6, CDK2 and CDK1, were not affected by treatment with pycnidione alone (lane 2) or with bleomycin and pycnidione (lane 4). These results indicate that pycnidione abrogated bleomycin-induced G2 arrest by decreasing the Chk1 and Chk2 levels.

Human Cancer Cell Line Panel Experiment To search for the molecular target of pycnidione, the compound was evaluated in a human cancer cell line panel (COMPARE analysis),²⁶⁾ in which values giving 50% inhibition (IC₅₀) of pycnidione against the growth of 39 human cancer cell lines (mean graphs) were measured (Fig. 5A), and the pattern was compared with those of typical anticancer agents with different mechanisms of action (Fig. 5B). Fingerprint pattern analysis revealed that the top drug in the rank order of correlation coefficients is ellipticine ($r=0.656$), a topoisomerase II inhibitor.³⁴⁾ And the second drug is carmofur ($r=0.544$), of which the mode of action is still controversial in terms of the blocking of DNA phosphorylation through inhibition of thymidylate synthetase or the distortion of gene expression after incorporation of fluoropyrimidines into RNA.³⁵⁾ However, the correlation coefficient between carmofur and pycnidione was too low to be relied on.

Importance of Tropolone Backbone in Pycnidione for G2 Arrest Abrogative Activity Epolone A (Fig. 6A) with a structure in which one tropolone moiety in pycnidione

was replaced for a benzene moiety had almost no effect on bleomycin-induced G2 arrest up to 0.96 μM (data not shown), suggesting that the presence of two tropolones is important for activity. To confirm this point, pycnidione was treated with diazomethane³⁶⁾ to produce four *O*-dimethyl derivatives D1 to D4 (Fig. 6B). As shown in Fig. 6C, none of the derivatives exhibited marked abrogation activity of bleomycin-induced G2 arrest. These results indicate that the presence of two tropolone backbones in pycnidione was important for the abrogation activity of bleomycin-induced G2 arrest.

DISCUSSION

Pycnidione abrogated bleomycin-induced G2 arrest in Jurkat cells and synergically potentiated the cytotoxicity of bleomycin (Fig. 2), leading to the death of Jurkat cells (Fig. 3) and several cancer cells (Fig. 5). These results indicate that pycnidione has ideal characteristics as a G2 checkpoint abrogator; therefore, we elucidated the mechanism of action in this study. First, we investigated the levels of six molecules, ATM/ATR, CDK1, Chk1, Chk2, Cdc25C and Wee1, involved in bleomycin-induced G2 arrest signal transduction as potential targets of pycnidione. In bleomycin-treated cells, the protein levels of Chk2 were decreased, while in pycnidione-treated cells, the levels of Chk1 were decreased (Fig. 4C). Furthermore, the concomitant treatment of bleomycin and pycnidione severely reduced the levels of Chk1 and Chk2. Therefore, we concluded that the abrogation of bleomycin-induced G2 arrest by pycnidione was caused by decrease in the levels of Chk1 and Chk2.

From the results of COMPARE analysis, we investigated whether pycnidione inhibits topoisomerases. Interestingly, pycnidione was found to inhibit topoisomerase II activity with an IC₅₀ value of 19 μM (data not shown), and also to weakly inhibit topoisomerase I activity (data not shown). Therefore, topoisomerases might be potential targets of pycnidione action although two problems were mentioned; 1) the IC₅₀ values between cell-based assay and enzyme assay had large differences, 2) unfortunately, ellipticine potentiated the cytotoxicity of bleomycin to Jurkat cells only 3.1 times (data not shown). It was reported that ellipticine has the different activity of directly binding to DNA³⁴⁾; however, pycnidione has no activity to bind DNA because the DNA mobility was not shifted in the presence of pycnidione (data not shown). Therefore, it is plausible that a lack of direct binding activity to DNA may be important for topoisomerase II inhibition to show the abrogation activity of bleomycin-induced G2 arrest.

Topoisomerases play critical roles in DNA metabolism by controlling the topology of DNA.³⁷⁾ Several topoisomerase inhibitors have been reported; NSC 320846 and F11782, inhibitors of topoisomerases I and II, induced cell death^{33,38)} and H2A.X phosphorylation,³³⁾ and R16, which was an inhibitor of topoisomerase II³⁹⁾ and an inducer of genotoxic damage similar to bleomycin,⁴⁰⁾ reduced Chk1 levels.⁴¹⁾ As demonstrated, pycnidione also induced cell death (Fig. 3), increased the phosphorylation of H2A.X (Fig. 4), and decreased Chk1 and Chk2 in combination with bleomycin (Fig. 4). Therefore, the decrease of the levels of Chk1 and Chk2 could be caused by the inhibition of topoisomerases. Recently, Feng *et al.* reported that the inhibition of topoisomerase II by R16 induced proteasome-dependent degradation of Chk1 without significant

A

B

Fig. 5. Anti-cancer Activity of Pycnidione in a Panel of 39 Human Cancer Cell Lines
(A) Dose-response curves of pycnidione against growth of JFCR-39 cells. x-Axis shows concentration of pycnidione and y-axis shows percentage growth. (B) MG-MID: mean of log X values (X =IC₅₀, TGI, and LC₅₀). Delta: logarithm of the difference between the MG-MID and log X of the most sensitive cell line. Range: logarithm of the difference between the log X of the most resistant cell line and the log X of the most sensitive cell line.

A **B** **C**

Fig. 6. Effect of *O*-Dimethylpyncnidione Derivatives on Topoisomerases

(A) Structure of epolone A. (B) Structures of *O*-dimethylpyncnidiones. (C) Jurkat cells (5.0×10^5 cells/mL) were incubated with *O*-dimethylpyncnidione in the presence of $30 \mu\text{g/mL}$ bleomycin. Distribution in the cell cycle was analyzed by flow cytometry 24 h after incubation. (D) DNA cleavage activity of human topoisomerase II(a) and I(b) was measured in the presence of the indicated concentration of *O*-dimethylpyncnidione using supercoiled plasmid DNA.

alteration of the Chk1 mRNA level.³⁹⁾ Furthermore, genotoxic stress induced by topoisomerase I inhibitor camptothecin triggered Chk1 destruction *via* the ubiquitin-proteasome pathway,⁴¹⁾ and MG132, a proteasome inhibitor, attenuated the potentiating effect of pyncnidione (Fig. 3B). These results strongly suggest that pyncnidione promotes the proteasome-mediated degradation of Chk1 and Chk2 proteins by inhibiting the topoisomerases.

G2 checkpoint is expected as a potential target to develop a new type of anticancer agent,⁴²⁾ and many abrogators of G2 checkpoint have been reported: MK-1775 (Wee1 inhibitor),⁴³⁾ STI571 (c-Abl kinase inhibitor),⁴⁴⁾ leptomycin B (Crm1 inhibitor),⁴⁵⁾ isogranulatimide (Chk1 inhibitors),⁴⁶⁾ UCN-01 (Chk1 inhibitors),¹¹⁾ Gö6976 (protein kinase C inhibitor),¹⁵⁾ debromohymenialdisine (Chk1 and Chk2 dual inhibitor),⁴⁷⁾ L-744,832 (farnesyl transferase inhibitor),⁴⁸⁾ and caffeine (ATM/ATR inhibitor)¹⁶⁾; however, no compounds, which decreased the levels

of Chk1 and Chk2, have been reported. Therefore, pyncnidione is expected to be developed as a new type of agent in combination with clinically used DNA-damaging anti-cancer drugs.

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