The plant alkaloid cryptolepine induces p21$^{WAF1/CIP1}$ and cell cycle arrest in a human osteosarcoma cell line

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Abstract. We previously established a bioassay method to screen for compounds that activate the promoter activity of p21$^{WAF1/CIP1}$, a potent inhibitor of cyclin-dependent kinases, in a p53-independent manner. As an activator of p21$^{WAF1/CIP1}$ promoter activity, we isolated cryptolepine (CLP: 5-methyl indolo (2,3b)-quiniine), an indoloquinoline alkaloid, from the traditional Ayurvedic medicinal plant *Sida cordifolia*. We show here that CLP induces the expression of p21$^{WAF1/CIP1}$ with growth arrest in p53-mutated human osteosarcoma MG63 cells. Four micromolar of CLP completely inhibited the growth of MG63 cells and caused G_2/M-phase arrest. CLP up-regulated the expression of p21$^{WAF1/CIP1}$ at both mRNA and protein levels in a dose-dependent manner. Using several mutant p21$^{WAF1/CIP1}$ promoter constructs, we found that the CLP-responsive element is an Sp1 site at -82 relative to the transcription start site of the p21$^{WAF1/CIP1}$ promoter. These findings suggest that CLP arrests the growth of MG63 cells by activating the p21$^{WAF1/CIP1}$ promoter through the specific Sp1 site in a p53-independent manner. In addition, CLP-mediated cell cycle arrest was reduced by the knockout of the p21$^{WAF1/CIP1}$ gene in human colon cancer HCT116 cells, suggesting that the cell cycle arrest by CLP was at least partially mediated through the induction of p21$^{WAF1/CIP1}$ expression. Although we need further study of chemotherapeutic effect *in vivo*, these results raise the possibility that CLP might be a suitable chemotherapeutic agent for treatment of osteosarcoma.

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Abbreviations: CLP, cryptolepine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EMSA, electrophoretic mobility shift assay

Key words: osteosarcoma, cryptolepine, p21$^{WAF1/CIP1}$, Sp1, cell cycle arrest

Introduction

Osteosarcoma is the most common form of primary malignant bone tumor that mainly occurs in juvenile patients. Although the development of effective adjuvant regimen of chemotherapy has substantially improved the prognosis of patients (1-4), >20% of patients still die as a result of tumor metastasis (5-10). One of the most serious causes of therapeutic failure is the resistance of the tumor cells to chemotherapeutic agents (11,12). To overcome the drug resistance, new anti-tumor agents or chemicals and new anti-tumor therapeutic approaches need to be developed.

p21$^{WAF1/CIP1}$ is a member of the cyclin-dependent kinase inhibitors and induces G_1- and G_2/M-phase cell cycle arrest (13-16). p21$^{WAF1/CIP1}$ induces differentiation of both normal and transformed cells and suppresses the growth of malignant cells, *in vitro* and *in vivo* (17,18). In osteosarcoma cells, p53 is frequently inactivated (19). Inactivated p53 confers resistance to chemotherapeutic agents (20,21). The transcription of p21$^{WAF1/CIP1}$ is directly activated by wild-type p53 protein (22), suggesting that inactivation of p53 decreased the expression of p21$^{WAF1/CIP1}$ in osteosarcoma cells. However, p21$^{WAF1/CIP1}$ is rarely mutated in human cancer cells. Therefore, p21$^{WAF1/CIP1}$ is an attractive molecular target to suppress cell growth even in p53-mutated cancer cells and p21$^{WAF1/CIP1}$-inducing agents by a p53-independent pathway might be effective for chemotherapy of osteosarcoma with poor prognosis.

Recently, we established a bioassay method using p53-negative human osteosarcoma MG63 cells to screen for compounds that activate the p21$^{WAF1/CIP1}$ promoter activity in a p53-independent manner (23). As an activator of p21$^{WAF1/CIP1}$ promoter activity, we isolated cryptolepine (CLP: 5-methyl indolo (2,3b)-quiniine), an indoloquinoline alkaloid, from the traditional Ayurvedic medicinal plant *Sida cordifolia*. CLP was first isolated from *Cryptolepis sanguinolenta* (24) that is native to Central and West

Africa, and also isolated from *Sida cordifolia* (25) in 1980. Anti-malaria activity of CLP was reported previously (26). In addition, it has been reported that CLP can suppress the growth of cancer cells *in vitro* by inhibiting cell cycle progression and causing apoptosis in human HL-60 leukemia (27) and B16 melanoma cells (28,29). However, the molecular mechanisms for the effect in the growth arrest of cancer cells by CLP have not yet been studied.

Using a series of mutant p21$^{WAF1/CIP1}$ promoter constructs, we found that the Sp1 site at -82 relative to the transcription start site is involved in the activation of the p21$^{WAF1/CIP1}$ promoter in MG63 cells by CLP.

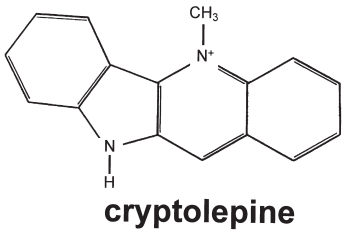
In this study, we show for the first time that CLP is a potent inducer of p21$^{WAF1/CIP1}$ in human osteosarcoma cells. CLP activated the p21$^{WAF1/CIP1}$ promotor through the Sp1 site, indicating that p53 is not required for the transcriptional activation of p21$^{WAF1/CIP1}$ by CLP. This result raises a possibility that CLP might be a promising candidate for molecular-targeting chemotherapy against p53-mutant malignant tumors.

Materials and methods

Luciferase assay for screening p21$^{WAF1/CIP1}$ promoter activators. The luciferase assay was performed using a luciferase assay system (E1501, Promega Corporation, Madison, WI). The suspension of MG63 cells (5x10^4 cells/well), stably transfected with the human wild-type p21$^{WAF1/CIP1}$ promoter luciferase fusion plasmid, WWP-luc, were incubated in a 12-well plate for 24 h. Samples were added as 5 μl of EtOH solution, and then the cells were further incubated for 24 h. The cells were washed twice with PBS (-) solution. After the addition of 100 μl of 1x lysis buffer to each well, the plate was shaken at room temperature for 15 min, then centrifuged at 2,000 x g at 4˚C. Supernatant (10 μl) was removed to a 96-well plate (96F untreated white microwell SH) for luminescence measurements. Luciferase assay substrate (50 μl) was added to each well to allow light production. The light intensity was measured using a Micro Lumat Plus LB96V (Berthold Technologies, Bad Wildbad, Germany). The activation of the p21$^{WAF1/CIP1}$ promoter was evaluated by the relative luciferase activity of the sample compared with that of control (cells treated with EtOH).

Isolation and identification of cryptolepine. CLP was isolated from the traditional Ayurvedic medicinal plant *Sida cordifolia* (purchased from a local market in Colombo, Sri Lanka in 2001). Briefly, the dried plants were coarsely cut into pieces and soaked in methanol overnight. The resulting methanol extract was subjected to solvent partition ($CHCl_3$/aq HCl) to give an alkaloid portion. The active alkaloids were separated by high-performance liquid chromatography (HPLC, MeOH: H_2O: TFA) to obtain an active compound. The active compound was identified as CLP by comparison of the mass and NMR data with the authentic compound (Fig. 1). CLP was dissolved in DMSO for all experiments.

Cell culture. Human osteosarcoma MG63 cells and human colon adenocarcinoma wild-type HCT116 cells and p21$^{WAF1/CIP1}$-null HCT116 cells, provided from Dr Vogelstein (The Johns Hopkins University, Baltimore), were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and incubated at 37˚C in a humidified atmosphere of 5% CO_2.



cryptolepine

Figure 1. Structures of cryptolepine.

Cell growth. For the cell growth study, MG63 cells were seeded at a density of 1x10^4 cells in a 12-well plate. Twenty-four hours after the seeding, CLP was added at various concentrations. From 24 to 72 h after the treatment, the number of viable cells was counted by a trypan blue dye exclusion test. The data are presented as the means ± SD of at least three independent experiments.

Cell viability. The effect of CLP on the viability of HCT116 cells was determined by WST-8 assay. Wild-type HCT116 cells and p21$^{WAF1/CIP1}$-null HCT116 cells were seeded at a density of 5x10^3 cells in a 96-well plate. Twenty-four hours after the seeding, CLP was added at various concentrations. After incubation for 48 h, WST-8 solution was added to each well and incubated 4 h. The absorbance was recorded on a microplate reader at the wavelength of 540 nm. The effect of CLP was assessed as percent cell viability where DMSO-treated cells were taken as 100% viable. The data is presented as the means ± SD of at least three independent experiments.

Analysis of cell cycle progression. Unsynchronized cells were exposed to CLP for 24 h and harvested from culture dishes. After washing with PBS, the cells were suspended in PBS containing 0.1% Triton X-100, treated with RNase A, and the nuclei were stained with propidium iodide (PI). DNA content was measured using a FACSCalibur flow cytometer with CellQuest software (Becton-Dickinson, Franklin Lakes, NJ). For all assays, 10,000 events were counted. The ModFit LT V2.0 software package (Verity Software, Topsham, ME) was used to analyse the data.

RNA isolation and real-time quantitative RT-PCR. Total RNA was isolated from MG63 cells treated with various concentrations of CLP using Sepasol-RNA I (Nakalai Tesque Inc., Kyoto, Japan) according to the manufacturer's instructions. Total RNA (10 μg) was reverse transcribed to cDNA in a 20 μl reaction volume, with Superscript II Reverse transcriptase (Invitrogen Corporation, Carlsbad, CA), using oligo $(dT)_{12-18}$ primers. The reaction mixture was incubated at 42˚C for 50 min, then at 70˚C for 15 min to stop the reaction. An equivalent volume (1 μl) of cDNA solution was used for the quantification of specific cDNAs by real-time quantitative RT-PCR. The primer sequences used were as

follows: for the p21$^{WAF1/CIP1}$ gene (318 bp), 5'-GCGGATTA GGGCTTCCTCTT-3' and 5'-GGCAGACCAGCATGACA GATT-3', for GAPDH (181 bp), 5'-CAACTATTTCGGTT GTTGC-3' and 5'-GCCAGTGGACTCCACGAC-3' (Greiner Japan, Tokyo, Japan). Quantitative real-time RT-PCR was carried out using an Applied Biosystems 7500 Sequence Detector (Applied Biosystems, Foster, CA). The expression level of p21$^{WAF1/CIP1}$ was normalized to the level of GAPDH mRNA of the same sample. The data were analyzed using Student's t-test. Differences were considered to be statistically significant from the controls at $p<0.05$.

Western blot analysis. Protein extraction, nuclear extraction, and Western blot analysis was performed as previously described (23), using rabbit polyclonal anti-p21$^{WAF1/CIP1}$ antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), anti-Sp1 antibody (1:200; Santa Cruz Biotechnology), anti-Sp3 antibody (1:200; Santa Cruz Biotechnology) and mouse monoclonal anti-GAPDH (1:1,000) antibody (Immunotech, Marseille, France). Enhanced chemiluminescence (GE Science, Piscataway, NJ) was used for detection.

Plasmids. The full-length human p21$^{WAF1/CIP1}$ promoter-luciferase reporter construct pWWP, a series of mutant p21$^{WAF1/CIP1}$ promoter constructs including pWP124, pWP101, pWP101-mtSp1-3, pWP101-mtSp1-4, and pWP101-mtSp1-5/6, as well as the luciferase-reporter plasmid Sp1-luc and the mutant Sp1-luc were generated as described previously (23,30). pWP124 contains all six Sp1 consensus binding sites between -124 and -61 of the p21$^{WAF1/CIP1}$ promoter, and pWP101 contains four Sp1 sites termed Sp1-3, Sp1-4, and Sp1-5/6 between -101 and -61 of the p21$^{WAF1/CIP1}$ promoter.

DNA transient transfection and luciferase assay. MG63 cells ($3x10^4$ cells) were incubated in a 12-well plate, and 1.0 μg per well of reporter plasmid DNA was transfected using a CellPhect transfection kit (GE Science). Twenty-four hours after transfection, the cells were treated with medium containing CLP, and 24 h after the start of treatment the cells were collected for luciferase assay. Luciferase assay was performed as described previously (15), and the activity was determined as luminescence units normalized for the amount of protein in the cell lysates. PGVB2, which is an empty vector, was used as a control for the lucifearse assay. All the luciferase assays were carried out in triplicate. The data were analyzed using Student's t-test. Differences were considered to be statistically significant from the controls at $p<0.05$.

Electrophoretic mobility shift assay. Annealed oligonucleotides containing the sequence between -87 and -72 relative to the transcription start site of the p21$^{WAF1/CIP1}$ promoter (5'-AGCTCGGGTCCCGCCTCCTT-3' and 5'-TC GAAAGGAGGCGGGACCCG-3') were labeled with [α-^{32}P]-dCTP and used as a probe, and termed wt Sp1. The mutant oligonucleotides with the Sp1 site mutated (5'-AGCTCG GGTCAAAACTCCTT-3' and 5'-TCGAAAGGAGTTTT GACCCG-3') were used as competitors, and termed mt Sp1. Nuclear extracts were prepared from MG63 cells treated with 4 μM CLP or DMSO for 24 h, and nuclear extracts (7 μg) were incubated in reaction mixture and ^{32}P-end-labelled probe, as described previously (15). For competition assays, a 100-fold excess of unlabeled wt Sp1 or mt Sp1 was mixed with the reaction mixtures prior to the addition of the probe. The reaction mixtures were incubated at room temperature for 15 min, and further incubated for 20 min in the presence or absence of specific polyclonal antibodies against Sp1 (Santa Cruz Biotechnology) or Sp3 (Santa Cruz Biotechnology). The protein-DNA complex was resolved on a 5% non-denaturing polyacrylamide gel with 0.5x TBE buffer. The gel was then dried and exposed to X-ray film.

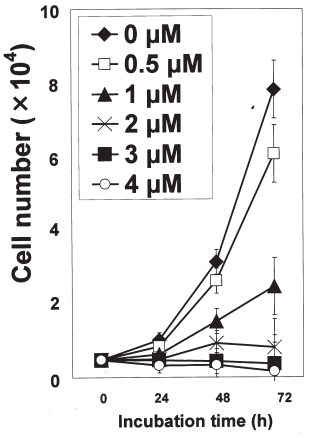


Figure 2. Effect of CLP on the growth of MG63. Twenty-four hours after seeding of MG63 cells, CLP at 0.5 (□), 1 (▲), 2 (x), 3 (■), or 4 (○) μM was added, and the cell number was compared with control culture with equivalent DMSO (◆) by counting the cells with trypan-blue dye exclusion test. The values shown are means (bars, SD) (n=3).

Results

CLP inhibits the growth of human osteosarcoma MG63 cells. We investigated the effects of CLP on the growth of human osteosarcoma MG63 cells. Fig. 2 shows the growth of MG63 cells in the presence of various concentrations of CLP. A dose-dependent inhibition of the cell growth was observed at concentrations of ≥0.5 μM. Seventy-two hours after the addition of CLP, the growth of cells was inhibited to 77.6, 31.6, 10.3, 4.7, and 2.1% of the control level by 0.5, 1, 2, 3 and 4 μM CLP, respectively.

CLP arrests MG63 cells at the G_2/M-phase in the cell cycle progression. To elucidate the effect of CLP on the cell cycle progression of MG63 cells, the DNA content of nuclei of MG63 cells was measured by flow cytometric analysis. As shown in Fig. 3A, FACS analysis revealed that a 24-h expose to CLP increased the population of G_2/M phase cells in a dose dependent manner. Cells at the G_2/M phase increased from 20.1% in medium alone to 24.8, 28.3, 41.0 and 68.9% by 1, 2, 3 and 4 μM CLP, respectively (Fig. 3B).

CLP up-regulates p21$^{WAF1/CIP1}$ expression in MG63 cells. To investigate whether p21$^{WAF1/CIP1}$ is involved in CLP-induced cell cycle arrest in MG63 cells, we examined the expression of p21$^{WAF1/CIP1}$ protein and mRNA after CLP treatment. As shown in Fig. 4A, we found that CLP increased p21$^{WAF1/CIP1}$

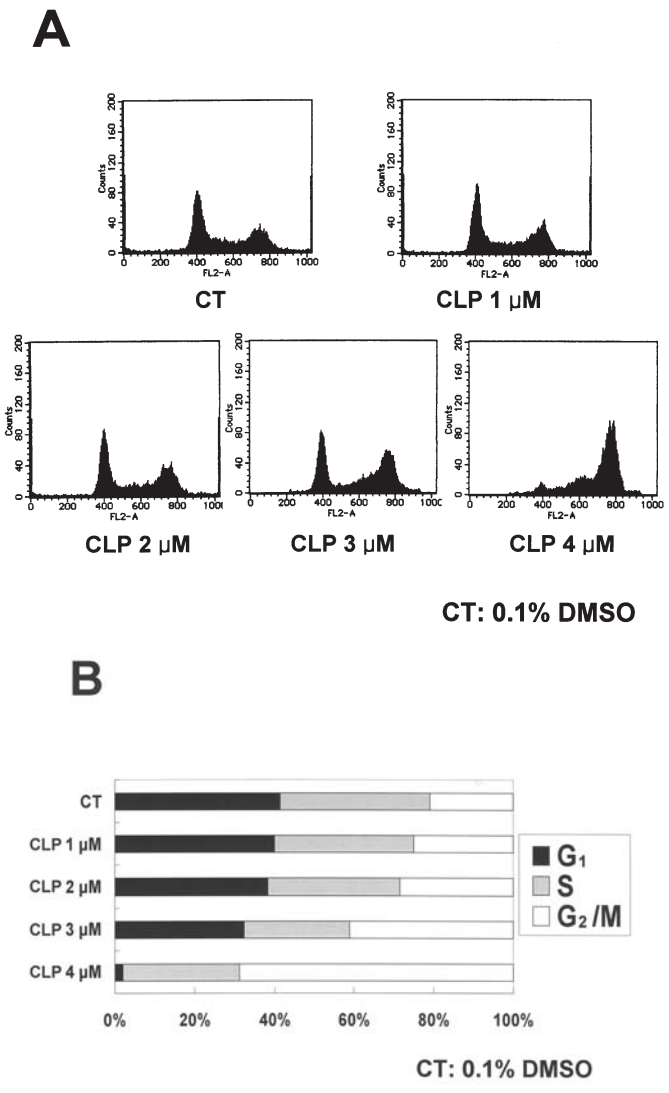


Figure 3. The effect of CLP on the cell cycle progression in MG63 cells. (A) MG63 cells were treated with CLP at the indicated concentrations for 24 h. The DNA content of propidium iodide-stained nuclei was analysed by FACSCalibur flow cytometry as described in Materials and methods. The experiments were repeatedly performed to confirm the results. (B) The percentage of cells in phases G_1 (black), S (gray), and G_2/M (white).

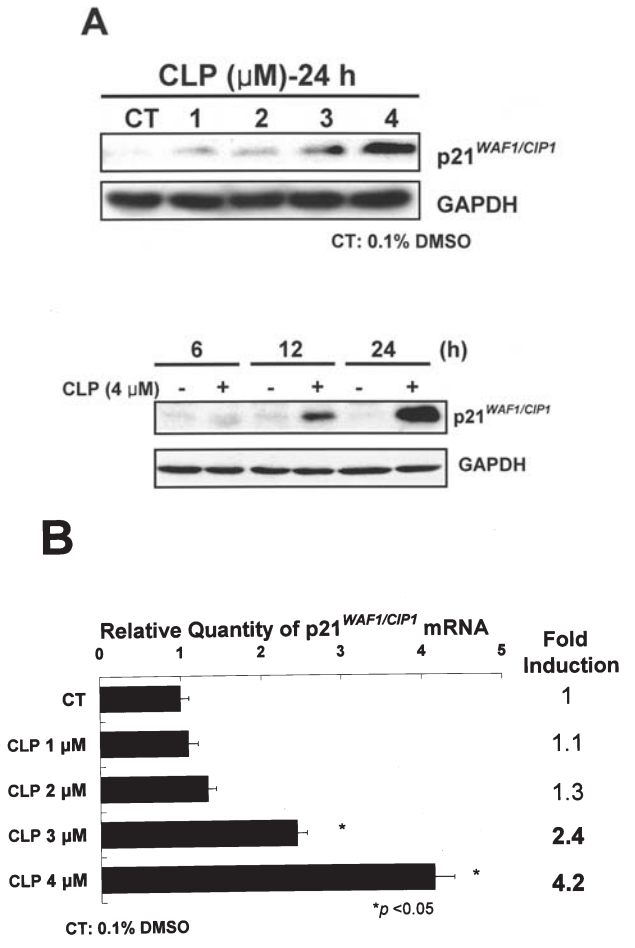


Figure 4. Up-regulation of p21$^{WAF1/CIP1}$ expression by CLP in MG63 cells. (A) CLP up-regulated p21$^{WAF1/CIP1}$ protein expression. MG63 cells were treated with CLP at the indicated concentrations for 24 h or with 4 μM CLP for the indicated periods. Western blotting was then performed as described in Materials and methods. GAPDH was used to ensure equal gel loading. (B) CLP up-regulated p21$^{WAF1/CIP1}$ mRNA expression. MG63 cells were treated with CLP at the indicated concentrations for 24 h. Real-time quantitative RT-PCR was performed as described in Materials and methods. The expression level of p21$^{WAF1/CIP1}$ was normalized by the level of GAPDH mRNA of the same sample. Data are shown as means (bars, SD) (n=3). *p<0.05.

protein expression in a dose-dependent manner using Western blotting. In addition, we demonstrated that CLP also induced p21$^{WAF1/CIP1}$ mRNA expression in a dose-dependent manner using real-time quantitative RT-PCR (Fig. 4B). The data showed that CLP induced both p21$^{WAF1/CIP1}$ protein and mRNA expression through a p53-independent pathway because p53 is inactivated in MG63 cells.

CLP stimulates p21$^{WAF1/CIP1}$ promoter activity. Next, we investigated whether CLP can stimulate activity of the promoter of the p21$^{WAF1/CIP1}$ gene using transient transfection of a p21$^{WAF1/CIP1}$ promoter-luciferase reporter plasmid, pWWP, or an empty vector PGVB2. As shown in Fig. 5A, CLP increased p21$^{WAF1/CIP1}$ promoter activity in a dose-dependent manner, indicating that CLP increases p21$^{WAF1/CIP1}$ expression through the activation of the p21$^{WAF1/CIP1}$ promoter in MG63 cells.

Analysis of the CLP-responsive elements in the p21$^{WAF1/CIP1}$ promoter. Furthermore, we tried to determine which regions of the p21$^{WAF1/CIP1}$ promoter are responsive to CLP activation. As shown in Fig. 5B, deletion up to -101 bp relative to the transcription start site did not reduce the response to CLP. We also previously generated a series of mutants of pWP101 having mutations in the each binding site for transcription factor Sp1 (23). We termed them pWP101-mtSp1-3, pWP101-mtSp1-4, and pWP101-mtSp1-5/6, respectively. These constructs were transiently transfected into MG63 cells, and their luciferase activities were assayed in the absence or presence of 4 μM CLP. As shown in Fig. 5C, the activation by CLP in pWP101-mtSp1-3 decreased to 1.3-fold from 4.3-fold activation in wild-type pWP101. The activation by CLP in pWP101-mtSp1-4 was slightly lower than wild-type pWP101 at 2.5-fold (Fig. 5C). The basal activity of pWP101-mtSp1-5/6 was reduced to background levels, and the activation by CLP was completely abolished (Fig. 5C). These results indicate that the Sp1-3 site is the main CLP-

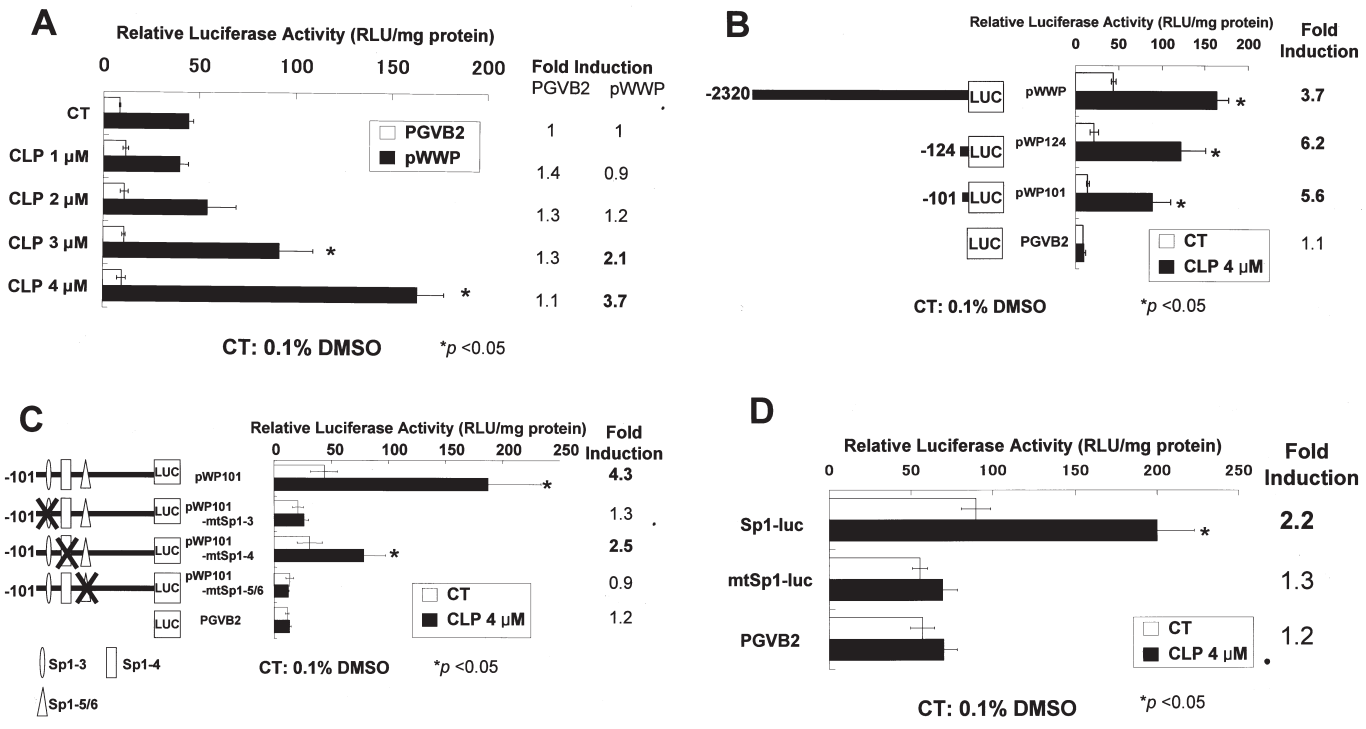


Figure 5. Activation of p21$^{WAF1/CIP1}$ promoter avtivity by CLP in MG63 cells. (A) CLP stimulated p21$^{WAF1/CIP1}$ promoter activity. MG63 cells were transiently transfected with the pWWP reporter plasmid, and luciferase activity was measured after incubation with CLP at the indicated concentrations for 24 h. Each of the raw light units (RLU) from the cell lysates were standardized to the concentrations of protein. Each fold induction by CLP was calculated relative to that of DMSO and indicated on the right. Data are shown as means (bars, SD) (n=3). *p<0.05. (B) Deletion analysis of the p21$^{WAF1/CIP1}$ promoter in MG63 cells was performed. The plasmids pWP124 and pWP101, shown on the left, were transiently transfected into MG63 cells, and luciferase activity was analysed 24 h after treatment with or without 4 μM CLP. Relative luciferase activity is shown as raw light units (RLU) per 1 mg of protein. Each fold induction by CLP was calculated and indicated on the right. Data are shown as means (bars, SD) (n=3). *p<0.05. (C) Mutation analysis of the p21$^{WAF1/CIP1}$ promoter in MG63 cells was performed. Three different mutants of pWP101, pWP101-mtSp1-3, pWP101-mtSp1-4, and pWP101-mtSp1-5/6, shown on the left, were transiently transfected into MG63 cells, and CLP-induced luciferase activity was analysed. Relative luciferase activity is shown as raw light units (RLU) per 1 mg of protein. Each fold induction by CLP was also calculated and indicated on the right. Data are shown as means (bars, SD) (n=3). *p<0.05. (D) The activation of the promoter activity of Sp1-luc in MG63 cells was analysed. MG63 cells were transiently transfected with either the wild-type Sp1-luc reporter plasmid or the mutant Sp1-luc reporter plasmid, and luciferase activity was analysed after 24-h treatment with or without 4 μM CLP. Each fold induction by CLP was also calculated and indicated on the right. Data are shown as means (bars, SD) (n=3). *p<0.05.

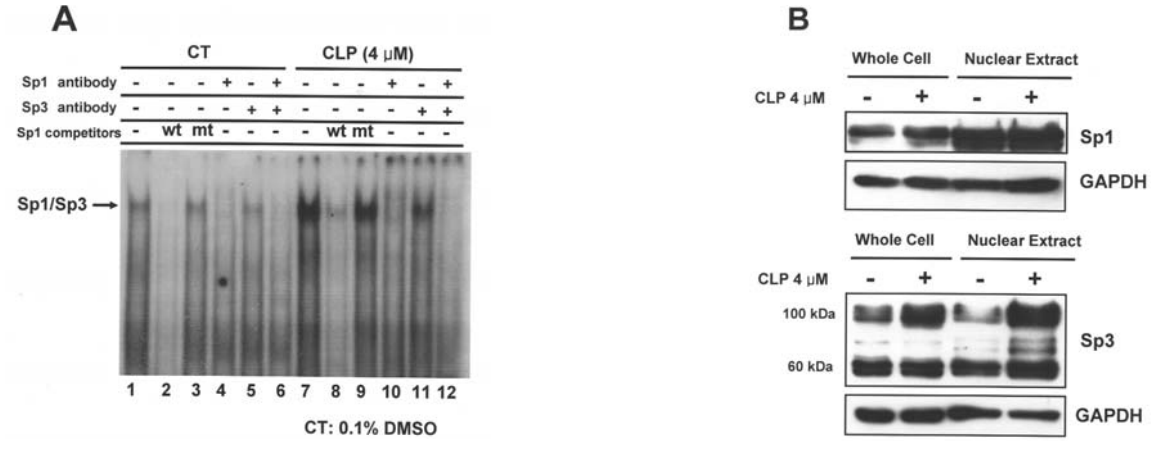


Figure 6. Sp1 and Sp3 can interact with the main CLP responsive element. (A) EMSA was carried out with nuclear extracts prepared from CLP-treated (lanes 7-12) or untreated (lanes 1-6) MG63 cells. The labeled oligo-nucleotide containing the sequence of the main CLP-responsive element between -87 and -72 from the transcription site, which includes the Sp1-3 site of the p21$^{WAF1/CIP1}$ promoter, was used as a probe. An excess of unlabeled wild-type Sp1 (wt, lanes 2 and 8) or mutant Sp1 (mt, lanes 3 and 9) oligonucleotide was added as competitor. Anti-Sp1 antibody (lanes 4 and 10), anti-Sp3 antibody (lanes 5 and 11), or both anti-Sp1 and anti-Sp3 antibodies (lanes 6 and 12) were used as indicated. The complex containing Sp1 and Sp3 was indicated by an arrow on the left. (B) MG63 cells were treated with or without 4 μM CLP for 24 h. Western blotting was then performed as described in Materials and methods. GAPDH was used to ensure equal gel loading.

responsive element and the Sp1-4 site is also weakly CLP-responsive. To confirm that Sp1 elements are indeed activated by CLP, the reporter plasmid Sp1-luc containing three consensus Sp1 binding sites or mtSp1-luc containing mutant

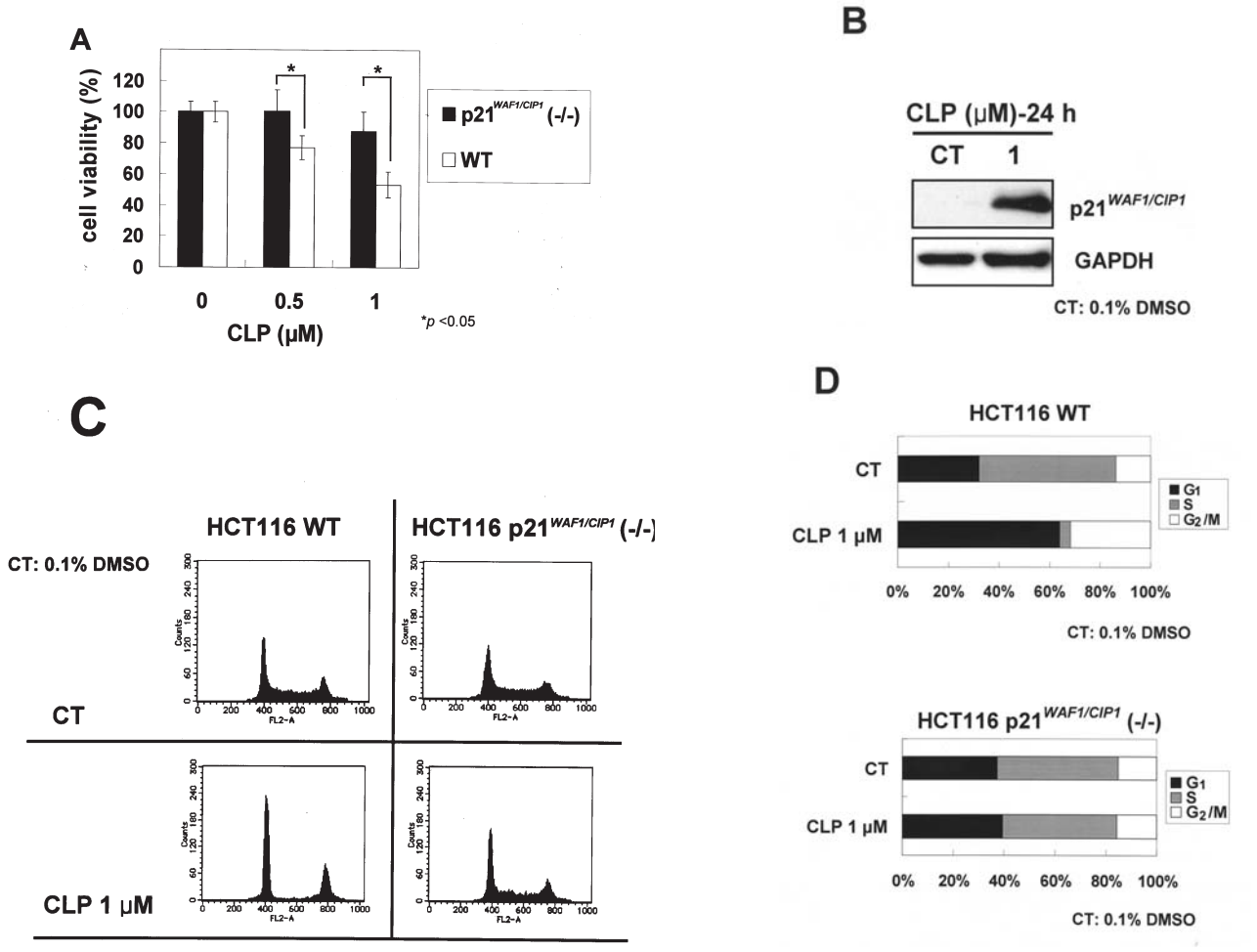


Figure 7. Knockout of p21$^{WAF1/CIP1}$ reduced CLP-mediated cell cycle arrest in human colon cancer HCT116 cells. (A) Wild-type and p21$^{WAF1/CIP1}$ deleted HCT116 cells were treated with CLP for 48 h at the indicated concentrations. Cellular viability was assessed by WST-8 assay. Data are shown as means (bars, SD) (n=3). (B) CLP up-regulated p21$^{WAF1/CIP1}$ protein expression in wild-type HCT116 cells. Wild-type HCT116 cells were treated with or without 1 μM CLPs for 24 h. Western blotting was then performed as described in Materials and methods. GAPDH was used to ensure equal gel loading. (C) Wild-type and p21$^{WAF1/CIP1}$ deleted HCT116 cells were treated with or without 1 μM CLP s for 24 h. The DNA content of propidium iodide-stained nuclei was analysed by FACSCalibur flow cytometry as described in Materials and methods. The experiments were repeatedly performed to confirm the results. D) The percentage of cells in phases G_1 (black), S (gray), and G_2/M (white).

Sp1 binding sites were transfected into MG63 cells. As shown in Fig. 5D, CLP activated the Sp1-luc, whereas mtSp1-luc was not activated.

Identification of proteins interacting with the main CLP responsive element. To determine whether Sp1 can interact with the Sp1-3 site, the main CLP-responsive element of p21$^{WAF1/CIP1}$ promoter, an electrophoretic mobility shift assay (EMSA) was performed using an oligonucleotide containing the wild-type Sp1-3 site, between -87 and -72 from the transcription start site. Nuclear extracts were purified from either CLP (4 μM) treated or untreated MG63 cells. As shown in Fig. 6A, a DNA-protein binding complex was detected, which was competed away by an excess of unlabeled wild-type Sp1 oligonucleotides but not by an excess of unlabeled oligonucleotides with a mutated Sp1 site. In addition, EMSA was performed with the nuclear extracts preincubated with Sp1- or Sp3-antibody. The retarded band disappeared in the presence of the Sp1-antibody. Furthermore, the band decreased in the presence of the Sp3-antibody (Fig. 6A). The results demonstrate that Sp1 and Sp3 bind to the wild-type Sp1-3 site. Moreover, CLP treatment enhanced the intensity of the Sp1/Sp3 band (Fig. 6A). However, CLP did not induce Sp1 protein expression in either the whole cell or nuclear extracts, though CLP induced Sp3 protein expression both in the whole cell and nuclear extracts (Fig. 6B).

p21$^{WAF1/CIP1}$ up-regulation contributes to the effect of CLP-induced cell cycle arrest in HCT116 cells. We tested whether or not the up-regulation of p21$^{WAF1/CIP1}$ expression was significant on CLP-induced cell cycle arrest in malignant tumor cells, using wild-type and p21$^{WAF1/CIP1}$ deleted human colon cancer HCT116 cells. We compared the anti-proliferative effects of CLP on wild-type and p21$^{WAF1/CIP1}$-null HCT116 cells. As shown in Fig. 7A, CLP (1 μM) treatment of wild-type HCT116 cells resulted in 47% decrease in cell viability. However, on p21$^{WAF1/CIP1}$-null HCT116 cells, CLP (1 μM) treatment resulted in 12% decrease in cell viability. In wild-type HCT116 cells, we showed that CLP induced p21$^{WAF1/CIP1}$ protein expression (Fig. 7B) and G_1 phase cell

cycle arrest (Fig. 7C and D). However, the knockout of p21$^{WAF1/CIP1}$ expression prevented CLP-induced cell cycle arrest (Fig. 7C and D). These results suggest that the up-regulation of p21$^{WAF1/CIP1}$ expression accounts at least in part for the CLP-induced cell cycle arrest in human malignant tumor cells.

Discussion

To improve the prognosis of osteosarcoma, new strategies are necessary. To screen new agents for chemotherapy against osteosarcoma, we used promoter screening of p21$^{WAF1/CIP1}$ to find a stimulator of p21$^{WAF1/CIP1}$ promoter activity in a p53-independent manner, inducing cell growth arrest in human osteosarcoma cells with inactivated p53. In the present study, we isolated CLP from the traditional Ayurvedic medicinal plant *Sida cordifolia* as a stimulator of p21$^{WAF1/CIP1}$ promoter activity, and we have shown that treatment of MG63 cells with CLP induces p21$^{WAF1/CIP1}$ mRNA and protein expression, resulting in G_2/M phase arrest of the cell cycle progression in a p53-independent manner because functionally-inactivated mutations of the p53 gene exist in MG63 cells. Recent studies reported that conventional anti-osteosarcoma agents such as doxorubicin, cisplatin, and etoposide have anti-tumor effects mainly in a p53-dependent manner (20). Therefore, the p53-independent pathway of p21$^{WAF1/CIP1}$ induction by CLP may be effective for the chemotherapy of osteosarcoma with resistance to conventional agents due to inactivated p53.

In addition, a series of mutation analyses of the p21$^{WAF1/CIP1}$ promoter have revealed that the main CLP-responsive element is the Sp1 site between -82 and -77 relative to the transcription start site (the Sp1-3 site in this report) (Fig. 5). EMSA using MG63 cells has shown that Sp1 and Sp3 can specifically interact with this main CLP-responsive element (Fig. 6). Furthermore, the intensity pattern of the bands increased in the presence of CLP (Fig. 6). In this study, CLP induced Sp3 protein expression, indicating that CLP can increase the interaction of Sp3 with the CLP-responsive element of the p21$^{WAF1/CIP1}$ promoter. However, CLP could not increase the expression of Sp1 protein, raising the possibility that Sp1 modification, such as phosphorylation, or other factors may be involved in the activation of the p21$^{WAF1/CIP1}$ promoter in response to CLP.

Several studies have reported the p53-independent induction of p21$^{WAF1/CIP1}$ expression (23,30-33). Recently, using promoter screening of p21$^{WAF1/CIP1}$, we also isolated aaptamine from a marine sponge as a stimulator of p21$^{WAF1/CIP1}$ promoter activity (34). Interestingly, this promoter analysis of the p53-independent pathway has also reported that the main responsive element of other p21$^{WAF1/CIP1}$-inducing agents, such as transforming growth factor ß (TGF-ß), histone deacetylase inhibitors (HDACIs), and aaptamine, included the same Sp1 site between -82 and -77 relative to the transcription start site (the Sp1-3 site) (31-34). However, the activation of the p21$^{WAF1/CIP1}$ promoter by TGF-ß and HDACIs was not caused by increasing the binding of Sp1 and Sp3 (31-33). This study indicated that the activation of the p21$^{WAF1/CIP1}$ promotor by CLP is due to increasing the binding of Sp1 and Sp3.

Moreover, using p21$^{WAF1/CIP1}$-null HCT116 cells, we demonstrated that the knockout of p21$^{WAF1/CIP1}$ expression reduced the antiproliferative effect of CLP and the induction of G_1 phase cell cycle arrest by CLP (Fig. 7). This finding indicates that the up-regulation of p21$^{WAF1/CIP1}$ expression by CLP is, at least in part, a mechanism for the induction of cell cycle arrest.

Whereas CLP-induced cell cycle arrest and apoptosis were previously reported, the mechanisms have not been elucidated. In this study, we demonstrated for the first time that CLP induces cell cycle arrest through p21$^{WAF1/CIP1}$ expression, indicating that CLP might be an attractive compound for molecular-targeting chemotherapy or chemoprevension.

In conclusion, we show that CLP induces growth arrest in MG63 cells through the p53-independent activation of p21$^{WAF1/CIP1}$, and the activator is mediated through the specific Sp1 site in the promoter region. These results raise a possibility that treatment with CLP is promising for the chemotherapy of osteosarcoma. Furthermore, the method of screening new candidates, using the promoter activity of p21$^{WAF1/CIP1}$ in a p53-independent manner, might be good tool to find potent compounds for molecular-targeting chemotherapy or chemoprevention against malignant tumors.

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