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Inhibition of Cell-Cycle Progression in Human Promyelocytic Leukemia HL-60 Cells by MCS-C2, Novel Cyclin-Dependent Kinase Inhibitor

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Abstract To elucidate the action mechanism of MCS-C2, a novel analogue of toyocamycin and sangivamycin, its effect on the expression of cell cycle-related proteins in the human myelocytic leukemia cell line HL-60 was examined using Western blotting and a flow cytometric analysis. MCS-C2, a selective inhibitor of cyclin-dependent kinases, was found to inhibit cell growth in a time- and dose-dependent manner, and inhibits cell cycle progression by inducing the arrest at G1 and G2/M phases, in HL-60 cells. The flow cytometric analysis revealed an appreciable arrest of cells in the G2/M phase of the cell cycle after treatment with MCS-C2. The HL-60 cell population increased gradually from 13% at 0 h, to 28% at 12 h in the G2/M phase, after exposure to 2 μM MCS-C2. Furthermore, Western blot analysis demonstrated that MCS-C2 induced the cell cycle arrest at G1 phase through the inhibition of pRb phosphorylation. Hypophosphorylated pRb accumulated after treatment with 5 μM MCS-C2 for 12 h, whereas, the level of hyperphosphorylated pRb was reduced. Thus, treatment of the cell with MCS-C2 suppressed the hyperphosphorylated form of pRb with a commensurate increase in the hypophosphorylated form.

Key words: MCS-C2, toyocamycin, cell-cycle arrest, HL-60, cyclin-dependent kinase

Cyclin-dependent kinases (CDKs) play important roles in the signal transduction pathways that control the proliferation and differentiation of eukaryotic cells. The eukaryotic cell-cycle progression is regulated by the sequential activation and subsequent inactivation of a series of CDKs at different phases [18]. The activities of CDKs are positively regulated by cyclins and negatively regulated by cyclin-dependent kinase inhibitors (CKIs). A cyclin-CDK complex hyperphosphorylates the retinoblastoma protein (pRb), leading to its release from E2F [21]. The transcription factor E2F released then activates the genes responsible for cellular proliferation by progression through the G1 phase into the S phase. The impairment of a growth stimulatory signaling pathway has been shown to induce the expression of CKI, which binds to and subsequently inhibits cyclin-CDK activity. This then interferes with the hyperphosphorylation of pRb by keeping it in a hypophosphorylated form and bound to E2F [12], thereby blocking cell proliferation and inducing cell growth arrest. As such, inhibitors of the cell-cycle progression have the potential of being used as antiproliferation agents [16].

In the course of screening for a novel inhibitor of CDK2 and CDC2, we isolated toyocamycin from a culture broth of *Streptomyces* sp. LPL931 [20]. Toyocamycin was reported as an antibiotic in 1966 [2], and later shown to inhibit RNA synthesis in mammalian cells [23]. Toyocamycin was previously tested for cancer treatment, yet, unfortunately, its high toxicity proved to be a problem [24]. Therefore, many toyocamycin analogues have since been synthesized

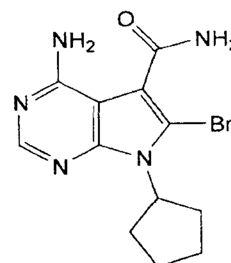

Fig. 1. Chemical structure of MCS-C2.

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to improve the selectivity profile and evaluated for their antitumor and antiviral activities [4, 5, 7, 13, 14, 22]. Although some toyocamycin analogues are potent inhibitors of many cancer cells, unfortunately, they are also toxic to normal human cells. Accordingly, in an attempt to search for a specific inhibitor that inhibits CDKs with minimum side effects on other Ser/Thr protein kinase activities, the current study was undertaken to synthesize an analogue of toyocamycin, MCS-C2 (Fig. 1), and its CDK inhibitory activity was evaluated by investigating the effect of MCS-C2 on the cell-cycle progression and regulatory molecules of HL-60 cells.

MATERIALS AND METHODS

Chemicals

Phosphate-buffered saline (PBS) and RPMI 1640 medium were purchased from GIBCO, Ltd. (Grand Island, NY, U.S.A.). The mouse monoclonal antibodies against pRb, E2F1, cyclinD1, cyclinA, cyclinB1, CDC2, CDK6, p16, p21, p27, and β-actin and the polyclonal antibody against CDK4 were all purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). The enhanced chemiluminescence (ECL) kit was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). The protein assay kit was from Bio-Rad Laboratories (Hercules, CA, U.S.A.). The kinase assay kits for PKA and PKC were purchased from Promega Corporation (Madison, WI, U.S.A.). All other materials were obtained from Sigma (St. Louis, MO, U.S.A.).

Cell Line and Cell Culture

The human myelocytic leukemia HL-60 cell line was obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). The HL-60 cells were cultured in the RPMI 1640 medium supplemented with 10% fetal bovine serum and 100 units/ml penicillin, at 37°C in a humidified atmosphere of 5% CO_2-air. The cell density in the culture did not exceed 5×10^5 cells/ml [10, 11, 15, 17].

Cell Viability Test

The HL-60 cells were seeded at a density of 5×10^3 cells/ml in a 96-well culture dish and treated with various concentrations (0.5-5 μM) of MCS-C2 at various incubation periods (12, 24, and 48 h). The viability was assessed by the conventional colorimetric dye reduction method, based on the reduction of MTT (Promega Corp.). The plate was read at 570 nm using an ELISA reader (Molecular Devices Corp., Sunnyvale, U.S.A.). All assays were performed in triplicate.

Kinase Activity Assay

For CDC2 kinase assay, HeLa cells were synchronized in the M-phase, washed with PBS, and resuspended in an extraction buffer (50 mM Tris-HCl/pH 7.4, 250 mM NaCl, 1 mM EDTA, 50 mM NaF, 1 mM DTT, 0.1% Triton X-100, one tablet of a protease inhibitor, and 0.5 mM PMSF). The resulting lysate was used as the enzyme source for CDC2. A kinase reaction mixture (25 μl), containing 2.5 μg of histone H_1 (1 μg/ml) as the substrate, 10 μg of crude CDC2 fraction from the HeLa cells, 2.5 μl of 10× kinase buffer (250 mM Tris-HCl/pH 7.4, 2 mM $MgCl_2$, 20 mM DTT, 50 mM β-glycerophosphate, and 1 mM Na_3VO_4), 1 μCi of [r-^{32}P] ATP (5 Ci/μmol), and 2.5 μl of 0.5 mM ATP, was incubated for 15 min at 30°C. After the incubation, the reaction was stopped by the addition of 12 μl of 7.5 M guanidine hydrochloride. Subsequently, the reaction mixture was centrifuged, and 15 μl aliquots of the supernatant were spotted onto Whatman P-81 phosphocellulose paper. After 10 min, the paper was washed 3 times with 1% phosphoric acid for 15 min, dried, and counted for radioactivity using a liquid scintillation counter (Wallac, Perkin Elmer, Boston, U.S.A.).

The PKC and PKA kinase assays were carried out using a SignaTECT Protein Kinase C assay kit and SignaTECT cAMP-dependent Protein Kinase assay kit (Promega), respectively, following the manufacturer's manuals. All assays were performed in triplicate.

Cell-Cycle Analysis

After the drug treatment, the cells (1×10^6 cells/ml) were washed twice with cold PBS and fixed in 70% ethanol. For the flow cytometric analysis, the cells were incubated with 0.1 mg/ml RNase A at 37°C for 30 min, stained with a solution containing 0.2 mg/ml propidium iodide for 1 h at 4°C, and then analyzed with a FACS Calibur flow cytometer (Becton Dickinson, San-Diego, CA, U.S.A.) using Cell Quest software.

Preparation of Total Cell Extracts and Immunoblot Analysis

The HL-60 cells were plated onto 60-mm dishes at a density of 2×10^5 cells/ml with or without MCS-C2 (5 μM, 0-12 h) and then harvested. To prepare the whole-cell extract, the cells were washed with PBS and suspended in a protein lysis buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 1 mM sodium orthovanadate, 100 μg/ml PMSF, and proteinase inhibitors). The protein content was determined with a Bio-Rad protein assay reagent using bovine serum albumin as the standard. The protein extracts (30-50 μg) were analyzed using 8-14% SDS-PAGE and transferred to a PVDF membrane (Millipore Corp., Bedford, MA, U.S.A.). The membranes were blocked with 5% (w/v) non-fat dry milk and then incubated with the indicated antibodies in TBS (10 mM Tris-HCl, 150 mM NaCl/pH 7.6) containing 0.1% Tween-20 with gentle shaking at 4°C for 2-12 h. The secondary antibody was a peroxidase-conjugated goat anti-

mouse antibody. The signals were detected using an ECL Western blotting kit.

Statistical Analysis

The data are reported as mean±standard deviation of three independent experiments, and were evaluated by Student's *t*-test. Values of $P<0.05$ were considered to be statistically significant.

RESULTS

Growth Inhibition by MCS-C2

To determine the MCS-C2-induced cell growth inhibition of a human cancer cell line, the effect of MCS-C2 on the viability of the human leukemia HL-60 cell line was assessed using an MTT assay. As shown in Fig. 2, MCS-C2 inhibited the growth of HL-60 cells, when treated with 0.5 to 5 mM of MCS-C2, in a time-dependent and dose-dependent manner (IC_{50}: 0.21 μM). Concentrations of 3 and 5 μM of MCS-C2 significantly inhibited the growth of HL-60 cells after 12 ($P<0.05$), 24, and 48 h ($P<0.01$), when compared to the control group.

Based on previous reports that toyocamycin and its analogues are highly toxic to mammalian cells due to its nonspecific inhibition of various protein kinases, such as PKC and PKA, selectivity of MCS-C2 to inhibit the cell-cycle regulator kinase, namely CDC2, was tested. As shown in Fig. 3, MCS-C2 consistently produced a dose-dependent decrease in CDC2 activity (IC_{50}: 0.38 μM). However, MCS-C2 showed no significant inhibition of

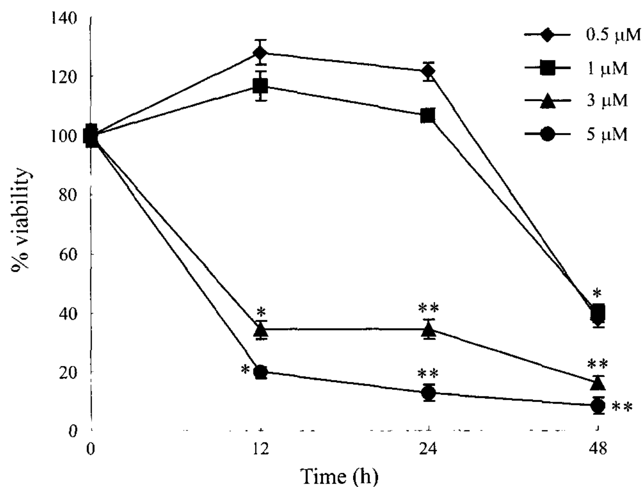

Fig. 2. Effect of MCS-C2 on growth of human leukemia HL-60 cells.

The cells were treated with various concentrations of MCS-C2 for 12, 24, and 48 h, as described in the text. The results represent the mean±SD of three independent experiments and a significant difference was established at $P<0.05$. *$P<0.05$, **$P<0.01$ compared with the control group (0.1% DMSO) for the indicated time.

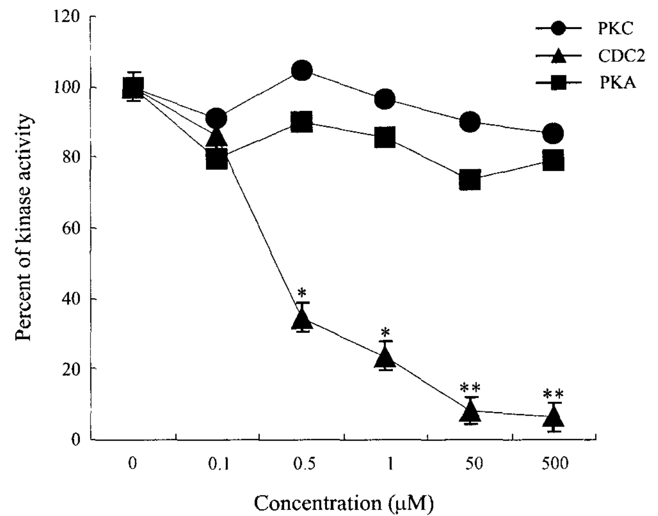

Fig. 3. Selective inhibitory effects of MCS-C2 on CDC2 activity. IC_{50}=0.38 μM (CDC2).

PKC and PKA even at a high concentration (500 μM). Accordingly, these results suggested that MCS-C2 could selectively inhibit CDC2 kinase at 0.38 mM without affecting other protein kinases.

Effect of MCS-C2 on Cell Cycle Progression

To elucidate the mechanism of the MCS-C2-induced inhibition of human leukemia cell growth, the cell-cycle progression was analyzed using flow cytometry. The HL-60 cells were cultured with MCS-C2 at the indicated time, washed, stained with propidium iodide (PI), and then the cell cycle was analyzed. As shown in Fig. 4, the flow cytometric analysis revealed an appreciable arrest of the cells in the G2/M phase of the cell cycle after treatment with 2 μM MCS-C2. The HL-60 cell population increased gradually from 13% at 0 h to 28% after 12 h in the G2/M phase, after the exposure to 2 μM of MCS-C2. Furthermore, an eventual progression to apoptosis was observed after 12 h (18%). However, the percentage of S phase cells was not significantly affected. Accordingly, treatment of HL-60 cells with MCS-C2 induced G2/M phase arrest of the cell cycle progression.

Effect of MCS-C2 on pRb

Since pRb plays a role in controlling cell-cycle progression, the expression level and phosphorylation state of pRb in the MCS-C2-treated HL-60 cells were examined using a Western blot analysis. As shown in Fig. 5, hypophosphorylated pRb (with faster migration on the gel) accumulated after treatment with 5 μM MCS-C2 for 12 h, whereas the amount of hyperphosphorylated pRb (with slower migration on the gel) was reduced. Thus, treatment of the cells with MCS-C2 was found to suppress the amount of hyperphosphorylated form of pRb with commensurate

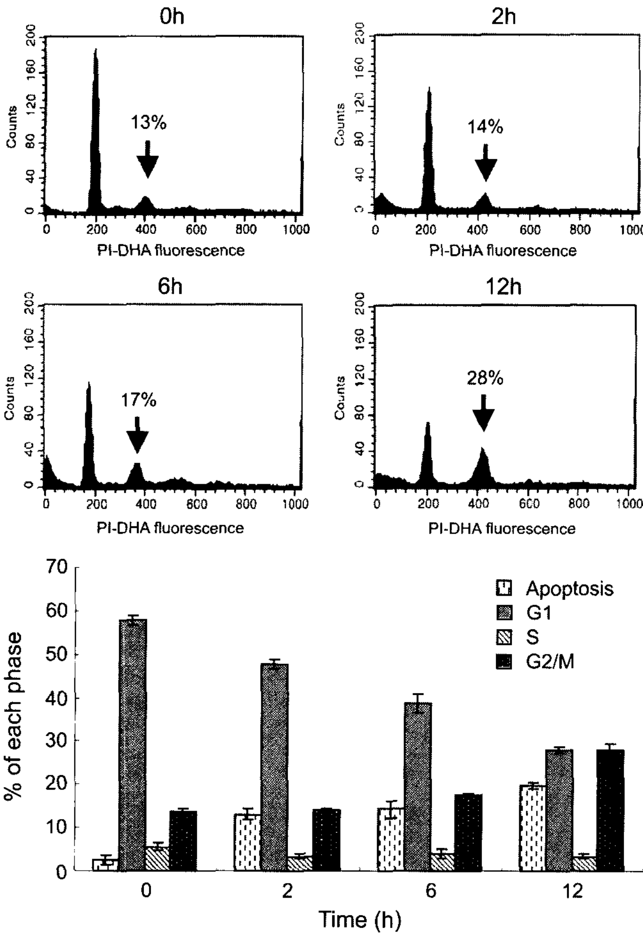

Fig. 4. Quantification of apoptosis by flow cytometry.
HL-60 cells were treated with 5 μM MCS-C2 for the indicated times. The cells were then stained with propidium iodide (PI) and the nuclei analyzed for their DNA content by flow cytometry using Cell Quest software. A total of 10,000 nuclei were analyzed from each sample.

increase in the hypophosphorylated form. The expression levels of E2F-1 were also reduced. β-Actin served as the internal control.

Effect of MCS-C2 on CDKs, Cyclins, CKIs

Recent studies have shown that cyclins and CDK complexes have important regulatory roles during cell cycle progression. CDK4 and CDK6 phosphorylate pRb in the mid-late phase in the G1 phase. Thus, the expression levels of CDK4, CDK6, and cyclins were examined. As shown in Fig. 6, the levels of CDK4 and CDK6 decreased 8 h after the treatment with 5 μM MCS-C2, and there was almost no signal for the CDK4 and CDK6 proteins after 12 h treatment with 5 μM MCS-C2. The level of the cyclinD1 protein was gradually reduced in a time-dependent manner after the treatment with 5 μM MCS-C2.

To determine the MCS-C2-inducing G2/M phase arrest, the expression levels of CDC2, cyclinB1, and cyclinA

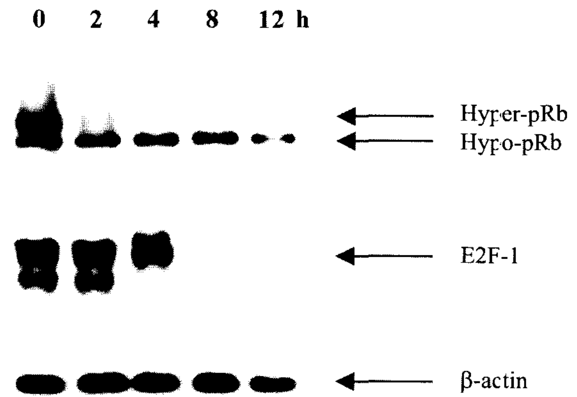

Fig. 5. Effect of MCS-C2 on pRb phosphorylation and E2F-1 release.
Total cell lysates from HL-60 cells treated with 5 μM MCS-C2 were electrophoretically separated on an 8% polyacrylamide gel and immunoblotted with an antibody against pRb, E2F-1, and β-actin, which served as the internal control.

were assessed. As expected, the expression levels were significantly reduced 8 and 12 h after the treatment with 5 μM MCS-C2 (Fig. 6).

Since cyclin-CDK complexes are in turn regulated by CKIs, which generally inhibit cell-cycle progression, the expression levels of p16, p21, and p27 involved in the G1 phase arrest were examined. Interestingly, Western blot analysis revealed that the expression levels of CKIs showed no change after the treatment with 5 μM MCS-C2 (data not shown).

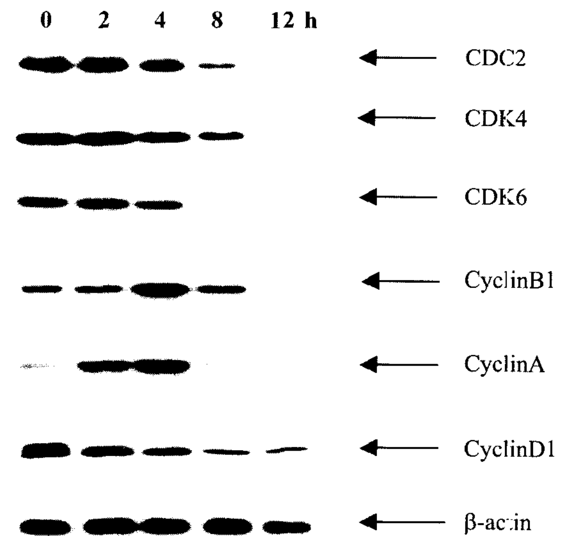

Fig. 6. Effect of MCS-C2 on protein expression of CDKs and cyclins.
Total cell lysates from HL-60 cells treated with 5 μM MCS-C2 were analyzed using an 8–10% polyacrylamide gel for CDKs and cyclins and subsequently immunoblotted with an antibody against CDKs, cyclins, and β-actin, which served as the internal control.

DISCUSSION

To elucidate the mechanism behind the antitumor activity of MCS-C2, the effects of MCS-C2 on viability [8] and cell-cycle progression of HL-60 cells were investigated. As shown in the current study, the inhibitory effect of MCS-C2 on the viability of HL-60 cells was due to the induction of both apoptosis and cell-cycle arrest. The result also demonstrated that MCS-C2 inhibited the growth of HL-60 cells in a time-dependent and dose-dependent manner. Furthermore, Western blot analysis revealed that MCS-C2 induced the cell-cycle arrest in the G1 phase through the inhibition of pRb phosphorylation and in the G2/M phase through direct inhibition of CDC2. Although no cell-cycle arrest by MCS-C2 in the G1 phase was detected by flow cytometric analysis, which identified G2/M phase arrest, a Western blot analysis clearly showed G1 phase arrest. The reason of why the G1 phase arrest by MCS-C2 was not detected should be clarified by further studies.

pRb phosphorylation plays a central role in controlling cellular proliferation by regulating the G1/S transition of the cell cycle. Upon commitment of a cell to continuous proliferation, pRb is phosphorylated by the activity of G1 CDKs, such as CDK4/6 and CDK2, thereby liberating the factors which govern the S phase entrance. Among the released proteins, the E2F family of transcription factors has a central position [12]. E2F induces the gene expression necessary for DNA synthesis and also contributes to the regulation of the cyclinD1 and E genes. These genes constitute the main regulators of the CDKs that phosphorylate pRb by forming an autoregulatory loop between pRb and the G1 cyclins [3]. The current results demonstrated that the treatment of HL-60 cells with MCS-C2 resulted in an accumulation of hypophosphorylated pRb. In addition, MCS-C2 treatment was found to reduce cyclinD1.

CKIs also negatively regulate progression through the cell cycle and restriction points. There are two families of structurally distinct CKIs: the CIP/KIP family, such as p21 and p27, which inhibit a broad range of CDKs by selectively binding and inhibiting fully associated cyclin-CDK complex, and the INK family, such as p16, which binds specifically to CDK4 and/or CDK6 and inhibits a complex formation with cyclinD [6, 9, 19]. According to the current data, MCS-C2 treatment induced no change in the expression levels of CKIs, including p16, p21, and p27. Since the regulation of pRb by phosphorylation is indeed quite complex [1], it would appear that MCS-C2 induced cell-cycle arrest in the G1 phase and affected G1 regulatory factors, thereby inhibiting the phosphorylation of pRb in HL-60 cells. However, the detailed mechanism needs to be further studied.

In conclusion, MCS-C2 was found to inhibit the growth of human leukemia HL-60 cells through induction of G1 cell-cycle arrest by inhibiting pRb phosphorylation, and G2/M phase arrest by directly inhibiting CDC2 and cyclin B1.

Acknowledgment

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