

p53-Independent Induction of G₁ Arrest and p21^{WAF1/CIP1} Expression by Ascofuranone, an Isoprenoid Antibiotic, through Downregulation of c-Myc

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Abstract

Ascofuranone has been shown to have antitumor activity, but the precise molecular mechanism by which it inhibits the proliferation of cancer cells remains unclear. Here, we study the effects of ascofuranone on cell cycle progression in human cancer cells and find that ascofuranone induces G₁ arrest without cytotoxicity with upregulation of p53 and p21^{WAF1/CIP1} while downregulating c-Myc and G₁ cyclins. Chromatin immunoprecipitation assay and RNA interference studies with cells deficient in p53 and p21 show that ascofuranone induces p21^{WAF1/CIP1} expression and subsequent G₁ arrest through the release of p21^{WAF1/CIP1} promoter from c-Myc-mediated transcriptional repression, independent of p53. Ascofuranone-induced p21^{WAF1/CIP1} associates with CDK2 and prevents CDK2-cyclin E complex formation, leading to the inactivation of E2F transcriptional activity. These results suggest that ascofuranone upregulates p21^{WAF1/CIP1} through p53-independent suppression of c-Myc expression, leading to cytostatic G₁ arrest. Thus, ascofuranone represents a unique natural antitumor compound that targets c-Myc independent of p53. *Mol Cancer Ther*; 9(7); 2102–13. ©2010 AACR.

Introduction

The detailed mechanisms of cell cycle regulatory systems have been studied in depth over the past two decades. The somatic cell cycle is divided into four distinct phases: DNA synthesis (S), mitosis (M), a gap between M and S phase (G₁), and a gap between S and M phase (G₂). In addition to these phases, there is a nondividing, quiescent state known as G₀ phase (1). Cell cycle transitions between phases involve multiple checkpoints, and the cell cycle can be arrested at the G₁ or G₂ checkpoints through the assessment of cell size, extracellular growth signals, and genomic integrity. Cell cycle progression in eukaryotic cells is highly ordered and tightly regulated by the activity of the CDK-cyclin complexes (2, 3). In the G₁ phase of the cell cycle, the CDK4/6-cyclin D and CDK2-cyclin E complexes mediate the phosphorylation of pRb. Upon phosphorylation, pRb releases and activates several proteins involved in cell cycle transition including the E2F family of transcription factors at the G₁-S transition phase (4, 5).

Moreover, cell cycle progression is controlled by several positive and negative regulators such as CDK-cyclin complexes and cyclin-dependent kinase inhibitors (CDKI; refs. 6–8). CDK-cyclin complexes are positive regulators that induce cell cycle progression, whereas two types of CDKIs, the INK4 family and the CIP/KIP family, are important negative regulators that stop cell cycle progression in response to appropriate regulatory signals. The INK family is composed of four members: p16^{INK4A}, p15^{INK4B}, p18^{INK4C}, and p19^{INK4D} (9). These proteins inhibit the kinase activity of CDK4/6-cyclin D complexes by binding to CDK4 and CDK6, preventing their association with D-type cyclins (cyclin D1, D2, and D3; ref. 10). The CIP/KIP family is composed of the three members: p21^{WAF1/CIP1}, p27^{KIP1}, and p57^{KIP2} (11). These proteins hinder the kinase activity of CDK2-cyclin E complexes by forming heterotrimeric complexes with CDK2, preventing their association with cyclin E (12).

Ascofuranone (Fig. 1A) is an isoprenoid antibiotic that was originally isolated as a hypolipidemic substance from a culture broth of a phytopathogenic fungus, *Ascochyta viciae* (13). Although ascochlorin-related compounds were originally reported to be antiviral antibiotics (14), they produce a variety of physiologic effects including hypolipidemic activity (15), suppression of hypertension (16), amelioration of type I and II diabetes (17), immunomodulation (18), and antitumor activity (19, 20). Ascochlorin and ascofuranone, one of its derivatives, inhibit oxidative phosphorylation by hindering ubiquinone-dependent electron transport in isolated mitochondria (21), and it has been suggested that macrophage activation by ascofuranone is related to inhibition of mitochondrial respiration (22, 23).

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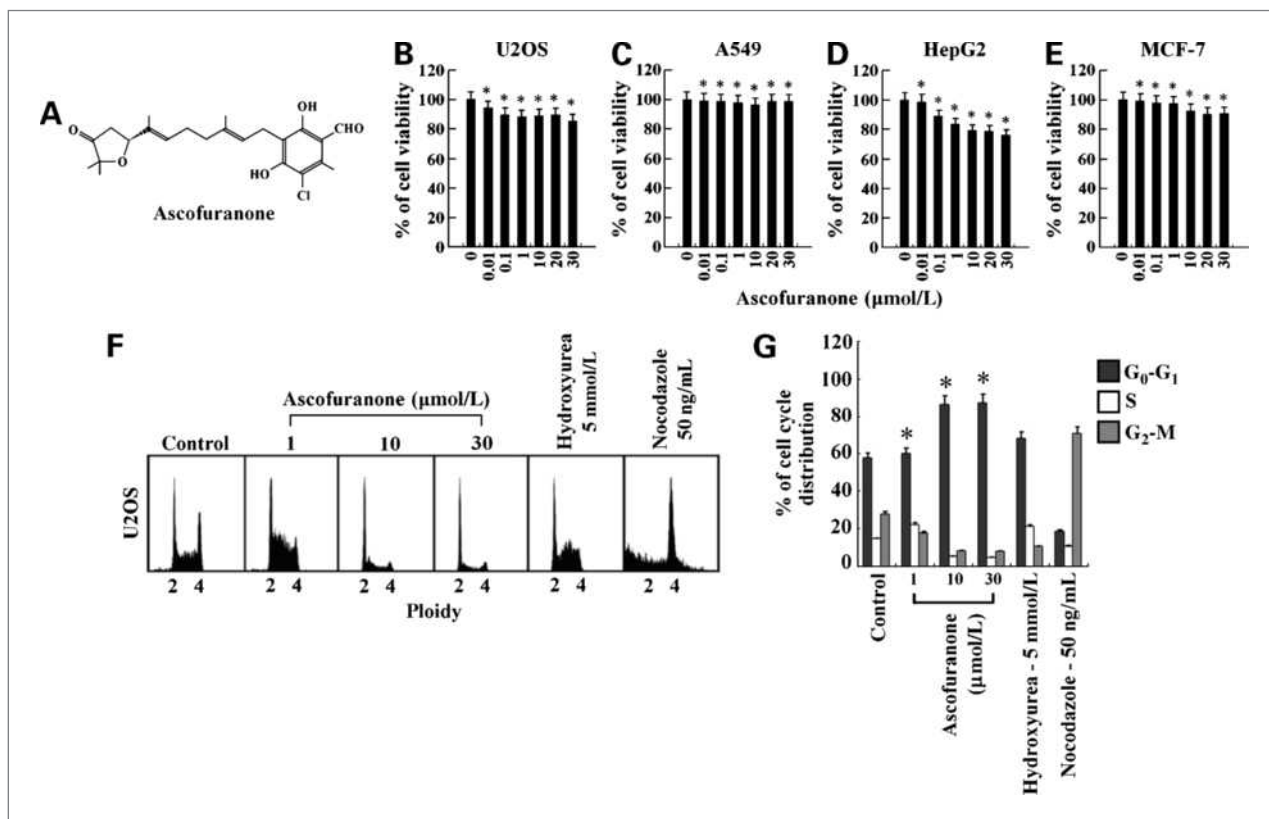


Figure 1. G₁ arrest induced by ascofuranone. **A**, chemical structure of ascofuranone. Dose-dependent effect of ascofuranone on the viability of U2OS (**B**), A549 (**C**), HepG2 (**D**), and MCF-7 (**E**) cells. Cells were treated with varying concentrations of ascofuranone for 24 h. Viability was determined by MTT assay. Columns, mean of triplicate assay experiments; bars, SD. *, $P < 0.05$, statistically significant compared with control group. **F**, **G**, arrest induced by ascofuranone. U2OS cells were treated with drugs for 24 h, and cell cycle distribution was determined by flow cytometry. **G**, data from the cell cycle distribution were quantified with a densitometer as the mean \pm SD of three independent experiments. *, $P < 0.05$ versus nonascofuranone-treated control group.

Ascochlorin and ascofuranone selectively suppress activator protein activity in human renal carcinoma cells and its downstream targets such as matrix metalloproteinase-9 through the suppression of the extracellular signal-regulated kinase-1/2 (ERK-1/2) signaling pathway (24, 25). Proteome analysis of ascochlorin-treated human osteosarcoma cells indicates a decrease in expression of several genes in the mitogen-activated protein kinase signaling cascade, including epidermal growth factor receptor and ERK-1/2 (26). Consequently, ascochlorin and ascofuranone suppress invasion of tumor cells *in vitro* (24, 25), transforming growth factor- β -induced factors involved in renal fibrosis including extracellular matrix proteins and PAI-1 (27), and growth of estrogen receptor-negative human breast cancer cell lines that exhibit high activator protein activity (28).

We recently found that ascochlorin and ascofuranone activate p53 and enhance transcription of its downstream targets including p21^{WAF1/CIP1} and Hdm2 (29). The manner of activation is distinct from DNA-damaging agents in that the antibiotics induce phosphorylation of serine 392 with negligible effect on serine 15, a major phosphorylation residue induced by genotoxins. The ability of as-

cochlorin derivatives to activate p53 is correlated to their respiratory inhibition, and respiratory inhibitors such as antimycin and rotenone activate p53 in a manner similar to ascofuranone. These results suggest that ascofuranone activates p53 by a mechanism involving mitochondrial respiration, distinct from DNA-damaging agents. In the present study, we report that ascofuranone induces transcription of p21^{WAF1/CIP1} through the suppression of c-Myc expression but not through p53 activation. Our results reveal that the induction of G₁ arrest by ascofuranone is associated with the p53-independent activation of p21^{WAF1/CIP1} through the disruption of c-Myc in human cancer cell lines.

Materials and Methods

Cells and materials

Human breast adenocarcinoma MCF-7 cells, human hepatocellular liver carcinoma HepG2 cells, human osteosarcoma U2OS cells, and human colorectal carcinoma HCT116 cells as well as their sublines deficient in p53 or p21^{WAF1/CIP1} were grown in DMEM supplemented with 1% antibiotic mixture for bacteria and fungi, and

10% fetal bovine serum, and incubated at 37°C in a humidified atmosphere of 5% CO₂. Human lung adenocarcinoma A549 cells were maintained in RPMI 1640 supplemented with fetal bovine serum and an antibiotic mixture. Ascofuranone was supplied by Chugai Pharmaceutical. Ascofuranone stock solution (10 mmol/L) was prepared using DMSO as solvent, stored at -20°C, and diluted with medium before use.

Cytotoxicity assays

Cell viability was determined with a MTT dye reduction assay kit (Roche Applied Science, Mannheim, Germany), which measures mitochondrial respiratory function. Cells (1 × 10⁴ cells per well) were cultured for 24 hours and then incubated with 100 µg per well MTT dye for 4 hours. The resultant formazan deposits were solubilized with 20 µL of 10% SDS, and the absorbance at 590 nm was measured. All data were calculated and expressed as percent A₅₉₀ of control cells (without treatment, set at 100%).

Cell cycle analysis

One million cells were suspended in staining buffer, 50 µg/mL propidium iodide, 0.2% Nonident P-40, and 4 mmol/L sodium citrate. The fluorescence of stained nuclei was analyzed by flow cytometry (Epics-XL, Coulter).

Western blot analysis

Cellular lysates were prepared by suspending cells in SDS sample buffer, 120 mmol/L Tris, 4% SDS, 20% glycerol, 0.1 mg/mL bromophenol blue, and 100 mmol/L DTT (pH 6.8) at a density of 0.5 × 10⁶ cells/mL. After brief sonication, the lysates were heated at 95°C for 5 minutes. The proteins were electrotransferred to Immobilon-P membranes (Millipore Corp.). Western blotting of all samples was done as previously described (25) using first antibodies and the corresponding second antibodies for whole immunoglobulins from mouse or rabbit (Amersham Biosciences). The detection of specific proteins was carried out with an enhanced chemiluminescence Western blotting kit (Roche Diagnosis) following the manufacturer's instructions. Specific antibodies for p53 (DO-1), c-Myc (0.N.222), p27^{KIP1} (F-8), p21^{WAF1/CIP1} (C-19), cyclin D (HD11), cyclin E (C-19), cyclin B (GNS1), 14-3-3-σ (N-14), Bax (P-19), and CDK2 (M2) were purchased from Santa Cruz Biotechnology, Inc. An anti-β-actin (C4) antibody was obtained from Abcam Ltd. We quantified the actual levels of proteins by using the Multigauge v. 3.0 software (Fujifilm). The gels were stained with Coomassie Brilliant Blue R-250 (0.25%) for 1 hour and then destained (all solutions from Bio-Rad) to check the loading amount of protein samples on the gels.

Quantitative PCR

Total RNA was extracted from cells with Trizol reagent (Invitrogen), and reverse-transcriptase reactions were done with a commercial kit (SuperScript II RNase H-

reverse transcriptase, Invitrogen) using 50 µg/mL RNA. Quantitative PCRs were done in a Prism 7900HT real-time PCR system (Applied Biosystems) with a commercial kit (Full Velocity SYBR Green QPCR master mix, Stratagene) in a reaction mixture containing 20% of the reverse transcriptase reaction product as template and 2.5% of each Taqman primer set [p21, Hs00355762; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Hs99999905; cyclin E1, Hs00233356; c-Myc, Hs00153408], obtained from Applied Biosystems. Relative mRNA expression levels were normalized to the value of GAPDH for each reaction.

Reverse transcription-PCR

Cells including MCF-7, HCT116 wild-type cells, and its sublines deficient in p53 or p21^{WAF1/CIP1} were incubated for indicated times with 10 µmol/L ascofuranone. Total RNA was extracted from cells with Trizol reagent (Invitrogen), and reverse-transcriptase reactions were done with a commercial kit (SuperScript II RNase H-reverse transcriptase, Invitrogen) using total RNA (1 µg) from MCF-7 cells and HCT116 wild-type cells and its sublines deficient in p53 or p21^{WAF1/CIP1} according to the manufacturer's protocol. The PCR primers for p53 were 5'-AGACCGGCGCACAGAGGAAG-3' (sense) and 5'-CTTTTGGACTTCAGGTGGC-3' (antisense), 5'-TCGGGGCTTTATCTAACTCG-3' (sense) and 5'-GCTGCTATGGGCAAAGTTC-3' (antisense) for c-Myc, 5'-ATGTCAGAACCGGCTGGGGA-3' (sense) and 5'-GCCGTTTTCGACCCTGAGAG-3' (antisense) for p21, and 5'-GCCATCGTCACCAACTGGGAC-3' (sense) and 5'-CGATTTCCCGCTCGGCCGTGG-3' (antisense) for β-actin. PCR products were resolved electrophoretically on a 1.0% (w/v) agarose gel and visualized by staining with ethidium bromide. The oligonucleotide sequences of the reaction products were confirmed by sequencing. We quantified the actual levels of proteins by using the Multigauge V. 3.0 software (Fujifilm).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assay was done as outlined by the commercial assay kit (Upstate Biotechnology). DNA-binding proteins were cross-linked to DNA and lysed in SDS lysis buffer containing 1× protease inhibitor. DNA was sheared to 200- to 500-bp fragments by 30-second sonications with a VC100 sonicator (Sonics & Materials, Inc.). The chromatin solution was precleared with salmon DNA/protein A agarose 50% slurry (Upstate Biotechnology) for 30 minutes at 4°C. The precleared supernatant was incubated with polyclonal antibodies [anti-c-Myc (N-262), anti-Sp1 (PEP2), and anti-Miz-1 (H-190), Santa Cruz Biotechnology, Inc.] overnight at 4°C. A proximal region in the p21^{WAF1/CIP1} promoter was amplified from the immunoprecipitated chromatin by PCR using the following primer set (30): sense, 5'-ACCGGCTGGCCTGCTGGAAC-3' and antisense, 5'-TCTGCCGCCGTCTCTCACCT-3'. PCR products were separated on a 2% agarose gel.

RNA interference

MCF-7 or HCT116 cells at 50% confluency were transfected with a final concentration of 50 nmol/L of negative control small interfering RNA (siRNA) or c-Myc-specific siRNA duplex (Dharmacon, Inc.) and p53-specific siRNA duplex (Santa Cruz Biotechnology, Inc.) with Trans IT-TKO (Mirus Bio Corp.) according to the manufacturer's instructions.

Immunoprecipitation

Eighty percent confluent MCF-7 cells were cotransfected with pCDNA3-p21/pCMV-CDK2 or pCMV-CDK2/pCMV-cyclin E. Two micrograms of each plasmid DNA were cotransfected into the cells using Lipofectamine reagent (Invitrogen) according to the manufacturer's instructions. Cells were washed with ice-cold PBS; whole-cell lysates were cleared by centrifugation at 14,000 g for 10 minutes; and the supernatant fraction was then used for immunoprecipitation. Aliquots containing 500 µg of protein were cleared with protein A/G-plus agarose beads (Santa Cruz Biotechnology, Inc.). Target proteins were immunoprecipitated from whole-cell lysates using specific antibodies after incubation for 12 hours followed by the addition of 20 µL protein A/G-plus agarose beads, and incubation was continued overnight at 4°C. Immunoprecipitates were washed and subsequently subjected to SDS-PAGE for Western blot analysis.

Luciferase assay

The human p21^{WAF1/CIP1} promoter construct, p21P-luc (p21P), has been previously described (31). For E2F1 promoter assays, the E2F reporter construct [E2F₄]-luc (32) was used. The expression plasmid for β-galactosidase (pCMV-β-gal) used for normalization was a kind gift from N. H. Heintz (University of Vermont, Burlington, VT). Transient transfection was carried out with the Lipofectamine reagent (Invitrogen). Cells were washed twice with PBS and lysed with 200 µL of 1× Reporter lysis buffer (Promega). Each lysate (50 µL) was examined for luciferase activity. Relative luciferase activity was determined after normalization with β-galactosidase activity.

Statistical analysis

All results are representative of at least three independent experiments done in triplicate; statistical significance between experimental and control values was calculated with Newman-Keuls' multicomparison test.

Results

The effect of ascofuranone on the proliferation of human cancer cell lines

To understand the mechanism by which ascofuranone exerts its antiproliferative effects, we first tested the cytotoxicity of ascofuranone on various human cancer cell lines using a MTT assay. Ascofuranone stock solution was prepared using DMSO as vehicle, and the vehicle itself had no effects on the proliferation of cells (data

not shown). The reduction of cell viability ranged from 6% to 15% in U2OS, 1% to 4% in A549, 1% to 25% in HepG2, and 1% to 10% in MCF-7 (Fig. 1B–E) after 24 hours of treatment with ascofuranone at 0.01 to 30 µmol/L. Because ascofuranone did not show cytotoxicity and because a previous study showed significant ascofuranone-induced upregulation of p21^{WAF1/CIP1} (29), we used flow cytometry to investigate whether ascofuranone might target cell cycle regulation in cancer cells. As illustrated in Fig. 1F, treatment of U2OS cells with ascofuranone resulted in a dose-dependent increase in cells at the G₁ phase. The percentage of cells in the G₁ phase was increased as follows (Fig. 1G): 2.6% increase (60.1%, *P* < 0.05) by 1 µmol/L ascofuranone treatment, 29.0% increase (86.5%, *P* < 0.05) by 10 µmol/L ascofuranone treatment, and 29.8% increase (87.3%, *P* < 0.05) by 30 µmol/L ascofuranone treatment compared with the nonascofuranone-treated control (57.5%, *P* < 0.05). Ascofuranone has partial effect on cell cycle at 1 µmol/L, and part of S-phase cells are leaked from G₁ phase. Similar increase of S-phase cells is also observed with hydroxyurea, an inhibitor of DNA synthesis, which increased 10.6% cells in G₁-phase cells (68.1%, *P* < 0.05) and 6.7% cells in S-phase cells (21.3%, *P* < 0.05) with 17.3% reduction of cells in G₂-M phase (10.6%, *P* < 0.05), whereas nocodazole, an inhibitor of mitosis, caused 42.7% increase of G₂-M phase cells (70.6%, *P* < 0.05) with 11.6% increase in the sub-G₁ fraction, indicating induction of apoptosis. In contrast, ascofuranone did not increase the sub-G₁ fraction. These results suggest that ascofuranone arrests cells in the G₁ phase. Based on these data, we chose a dosage of 10 µmol/L for further characterization of this anticancer agent.

The effects of ascofuranone on G₁-phase cell cycle regulatory proteins in human cancer cell lines

We next assessed the effect of ascofuranone on cell cycle regulatory genes of the G₁ phase by Western blot analysis. Cancer cells treated with 10 µmol/L ascofuranone for up to 24 hours displayed a marked increase in expression of p53 and p21^{WAF1/CIP1} (Fig. 2A–D). The increase in the expression of p53 and p21^{WAF1/CIP1} in cancer cells treated with 10 µmol/L ascofuranone for 24 hours was increased as follows: 5.3-fold increase in p53 and 2.1-fold increase in p21^{WAF1/CIP1} in U2OS cells, 3.1-fold increase in p53 and 10.4-fold increase in p21^{WAF1/CIP1} in A549 cells, 1.6-fold increase in p53 and 3.7-fold increase in p21^{WAF1/CIP1} in HepG2 cells, and 5.1-fold increase in p53 and 2.4-fold increase in p21^{WAF1/CIP1} in MCF-7 cells, compared with the nonascofuranone-treated control, respectively. A significant increase of p21 mRNA in these cell lines shown by quantitative PCR (Fig. 2E) as described earlier (29) indicates that this increase is a result of transcription activation as follows: 5.5-fold increase in A549 cells, 5.8-fold increase in HepG2 cells, 1.8-fold increase in MCF-7 cells, and 3.5-fold increase in U2OS cells, compared with the nonascofuranone-treated control respectively. Expression of another CDK, p27^{KIP1},

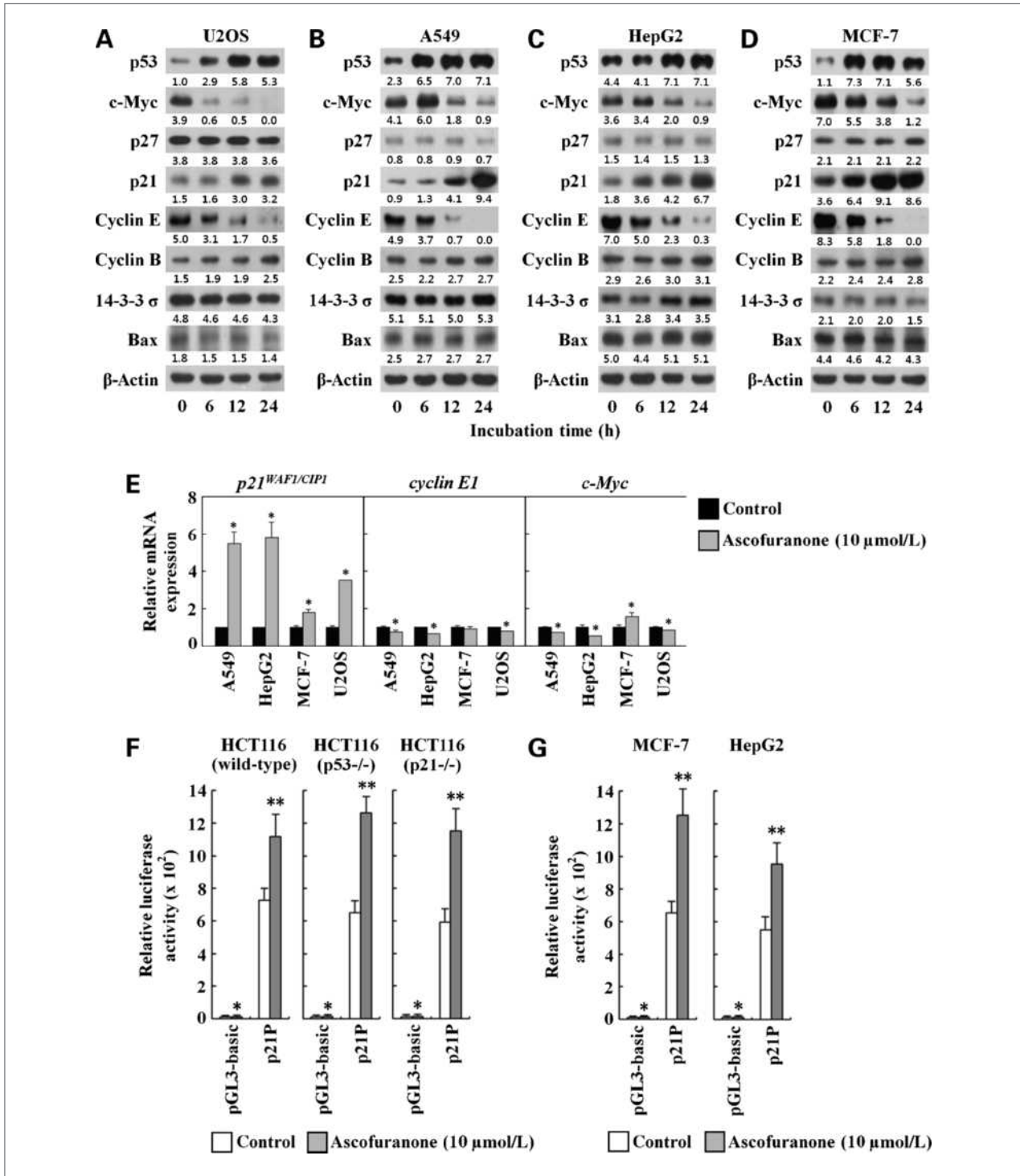


Figure 2. Induction of p21^{WAF1/CIP1} by ascofuranone. Protein expression of U2OS (A), A549 (B), HepG2 (C), and MCF-7 (D) cells by ascofuranone treatment. Cells were treated with 10 μ mol/L ascofuranone for the indicated time, and protein expression was determined by Western blot analysis. E, suppression of p21^{WAF1/CIP1} transcription by ascofuranone. Cells were treated with ascofuranone for 24 h. Relative mRNA for p21^{WAF1/CIP1} after normalization with GAPDH expression determined by quantitative PCR is shown. Columns, mean of three independent cultures; bars, SD. *, $P < 0.01$, statistically significant compared with nontreated control. Suppression of p21^{WAF1/CIP1} promoter activity of HCT116 and its sublines deficient in p53 or p21^{WAF1/CIP1} (F) or MCF-7 and HepG2 cells (G) by ascofuranone. Cells were transfected with p21^{WAF1/CIP1} reporter plasmid (p21P) or control plasmid (pGL3-basic) together with pCMV- β -gal. Ascofuranone was added 24 h after transfection. Cells were further cultured for 24 h, and enzyme activity in the cell lysate was determined. Relative luciferase activity after normalization for β -galactosidase activity is shown. Columns, mean of five independent experiments; bars, SD. Statistical significance was determined compared with nontreated control (*, $P < 0.05$; **, $P < 0.01$).

was not affected by ascofuranone. This result was corroborated by reporter experiments for the p21^{WAF1/CIP1} promoter, which showed a significant increase in luciferase gene expression by ascofuranone treatment (Fig. 2F and G). Results of the activity of p21^{WAF1/CIP1} promoter presented in Fig. 2F and G showed that 10 μmol/L ascofuranone treatment produced 1.5-fold increase in HCT116 +/+ cells, 1.9-fold increase in HCT116 p53^{-/-} cells, 1.9-fold increase in HCT116 p21^{-/-} cells, 1.9-fold increase in MCF-7 cells, and 1.7-fold increase in HepG2 cells, compared with the p21P-transfected control, respectively. Consistent with cytostatic G₁ arrest induced by ascofuranone, treatment of cells with ascofuranone resulted in a time-dependent decrease in the expression of cyclin E, involved in the G₁-S transition, without affecting factors involved in G₂ arrest (14-3-3σ and cyclin B) or the proapoptotic factor Bax (Fig. 2A–D). The change in expression of cyclin E in cancer cells treated with 10 μmol/L ascofuranone for 24 hours was decreased as follows: 23.0-fold decrease in U2OS cells, not expressed in A549 cells, 15.0-fold decrease in HepG2 cells, and not expressed in MCF-7 cells, compared with the nonascofuranone-treated control, respectively. We also found that c-Myc, a regulator of apoptosis and cell proliferation, was significantly suppressed by ascofuranone as follows: not expressed in U2OS cells, 4.6-fold decrease in A549 cells, 4.0-fold decrease in HepG2 cells, and 5.8-fold decrease in MCF-7 cells, compared with the nonascofuranone-treated control, respectively. In contrast, no marked change in mRNA levels for c-Myc as well as for cyclin E1 was observed (Fig. 2E), suggesting that posttranscriptional regulation is the main route for ascofuranone-mediated downregulation of these molecules. Ascofuranone affected neither RNA recovery per cell nor GAPDH expression significantly. Thus, ascofuranone has no general influence on cellular transcription.

Ascofuranone induces p21^{WAF1/CIP1} and G₁ arrest in a p53-independent manner

Because the tumor suppressor p53 is one of the major regulators of p21^{WAF1/CIP1} gene expression, we used a human colon cancer cell line, HCT116, and sublines deficient in p53 and p21 to explore the relationship between p53 activation and ascofuranone-induced G₁ arrest. As was the case in U2OS cells (Fig. 1F), ascofuranone treatment increased 30.1% cells in the G₁ phase (83.8%, $P < 0.05$) and reduced 17.8% in the G₂-M phase (10.7%, $P < 0.05$) compared with the nonascofuranone-treated control in HCT116 cells (Fig. 3A and B). Unexpectedly, however, ascofuranone induced G₁ arrest in a p53-deficient cell line (30.3% increase of G₁ phase) but not significantly in a p21^{WAF1/CIP1}-deficient line (5.6% increase of G₁ phase), suggesting that p53 is not involved in ascofuranone-induced G₁ arrest and that p21^{WAF1/CIP1} is necessary to induce G₁ arrest. Western blotting of these ascofuranone-treated cell lines showed that induction of p21^{WAF1/CIP1} expression as well as suppression of cyclin E, cyclin D, and c-Myc were evident in the absence of p53, and that

expression of cyclin E, cyclin D, and c-Myc was still significantly suppressed in the p21^{WAF1/CIP1}-deficient cell line (Fig. 3C). The expression of proteins related to cell cycle progression in HCT116 p21^{-/-} cells treated with 10 μmol/L ascofuranone for 24 hours was changed as follows: 17.5-fold increase in p53, 11.7-fold decrease in c-Myc, 21.0-fold decrease in cyclin D, 2.3-fold decrease in cyclin E, and no significant change in p27 or cyclin B compared with the nonascofuranone-treated control, respectively. Furthermore, ascofuranone increased the p21^{WAF1/CIP1} promoter activity in the HCT116 sublines deficient in p53 or p21^{WAF1/CIP1}, suggesting that p53 is not a major factor responsible for the transcription activation of p21 by ascofuranone (Fig. 2F). It should be noted that dynamic of protein expression upon ascofuranone is substantially different in HCT116 p53^{-/-} and +/+ cells (Fig. 3C and D), presumably because of the coordinate modulation of cell cycle regulators by c-Myc and p53, both of which are affected by ascofuranone. As shown in Figs. 2E and 3F to I, ascofuranone had no effect on c-Myc transcription in these cell lines. These results suggest that c-Myc is an upstream repressor of p21^{WAF1/CIP1} expression in ascofuranone-treated cells, as has been shown in cells treated with transforming growth factor-β, phorbol ester, and genotoxins (32, 33).

Stability of c-Myc is regulated by the NH₂-terminal phosphorylation. ERK-mediated phosphorylation at Ser 62 stabilizes c-Myc, whereas Glycogen synthase kinase-3-mediated phosphorylation at Thr 58 accelerates ubiquitin-mediated degradation of the protein (34). Thus, PD98059, an inhibitor of ERK, destabilizes c-Myc. Wortmannin, an inhibitor of phosphoinositide 3-kinase (PI3K), also promotes degradation of c-Myc through the suppression of AKT, which phosphorylates and inactivates Glycogen synthase kinase-3 (35). Ascofuranone interferes the PI3K/ERK signal transduction pathway and mitochondria respiration. We observed that inhibitors for these cellular events including PD98059, Wortmannin, antimycin, and rotenone suppressed c-Myc expression in HCT116, and sublines deficient in p53 and p21 cells, ranging from 2.2-fold decrease to 12.3-fold decrease compared with the control, respectively (Fig. 4A–C), whereas the expressions of p21^{WAF1/CIP1} in HCT116, and subline deficient in p53 cells treated with those inhibitors except for oligomycin was increased >1.6-fold compared with the control, respectively (Fig. 4A–C). This result suggests that the inhibition of mitochondria respiration and PI3K/ERK pathway is involved in ascofuranone-mediated c-Myc suppression.

The binding activity of c-Myc at the promoter region of p21^{WAF1/CIP1} in ascofuranone-treated cells

c-Myc is directly recruited to the p21^{WAF1/CIP1} promoter by the DNA-binding protein Miz-1, and this association prevents p21^{WAF1/CIP1} expression (32, 33). To examine whether c-Myc binds the p21^{WAF1/CIP1} promoter in ascofuranone-treated HCT116 or MCF-7, we performed ChIP assays. We used antibodies against Miz-1

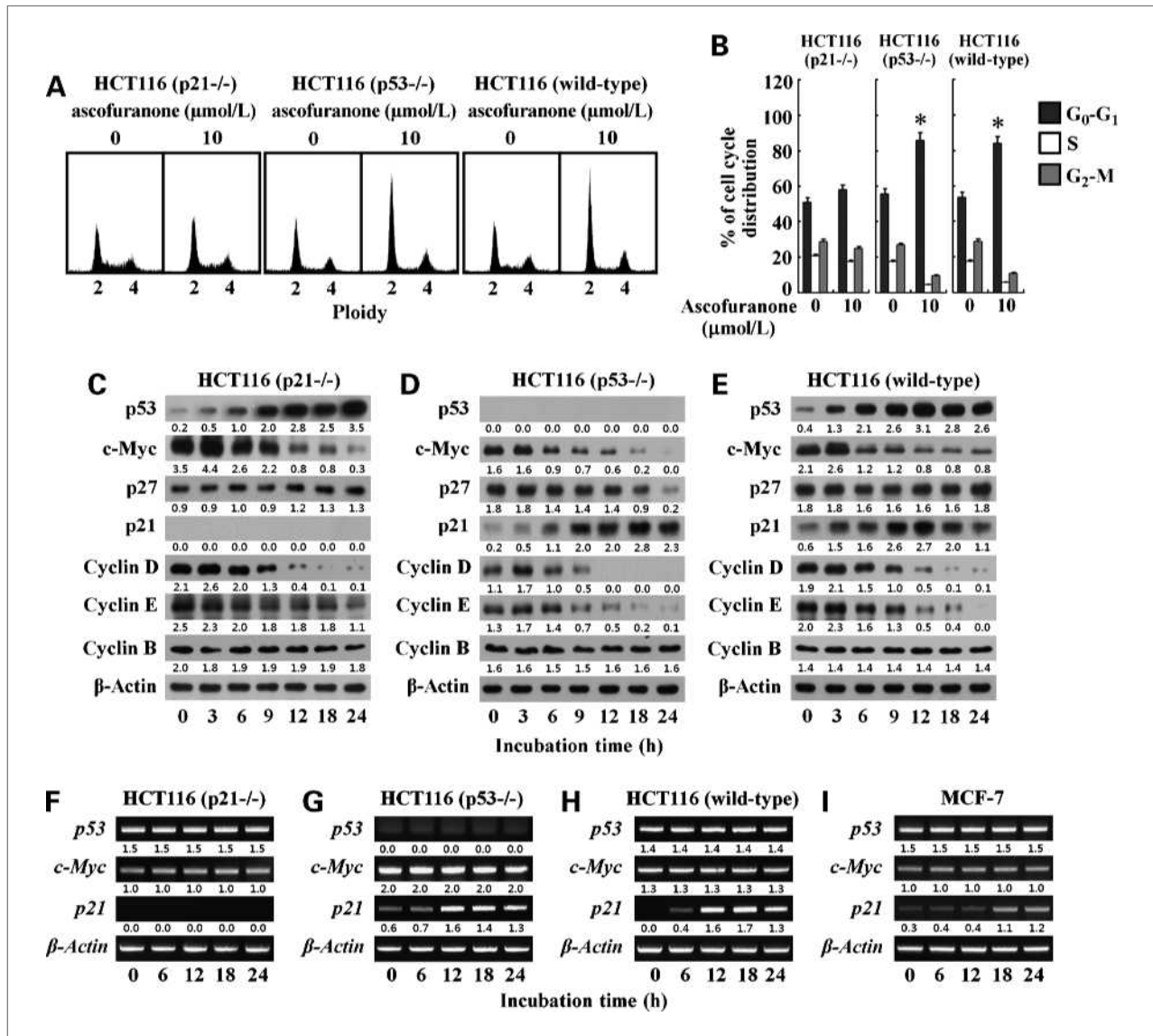


Figure 3. Ascofuranone-induced G_1 arrest is independent of p53. **A**, cell cycle progression of ascofuranone-treated cells. Cells were treated with ascofuranone for 24 h, and cell cycle distribution was determined by flow cytometry. **B**, data from the cell cycle distribution were quantified with a densitometer as the mean \pm SD of three independent experiments. *, $P < 0.05$ versus nonascofuranone-treated control group. Protein expression of HCT116 p21 $^{-/-}$ (**C**), HCT116 p53 $^{-/-}$ (**D**), and HCT116 wild-type cells (**E**) by ascofuranone treatment. Cells were treated with 10 $\mu\text{mol/L}$ ascofuranone for the indicated time, and protein expression was analyzed by Western blot. Reverse transcription-PCR analysis of mRNA expressions related to cell cycle regulatory genes was done using total RNA prepared from HCT116 p21 $^{-/-}$ (**F**), HCT116 p53 $^{-/-}$ (**G**), HCT116 wild-type (**H**), and MCF-7 cells (**I**) treated with 10 $\mu\text{mol/L}$ ascofuranone for indicated times.

and Sp1 to compare against c-Myc. After immunoprecipitation with these antibodies, the proximal region of p21^{WAF1/CIP1} promoter was amplified by PCR using specific primer sets (Fig. 4D–G). Following c-Myc immunoprecipitation, a strong signal detected in nontreated cells completely disappeared in ascofuranone-treated cells. We performed the ChIP assays in p53- or p21^{WAF1/CIP1}-deficient cells (Fig. 4D and E) and found that ascofuranone dissociated c-Myc from p21 promoter regardless of p53. In contrast, a similar signal was detected in the chromatin fragments immunoprecipitated with antibodies for Miz-1

and Sp1 in ascofuranone-treated and nontreated cells. No amplified product was detected with control IgG, verifying the specificity of the antibodies used in the present study. These results suggest that ascofuranone specifically impairs the c-Myc-mediated repression of the p21^{WAF1/CIP1} promoter through the downregulation of c-Myc expression.

Suppression of ascofuranone-induced G_1 arrest and p21^{WAF1/CIP1} induction by c-Myc knockdown

To confirm the activation of p21^{WAF1/CIP1} through the ascofuranone-induced downregulation of c-Myc, we took

advantage of siRNA with c-Myc-specific siRNA duplex or p53-specific siRNA duplex. We transfected HCT116 with siRNAs targeted for c-Myc or p53, or with nonspecific siRNAs as a negative control and analyzed the expression of c-Myc, p53, p21^{WAF1/CIP1}, and β -actin by

Western blot analysis. Each siRNA specifically eliminated the expression of the target molecule, and ascofuranone-induced p21^{WAF1/CIP1} expression was completely suppressed by c-Myc knockdown but not by p53 knockdown (Fig. 5A and B). The expression of p21^{WAF1/CIP1} in c-Myc

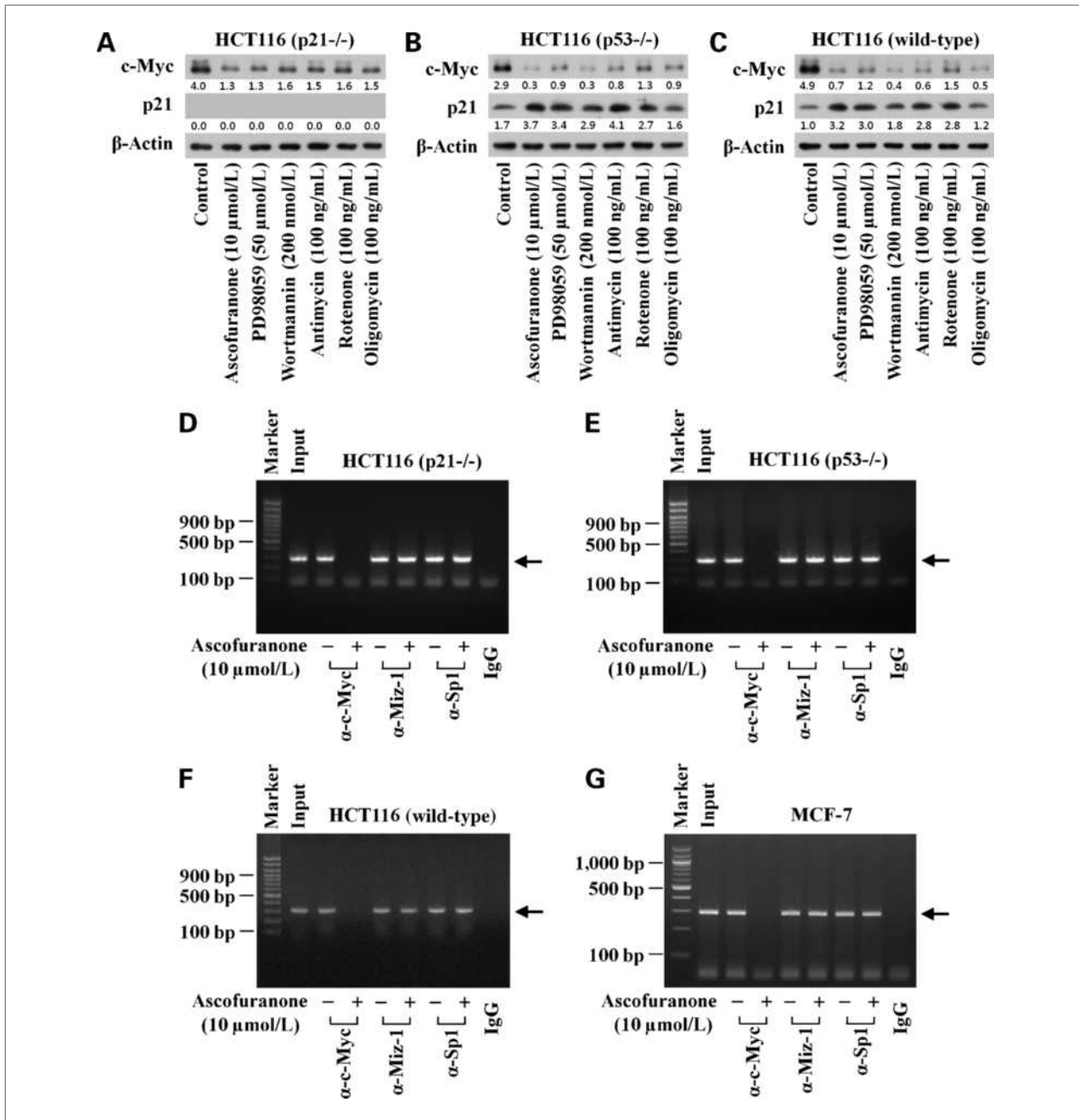


Figure 4. Regulation of c-Myc and its binding activity to p21^{WAF1/CIP1} promoter by ascofuranone. c-Myc and p21 expressions of HCT116 p21^{-/-} (A), HCT116 p53^{-/-} (B), and HCT116 wild-type cells (C) treated with ascofuranone and indicated inhibitors. Cells were treated with ascofuranone and the indicated inhibitors for 24 h, and protein expression was analyzed by Western blot. PD98059 and wortmannin are inhibitors for mitogen-activated protein/ERK kinase and PI3K. Antimycin and rotenone inhibit complex III and I in the electron transport chain. HCT116 p21^{-/-} (D), HCT116 p53^{-/-} (E), and HCT116 wild-type cells (F) or MCF-7 cells (G) were treated with ascofuranone for 24 h, and c-Myc, Miz-1, and Sp-1 bound to the p21 promoter were detected by ChIP assay. A mixture of rabbit and mouse IgGs was used as a negative control for immunoprecipitation. Input, DNA from cell lysates before immunoprecipitation. Arrows, the target PCR product. Left, molecular weights (bp) of marker DNA.

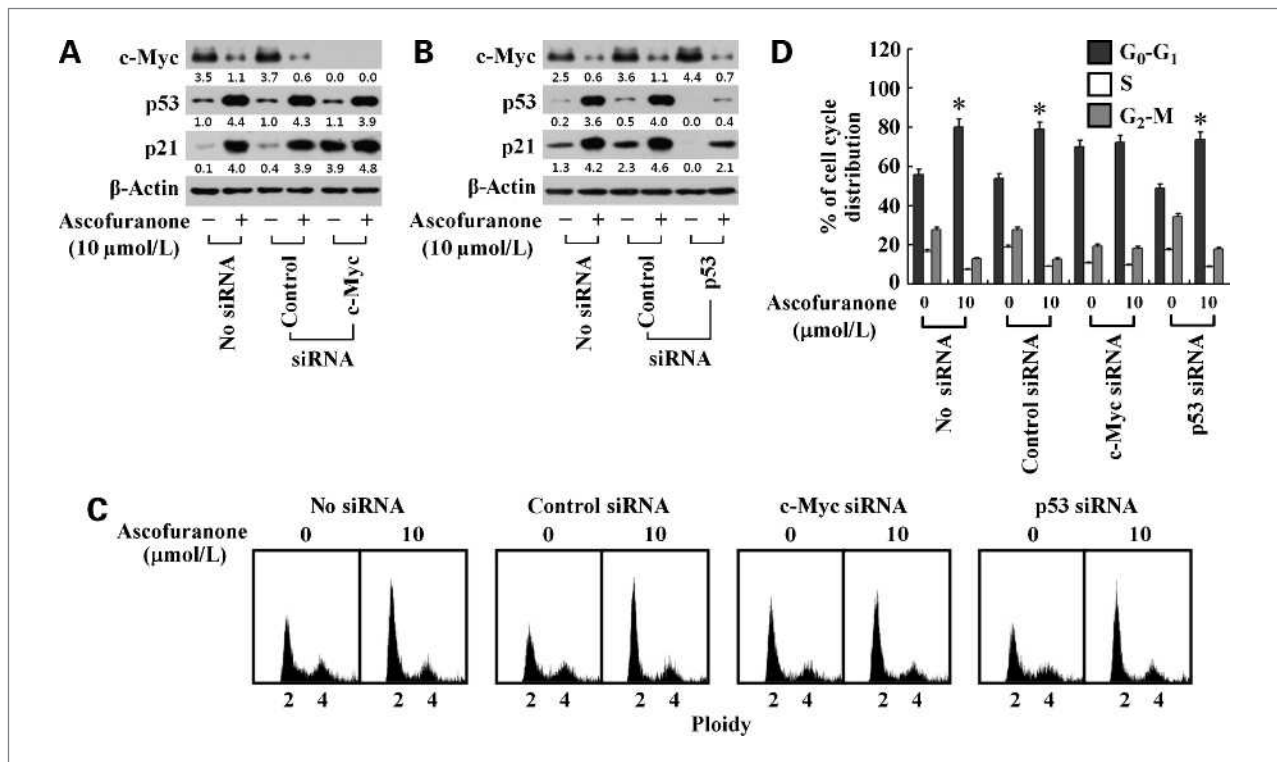


Figure 5. Ascofuranone-induced G₁ arrest is dependent on c-Myc but not on p53. Cells were transfected with 50 nmol/L of negative control siRNA or siRNA duplex specific for c-Myc and p53. Ascofuranone was added 24 h after transfection and further cultured for 24 h. Protein expression in c-Myc knockdown (A) or p53 knockdown (B), and cell cycle distribution (C) were analyzed by Western blot and by flow cytometry. D, data from the cell cycle distribution were quantified with a densitometer as the mean ± SD of three independent experiments. *, $P < 0.05$ versus nonascofuranone-treated control group.

knockdown cells treated with ascofuranone was not significantly increased (1.2-fold increase) compared with the nonascofuranone-treated control. In addition, the knockdown of c-Myc increased the basal expression level of p21^{WAF1/CIP1}, resulting in a 9.8-fold increase compared with the nonascofuranone-treated control siRNA. In addition, the expression of p21^{WAF1/CIP1} in p53 knockdown cells was highly increased (2.1 density in arbitrary units) compared with the nonascofuranone-treated control that completely eliminated its basal expression level. Taken together, these results suggest that c-Myc is a key negative regulator in determining p21^{WAF1/CIP1} expression level in normally growing cells.

To clarify the mechanism of ascofuranone-induced G₁ arrest, we analyzed cell cycle progression in HCT116 transfected with siRNAs (Fig. 5C and D). c-Myc knockdown with siRNA resulted in an increase in 16.3% of cells at the G₁ phase (70.0%, $P < 0.05$) compared with control cells transfected with nonspecific siRNA and completely eliminated ascofuranone-induced G₁ arrest. In contrast, p53 knockdown as well as nonspecific siRNA transfection neither increased G₁ phase population in nontreated cells nor suppressed ascofuranone-induced G₁ arrest. These results suggest that c-Myc, but not p53, plays a crucial role in ascofuranone-induced p21^{WAF1/CIP1} induction and subsequent G₁ arrest.

The effects of ascofuranone on biochemical events involved in the G₁ check point

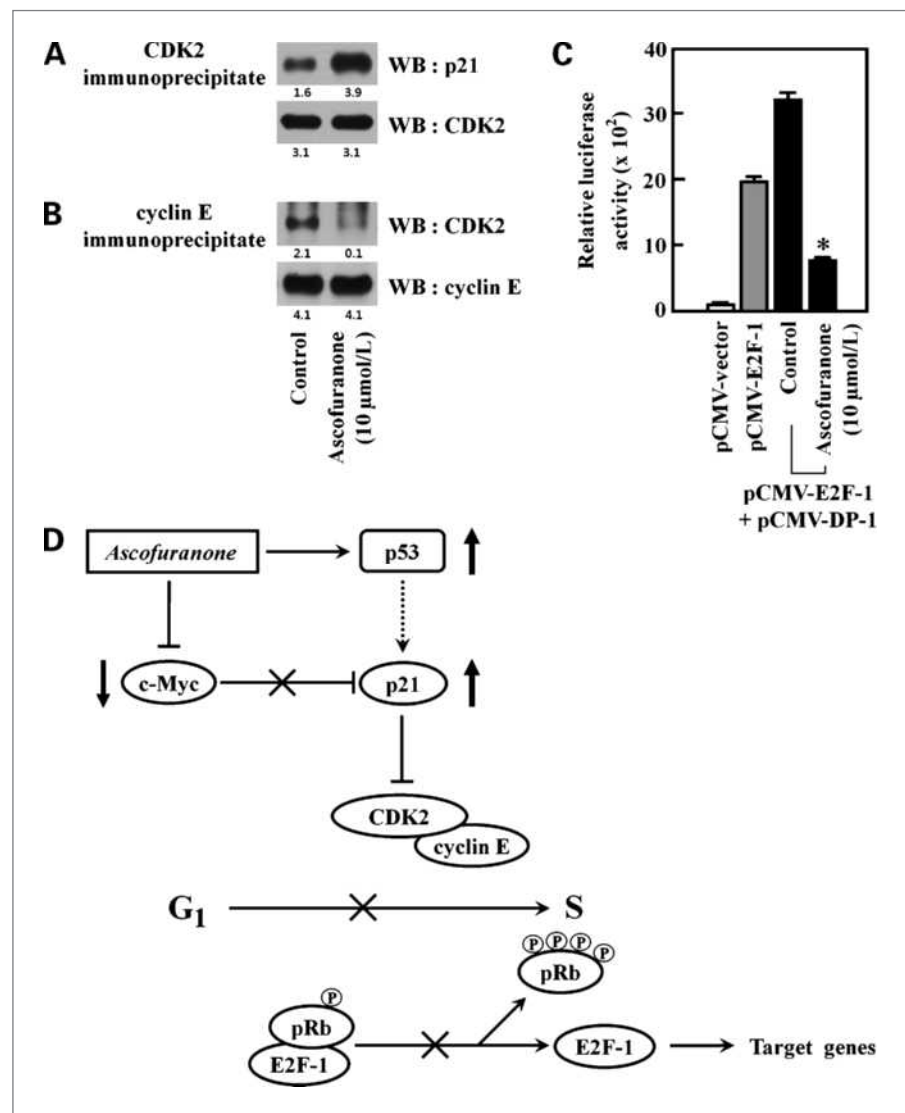
p21^{WAF1/CIP1} prevents the association of CDK2-cyclin E, resulting in the inactivation of CDK2 kinase (12), which subsequently depresses the hyperphosphorylation of pRb and resultant activation of E2F and transcription activation of its downstream target genes involved in cell cycle progression, leading to a blockade of the G₁-S transition (4, 5). Treatment with ascofuranone resulted in the 2.4-fold increase of p21^{WAF1/CIP1}-CDK2 complex in MCF-7 cells ectopically expressing p21^{WAF1/CIP1} and CDK2 as well as the 21.0-fold decrease of CDK2-cyclin E complex in cells ectopically expressing CDK2 and cyclin E (Fig. 6A and B). Ascofuranone also suppressed E2F transcription activity in 4.1-fold decrease in MCF-7 cells transfected with an E2F-reporter plasmid and expression plasmids for E2F-1 and its heterodimeric partner, DP-1 (Fig. 6C). Because transfection with the E2F-1 expression vector only partially activated transcription activity in 19.6-fold increase and the control vector plasmid did not, this reporter system specifically detects transcription activity of ectopically expressed E2F-1/DP-1 complex. Taken together, these results suggest that ascofuranone induces biochemical events involved in the G₁ checkpoint through the reduction of c-Myc-mediated repression of p21^{WAF1/CIP1} transcription.

Discussion

Ascofuranone has been shown to have significant anti-tumor activity against various transplantable tumors and has the ability to suppress melanoma and lung carcinoma metastasis in murine experimental models (19, 20). However, the precise molecular mechanism by which ascofuranone inhibits the proliferation of cancer cells is still not clear. Recently, we have reported that ascofuranone activates p53 and induces transcription of its downstream targets including p21^{WAF1/CIP1} (29), which prompted us to investigate cell cycle progression in ascofuranone-treated cells. In this study, we found that ascofuranone arrested human cancer cells in the G₁ phase. To gain insight into the molecular mechanisms underlying G₁ arrest induced by ascofuranone, we evaluated several proteins relevant to cell cycle progression and apoptosis in various cancer cells and found that ascofuranone

induced an increase in p53 and p21^{WAF1/CIP1}, and a decrease in G₁ cyclins including cyclins E and D and c-Myc without affecting p27^{KIP1}, 14-3-3 σ , Bax, or cyclin B. To assess the contribution of p53 and c-Myc to the effects of ascofuranone on cell cycle progression, we took advantage of HCT116 colon carcinoma variant cells (36) and knockdown technology using siRNA, and unexpectedly found that induction of p21^{WAF1/CIP1} and G₁ arrest was dependent on c-Myc but not on p53. These results suggest that suppression of c-Myc is present upstream of p21 induction and subsequent G₁ arrest in ascofuranone-treated cells. Several lines of evidence suggest that c-Myc is an essential gene involved in the regulation of proliferation, mitogenesis, differentiation, and programmed cell death (37). c-Myc represses the transcription of certain genes, including the CDKs p15^{INK4B}, p21^{WAF1/CIP1}, and p27^{KIP1} (38–41). Recent studies have reported that c-Myc is recruited to the p21^{WAF1/CIP1} promoter through

Figure 6. Ascofuranone inhibits biochemical events in G₁-S transition. Inhibition of CDK2-cyclin E complex formation by ascofuranone. MCF-7 cells were cotransfected with expression plasmids for p21^{WAF1/CIP1} and CDK2 (A), or those for cyclin E and CDK2 (B). WB, Western blot. Association of ectopically expressed molecules was analyzed by immunoprecipitation. C, suppression of E2F transcription activation by ascofuranone. MCF-7 cells were transfected with reporter plasmid for E2F together with expression plasmids for E2F-1, DP-1, and β -galactosidase. Ascofuranone was added 24 h after transfection; cells were further cultured for 24 h; and enzyme activity in the cell lysate was determined. Columns, mean of five independent experiments after being normalized for β -galactosidase activity; bars, SD. *, statistically significant compared with nontreated control ectopically expressing E2F-1 and DP-1 ($P < 0.01$). D, schematic representation of the mechanism for ascofuranone-induced G₁ arrest. Ascofuranone-induced cell cycle arrest at G₁ phase in human cancer cell lines. This effect was caused by inhibition of CDK activity through the increased association of p21^{WAF1/CIP1} with CDK2-cyclin E complexes, which occurred through transcriptional upregulation of p21^{WAF1/CIP1}. Ascofuranone downregulates c-Myc expression by a posttranslational mechanism, releasing p21^{WAF1/CIP1} promoter from c-Myc-mediated repression.



association with the DNA-binding protein Miz-1 and represses the transcription of p21^{WAF1/CIP1} in a manner independent of p53 (33). Thus, c-Myc switches from cytostatic to apoptotic through the repression of p21^{WAF1/CIP1} in daunorubicin-treated cells (42).

To show the involvement of c-Myc in ascofuranone-mediated p21^{WAF1/CIP1} induction, we elucidated the binding of c-Myc to the p21^{WAF1/CIP1} promoter region with a ChIP assay and clarified the crucial role of c-Myc in p21^{WAF1/CIP1} transcription by using RNA interference for c-Myc or p53. These results support our conclusion that the essential regulator of p21^{WAF1/CIP1} expression in ascofuranone-treated cells is not p53 but c-Myc. Ascofuranone activates p53 in a manner distinct of genotoxins (29). Ascofuranone induces the phosphorylation of Ser 392 of p53 with minimum effect on Ser15, whereas genotoxins markedly induce both phosphorylations. Ascofuranone similarly induces the transcription of Hdm2 and p21 among p53 targets, whereas genotoxins preferentially induce p21. Finally, genotoxins arrested U2OS cells in G₂ phase (data not shown), whereas ascofuranone arrests cell in the G₁ phase. Such biological differences observed between ascofuranone and genotoxins, despite their p53 activation observed using a reporter bearing synthetic p53 binding site may be due to the different environment around the p53 binding site in each promoter region of target genes. Modification of p53 induced by ascofuranone may affect the transcription activity of p21 minimally, and the depression of c-Myc-mediated transcription repression may predominate in the regulation of p21 promoter activity in ascofuranone-treated cells.

It is well known that cell cycle arrest can occur by loss of CDK activity (12, 43). The inactivation of CDKs by CDKIs including p16, p21^{WAF1/CIP1}, and p27^{KIP1} occurs through direct interaction with the ATP binding site of the CDK or through the blockade of CDK-cyclin complex formation (12, 44, 45). As shown in Fig. 6B, the specific loss of binding activity of the CDK2-cyclin E complex by ascofuranone inactivates CDK2 kinase, resulting in pRb family members in the G₁ phase of the cell cycle. The Rb family includes pRb/p105, p107, and Rb2/p130, referred to as "pocket proteins," which bind to and inactivate E2F family transcription factors, preventing cell cycle progression from the G₁ phase. Phosphorylation of pRb through G₁ cyclin-CDK complexes releases E2F family proteins from pRb and initiates the transcription of factors required for G₁-S transition. Ascofuranone also suppressed the transcription activity of ectopically expressed E2F. Ascofuranone decreased the expression of cyclins E and D in a p21^{WAF1/CIP1}-deficient cell line, whereas ascofuranone failed to arrest cells in the G₁ phase in this cell line. These results suggest that dissociation of the CDK2-cyclinE complex, but not reduction of G₁ cyclin expression, is prerequisite for G₁ arrest. The actions of ascofuranone on the cell cycle progression of human cancer cells are summarized in Fig. 6D.

Ascofuranone inhibits mitochondrial respiration (21, 23), and other respiratory inhibitors also induced the activation of p53 and transcription of its downstream targets

(29). Structure-activity relationships among ascofuranone-related compounds also indicate a correlation between respiration inhibition and p53 activation (29). These observations suggest that the ascofuranone-induced modulation of oxidative metabolism is involved in p21^{WAF1/CIP1} induction and c-Myc suppression. Indeed, respiratory inhibitors significantly suppressed c-Myc expression and increased p21 expression. Hypoxic conditions induce p21^{WAF1/CIP1} transcription and G₁ arrest through a mechanism involving hypoxia inducing factor-1 and c-Myc (45). In this response, hypoxia inducing factor-1 degraded in normoxic conditions by the ubiquitin/proteasome system is stabilized in hypoxic environments and displaces c-Myc bound to the proximal p21 promoter site through Miz-1, reducing the c-Myc-mediated transcription repression of the p21^{WAF1/CIP1} promoter. This mechanism, however, is less likely because a respiratory inhibitor, antimycin A, suppresses hypoxia inducing factor-1 expression (46).

c-Myc is degraded by the ubiquitin/proteasome system, which is regulated by phosphorylation of the NH₂-terminal of the protein (34). Because a signal transduction pathway downstream of epidermal growth factor receptor is involved in this phosphorylation, it may be possible that the inhibitory effect of ascofuranone in this signal transduction pathway (24–28, 47, 48) contributes to the degradation of c-Myc. In fact, we observed PD98059 and wortmannin suppressed c-Myc expression in this study. Taken together, these results suggest that modulation of signal transduction downstream of epidermal growth factor receptor, and mitochondria respiration is involved in ascofuranone-mediated c-Myc suppression, although further study is necessary to elucidate the precise mechanism for c-Myc downregulation by ascofuranone.

c-Myc is frequently overexpressed in human cancers because of genetic rearrangements such as gene amplification and chromosomal translocation (49). Deregulated c-Myc expression has been shown to drive vasculogenesis, reduce cell adhesion, and promote metastasis. Based on its novel effects on c-Myc, ascofuranone may provide a new therapeutic approach to the prevention and treatment of various cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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