*Highlighted paper selected by Editor-in-chief***Fungal Malformins Inhibit Bleomycin-Induced G2 Checkpoint in Jurkat Cells**Keiichi HAGIMORI,^a Takashi FUKUDA,^a Yoko HASEGAWA,^a Satoshi ŌMURA,^{*,a,b} and Hiroshi TOMODA^{*,c}^aKitasato Institute for Life Sciences & Graduate School of Infection Control Sciences, Kitasato University; ^cSchool of Pharmacy, Kitasato University; 5–9–1 Shirokane, Minato-ku, Tokyo 108–8641, Japan; and ^bKitasato Institute; 5–9–1 Shirokane, Minato-ku, Tokyo 108–8642, Japan.

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A DNA-damaging agent, bleomycin, arrests the cell cycle at the G2 phase of Jurkat cells, which are defective in the G1 checkpoint, while microtubule-disrupting colchicine arrests it at M phase. Fungal cyclopeptides, malformin A1 and malformin C, were found to abrogate bleomycin-induced G2 arrest (IC₅₀: 0.48 μ M and 0.9 nM, respectively), resulting in a drastic decrease in cells in G2 phase and increase in cells in subG1 phase. On the other hand, malformins showed little effect on the colchicine-induced M phase arrest in Jurkat cells (IC₅₀: 2.7 μ M and 24 nM, respectively). Malformin C (0.026 μ M) also abrogated bleomycin-induced G2 arrest in colon cancer-derived HCT-116 cells. These data strongly suggest that malformin C disrupted the cell cycle at the G2 checkpoint of cancer cells, leading to sensitization of the cancer cells to the anti-cancer reagent.

Key words malformin C; G2 checkpoint inhibitor; cell cycle; anti-cancer agent; bleomycin; fungal metabolite

Most conventional anti-cancer drugs used clinically kill not only cancer cells but also normal cells. Because these drugs are not specifically selective for cancer cells, patients suffer from adverse side effects due to the loss of healthy cells. Therefore, it is important to reduce such side effects caused by anti-cancer drugs.

DNA damage induces signal transduction pathways called checkpoints, which delay cell cycle progression and permit repair of DNA.¹⁾ Checkpoints arrest cells in G1 phase to prevent replication of damaged DNA and in G2 phase to prevent segregation of damaged chromosomes during mitosis. Normal human cells can repair DNA in the 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 like bleomycin, cisplatin and camptothecin, causing selective cell death of cancer cells due to their accumulation of mutation in G1 checkpoint genes. 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 ATM (ataxia telangiectasia-mutated) and ATR (A-T and rad3-related) members of the phosphoinositide kinase family.^{3,4)} A signal is then transmitted through the downstream protein kinases Chk1 and Chk2,^{3–8)} which are able to phosphorylate Cdc25C at Ser 216. This phosphorylation is thought to directly prevent Cdc25C from activating the Cdc2 kinase.⁹⁾ Chk1 and Chk2 can also phosphorylate and activate Wee1, a kinase that catalyzes Cdc2 inhibitory phosphorylation.^{10,11)} Several G2 checkpoint inhibitors have been reported to date. For example, UCN-01, a derivative of staurosporine, abrogates DNA damage-induced S and G2 arrest due to inhibition of Chk1 and perhaps Chk2.¹²⁾ 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 unwanted side effects in patients.^{13–15)} SB-218078¹⁶⁾ and Go6979¹⁷⁾ 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^{18,19)} and Wee1 kinase,²⁰⁾ 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 a part of 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.²¹⁾ On the basis of these findings, more potent analogs of this peptide have been developed as G2 checkpoint inhibitors.

Jurkat, a human T-cell leukemia-derived cell line, depends on the G2 checkpoint to repair DNA damage since it lacks functional *p53*.²¹⁾ We established a cell-based assay using Jurkat cells to screen culture broths of microorganisms for G2 checkpoint inhibitors.^{22,23)} During the course of the continuous screening program, two malformins, previously reported to cause malformation of the stems and petioles of bean plants and to be potentiators of fibrinolytic activity,^{24–26)} were isolated from the culture broth of strain *Aspergillus niger* FKI-2342 (Fig. 1). In this study, we describe the activity of malformins A1 and C as G2 checkpoint inhibitors.

MATERIALS AND METHODS

General Experimental Procedures *Aspergillus niger* FKI-2342 was isolated from a soil sample collected at Ooura Tensyudou, Nagasaki, Japan, and was used for production of malformins A1 and C.

Fig. 1. Structures of Malformins A1 and C

A human T-cell leukemia-derived cell line, Jurkat, was kindly provided by the Cell Resource Center for Biomedical Research, Tohoku University. Human colon adenocarcinoma cells (HCT-116) were kindly provided by CanBas Co., Ltd. (Numazu, Japan). Fetal bovine serum (FBS) was purchased from JRH Biosciences (Tokyo, Japan). Colchicine, trypsin-EDTA solution, propidium iodide, ribonuclease A and NP-40 were obtained from Sigma (St. Louis, MO, U.S.A.). Bleomycin was purchased from Nippon Kayaku Co., Ltd. (Tokyo, Japan). RPMI 1640 medium and McCoy's 5A medium were obtained from Invitrogen (Tokyo, Japan).

Production of Malformins A and C Malformins A1 and C were purified from the culture broth of *Aspergillus niger* FKI-2342. Briefly, the whole culture broth (3.7 l) was extracted with ethanol (3.7 l), concentrated by evaporation to remove the organic solvent, and re-extracted with ethyl acetate. The extract (1.66 g) was subjected to silica gel chromatography and purified by HPLC using an ODS column, giving pure malformins A1 (36.5 mg) and C (2.1 mg).

Cell Culture and Cell Cycle Analysis Jurkat cells were cultured in RPMI 1640 medium supplemented with 10% FBS at 37 °C in a humidified atmosphere of 5% CO₂. HCT-116 cells were cultured in McCoy's 5A medium supplemented with 10% FBS and 100 units/ml penicillin and 100 µg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO₂. The cell cycle status was analyzed according to the method described previously.^{23,24} Briefly, Jurkat cells (1.0×10^5 cells/200 µl) in a 96-well microplate were treated with malformins (0–25 µM) in the presence or absence of bleomycin (40 µM) or colchicine (0.25 µM) for 20 h at 37 °C. The cells were then suspended in 200 µl of 0.1% sodium citrate solution containing 50 µg/ml propidium iodide, 20 µg/ml ribonuclease A and 0.5% NP-40 (Krishan's solution). After HCT-116 cells (7.5×10^4 cells/500 µl) were incubated in a 24-well microplate for 24 h at 37 °C, they treated with malformins (0–25 µM) in the presence or absence of bleomycin (13 µM) or colchicine (0.25 µM) for 24 h at 37 °C. Then, the supernatant in each well was transferred to a test tube. Cells were washed with phosphate-buffered saline (PBS) and harvested by treatment with trypsin-EDTA followed by centrifugation at 1000 g for 5 min. The pellet was resuspended in 200 µl of Krishan's solution. The cell cycle status was determined by analysis of the DNA content using a FACSCalibur (Becton Dickinson, NJ, U.S.A.). Ten thousand cells were analyzed, and the distribution of cells in different phases (subG1, G1, S and G2/M) was calculated using the program ModiFit LT ver. 2.0 according to the manufacturer's protocol.

Immunoblotting Jurkat cells (5.0×10^5 cells/ml) were treated with malformin C (9.6 nM) in the presence or absence of bleomycin (40 µM) for 20 h at 37 °C. The cell suspension was centrifuged at 1000 g for 5 min. Then the cells were washed with PBS twice, suspended with 100 µl of lysis buffer (50 mM Tris-HCl buffer containing 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100, 1 µg/ml PMSF, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin A, 1 µg/ml Na₃VO₄, pH 7.4), and allowed to stand at 4 °C for 15 min. The cell lysates were centrifuged at 15000 g for 15 min to collect the supernatant, which were used for protein quantitation using BCA protein assay reagents (Pierce). Cell lysates (20 µg) were suspended with gel elec-

trophoresis sample buffer (125 mM Tris-HCl buffer containing 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.01% bromophenol blue, pH 6.8) and used for Western blot analysis of Cdc2 and phosphorylated Cdc2. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed under reducing conditions in a 12.5% acrylamide gel, and the proteins were electrotransferred from the gel to a polyvinylidene difluoride (PVDF) membrane (Millipore). The membrane was blocked in blocking buffer (20 mM Tris-HCl buffer containing 100 mM NaCl, 5% BSA, 0.1% Tween 20) at 37 °C for 1 h, and then was incubated with anti-Cdc2 antibody (Santa Cruz Biotechnology) (2 µg/ml) or anti-phosphorylated Cdc2 antibody (Cell Signaling) (0.2 µg/600 µl), and was incubated further with anti-rabbit peroxidase conjugated antibody (Amersham Bioscience Corp.) (0.2 µg/800 µl). The membrane was washed and the signals were detected using the enhanced chemiluminescence detection system (Amersham Bioscience Corp.) according to the manufacturer's instructions. Densitometric analysis and calculations were performed using Versadoc 5000 (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

RESULTS

Inhibition of Bleomycin-Induced G2 Arrest in Jurkat Cells by Malformin C

After Jurkat cells were cultured for 20 h, the cell cycle status was analyzed. The distribution of cells in subG1, G1, S and G2/M phases was 3.7%, 51%, 15% and 24%, respectively (Fig. 2a). When Jurkat cells were incubated with malformin C (1 nM), the distribution in subG1 (2.5%), G1 (51%), S (16%) and G2/M (25%) phases was almost unchanged (Fig. 2a). These data suggest that malformin C (1 nM) alone had no effect on the cell cycle of Jurkat cells. When Jurkat cells were treated with bleomycin (40 µM), the distribution in subG1 (19%), G1 (3.0%), S (5.2%) and G2/M (68%) phases was changed, resulting in cell cycle arrest at G2 phase (Fig. 2b). In cells treated with the combination of malformin C (1 nM) and bleomycin (40 µM), the cell cycle pattern was drastically changed. The distribution in subG1 was increased to 45%, indicating that malformin C induced cell death in DNA-injured Jurkat cells. On the other hand, when Jurkat cells were treated with colchicine (0.24 µM), the distribution in subG1 (35%), G1 (2.7%), S (2.2%) and G2/M (56%) phases was changed compared to that of untreated cells (Fig. 2c) and was almost the same as that of cells treated with the combination of colchicine plus malformin C (10 nM) (29%, 11.3%, 10.2% and 46%, respectively) (Fig. 2c). These data suggest that malformin C abrogated the DNA damage-induced G2 arrest in Jurkat cells and enhanced the cell death. The concentrations of the compounds required to decrease the cell population of G2/M in bleomycin- or colchicine-treated Jurkat cells by 50% (IC₅₀) was calculated, and the ratio of IC₅₀ (colchicine) vs. IC₅₀ (bleomycin) was obtained to evaluate the specificity of G2 checkpoint inhibition. As shown in Table 1, malformin C showed the highest specificity (ratio, 26.0) of G2 checkpoint inhibition. Malformin A1 and caffeine also caused G2 checkpoint inhibition, but they much less potently than malformin C.

Effect of Malformin C on Bleomycin-Treated HCT-116

The effect of malformin C was evaluated using a human colon adenocarcinoma cell line, HCT-116. The distribution

Fig. 2. Abrogation of Bleomycin-Induced G2 Arrest by Malformin C in Jurkat Cells

Jurkat cells treated with malformin C (0–10 nM) in the absence of other drugs (a) or in the presence of 40 μ M bleomycin (b) or 0.25 μ M colchicine (c). Distribution of cells in the cell cycle was analyzed by FACS after 20 h of incubation.

Table 1. Effect of Malformins and Caffeine on Bleomycin- or Colchicine-Treated Jurkat Cells

Compound	IC ₅₀ (μ M)		Ratio (colchicine/bleomycin)
	Bleomycin	Colchicine	
Caffeine	3900	11000	2.8
Malformin A1	0.48	2.7	5.6
Malformin C	0.0009	0.024	26

The concentration of a compound needed to decrease the cell population in G2/M to 50% of that in bleomycin- or colchicine-treated Jurkat cells was defined as IC₅₀. The specificity of the effect was calculated as the ratio of the IC₅₀ value of colchicine-treated cells vs. the IC₅₀ value of bleomycin-treated cells.

Fig. 3. Abrogation of Bleomycin-Induced G2 Arrest by Malformin C in HCT-116 Cells

HCT-116 cells treated with malformin C (0–38 nM) in the absence of other drugs (a) or in the presence of 13 μ M bleomycin (b) or 0.25 μ M colchicine (c). Distribution of cells in the cell cycle was analyzed by FACS after 24 h of incubation.

of HCT-116 cells in subG1, G1, S and G2/M phases without malformin C (control) was 7.2%, 47%, 18% and 24%, respectively (Fig. 3a). When HCT-116 cells were incubated with malformin C (38 nM), the distribution in subG1, G1, S and G2/M phases was almost the same as that in the control cells (Fig. 3a). When HCT-116 cells were treated with bleomycin (13 μ M), the distribution in subG1 (10%), G1 (7.3%), S (1.9%) and G2/M (69%) phases was changed, indicating that the cell cycle was arrested at G2 phase by bleomycin (Fig. 3b). In cells treated with malformin C (38 nM) plus bleomycin (13 μ M), the fraction of cells in G2 phase strongly decreased from 69 to 21% (Fig. 3b). On the

other hand, when Jurkat cells were treated with colchicine (0.25 μM), the distribution in subG1 (13%), G1 (3.3%), S (4.6%) and G2/M (72%) phases was changed (Fig. 3c). In cells treated with malformin C (38 nM) plus colchicines (0.25 μM), the cell cycle pattern was similar (24%, 4.7%, 6.5% and 59%, respectively) (Fig. 3c). IC_{50} (colchicine) and IC_{50} (bleomycin) were estimated as described above to evaluate the specificity. As shown in Table 2, malformin C caused specific G2 checkpoint inhibition (ratio, 13.0), indicating that malformin C abrogated the bleomycin-induced G2 arrest in HCT-116 cells.

Effect of Malformin C on the Phosphorylation of Cdc2

The events observed in Jurkat cells under the conditions described above were confirmed by comparison of the levels of Cdc2 and phosphorylated Cdc2 (pCdc2) (Fig. 4). When the ratio (pCdc2 vs. Cdc2) in the control cells was defined as 1, the ratio was estimated to be 0.8 in malformin C-treated cells (lane 3 in Fig. 4), indicating that malformin C alone had no cytotoxic effect on Jurkat cells. On the other hand, in Jurkat cells arrested in G2 phase by treatment with bleomycin

(40 μM), the pCdc2 level was increased, resulting in a ratio of 3 (lane 2 in Fig. 4). In Jurkat cells treated with a combination of malformin C (9.6 nM) plus bleomycin (40 μM), the pCdc2 level was significantly decreased, resulting in a ratio of 0.6 (lane 4 in Fig. 4).

DISCUSSION

As demonstrated in this study, malformins A1 and C inhibited the bleomycin-induced G2 phase arrest, leading to the death of Jurkat cells. They showed only a slight effect on the colchicine-induced M phase arrest, indicating that malformins A1 and C specifically abrogated the G2 checkpoint in the cell cycle. Furthermore, malformin C, the most potent G2 checkpoint inhibitor, was also effective against bleomycin-induced G2 arrest in adenocarcinoma HCT-116 cells. There were several potential target molecules of malformin C involved in the G2 checkpoint inhibition, namely, Chk1, Chk2, ATM, ATR, Wee1 and Cdc25C. First, we tested the level of Cdc2 and pCdc2, the final molecules involved in the G2 checkpoint. As shown in Fig. 4, we found that bleomycin increased the pCdc2 level in G2-arrested Jurkat cells, and that this increase was dramatically attenuated in Jurkat cells treated with a combination of bleomycin plus malformin C. Thus, we demonstrated the G2 checkpoint inhibition by malformin C at the protein level. Further studies are necessary to define the direct target of malformin C.

There are subtle structural differences between malformins A1 and C, which have Ile and Leu residues as components of the cyclic peptides, respectively (Fig. 1). These differences must account for our findings that malformin C is superior to malformin A1 regarding the potency and selectivity of G2 checkpoint inhibition. Various malformin derivatives should be synthesized for development as anticancer potentiators.

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Table 2. Effect of Malformins and Caffeine on Bleomycin- or Colchicine-Treated HCT-116 Cells

Compound	IC_{50} (μM)		Ratio (colchicine/bleomycin)
	Bleomycin	Colchicine	
Caffeine	3500	14000	4.0
Malformin A1	0.51	1.0	2.0
Malformin C	0.026	0.34	13

The concentration of a compound needed to decrease the cell population in G2/M to 50% of that in bleomycin- or colchicine-treated Jurkat cells was defined as IC_{50} . The specificity of the effect was calculated as the ratio of the IC_{50} value of colchicine-treated cells vs. the IC_{50} value of bleomycin-treated cells.

Fig. 4. Effect of Malformin C on Phosphorylated Cdc2 Level in Bleomycin-Treated or Untreated Jurkat Cells

Cell lysates were prepared from Jurkat cells (1×10^6 cells) cultured under the following conditions: No treatment (lane 1); 40 μM bleomycin (lane 2); 9.6 nM malformin C (lane 3); 40 μM bleomycin in combination with 9.6 nM malformin C (lane 4) for 20 h at 37 $^{\circ}\text{C}$. Relative phosphorylation was calculated as follows: The level of pCdc2 divided by that of Cdc2 in bleomycin-treated or untreated Jurkat cells.

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