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Ascochlorin, an isoprenoid antibiotic, induces G1 arrest via downregulation of c-Myc in a p53-independent manner

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ABSTRACT

Numerous anti-cancer agents inhibit cell cycle progression via a p53-dependent mechanism; however, many of these carcinostatic substances are toxic. Here, we show that ascochlorin, an isoprenoid antibiotic, is a non-toxic anti-cancer agent that induces G1 arrest via the induction of $p21^{WAF1/CIP1}$ in a c-Myc, but not a p53, dependent manner. Ascochlorin has a broad spectrum of anti-tumor and anti-metastatic activities, but the molecular mechanism by which it inhibits cell cycle progression of cancer cells remains to be elucidated. We demonstrated that cytostatic G1 arrest by ascochlorin is mainly associated with the upregulation of $p21^{WAF1/CIP1}$, and the downregulation of c-Myc. Furthermore, we used a chromatin immunoprecipitation assay, RNA interference, and p53-deficient cells to verify that $p21^{WAF1/CIP1}$ induction by ascochlorin is related to transcriptional repression of c-Myc. Ascochlorin abolished pRB hyperphosphorylation, which resulted in the inactivation of E2F transcriptional activity. These results suggest that ascochlorin induces G1 arrest via the p53-independent suppression of c-Myc. Thus, we reveal a role for ascochlorin in inhibiting tumor growth via G1 arrest, and identify a novel regulatory mechanism for c-Myc.

1. Introduction

Cancer cells undergo cytostasis or apoptosis in response to DNA damage. Upon DNA damage, cancer cells can recognize damaged DNA via members of the phosphoinositide-3-kinase(PI3K)-related protein kinase family, such as ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3-related (ATR), and the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) [1], which bind to damaged DNA, phosphorylate it, and activate p53. The pivotal roles of p53 in the regulation of DNA damage responses are well documented, and p53 is known to govern the downstream target genes responsible for cell cycle arrest ($p21^{WAF1/CIP1}$) or apoptosis (Bax, Puma, and Noxa) [2]. However, p53 can also be activated by oncogenes, such as Ras and Myc, independently of DNA damage [3]. The oncogenes activate p53 via $p14^{ARF}$, which binds to Hdm2. Based on the novel roles of p53, anti-cancer agents that activate p53 without resulting in DNA damage may be ideal carcinostatic substances.

The anti-cancer properties of the antibiotic ascochlorin (Fig. 1A) have been characterized in a number of cancer cell lines. Ascochlorin was originally isolated as a hypolipidemic substance from a culture broth of the phytopathogenic fungus, *Ascochyta viciae* [4]. Although ascochlorin-related compounds were originally reported to be antiviral antibiotics [5], they exhibit a variety of physiological effects, including the promotion of hypolipidemic activity [6], suppression of hypertension [7], amelioration of type I and II diabetes [8], immunomodulation [9], and anti-tumor activity [10,11].

In the present study, we report that ascochlorin can induce cell cycle arrest at the G1 phase in human cancer cell lines without causing damage to the DNA. Ascochlorin activates $p21^{WAF1/CIP1}$ by disrupting c-Myc in a p53-independent manner. Our results suggest that the induction of G1 arrest by ascochlorin is associated with the phosphorylation status of pRB and transcriptional activity of E2F, and emphasize that G1 arrest by the non-toxic anti-cancer agents is caused by inhibiting c-Myc in a p53-independent manner in human cancer cell lines.

2. Materials and methods

2.1. Cells and materials

U2OS, HCT116 cells, and HCT116 sub-lines deficient in p53 or $p21^{WAF1/CIP1}$ were grown in DMEM high glucose medium (GIBCO-BRL, Invitrogen Corp., Carlsbad, CA, USA), 1% Antibiotics (penicillin and streptomycin; GIBCO-BRL, Invitrogen Corp., Carlsbad, CA, USA), and 10% heat-inactivated fetal bovine serum (FBS) (GIBCO-BRL, Invitrogen Corp., Carlsbad, CA, USA), and incubated at 37 °C in a

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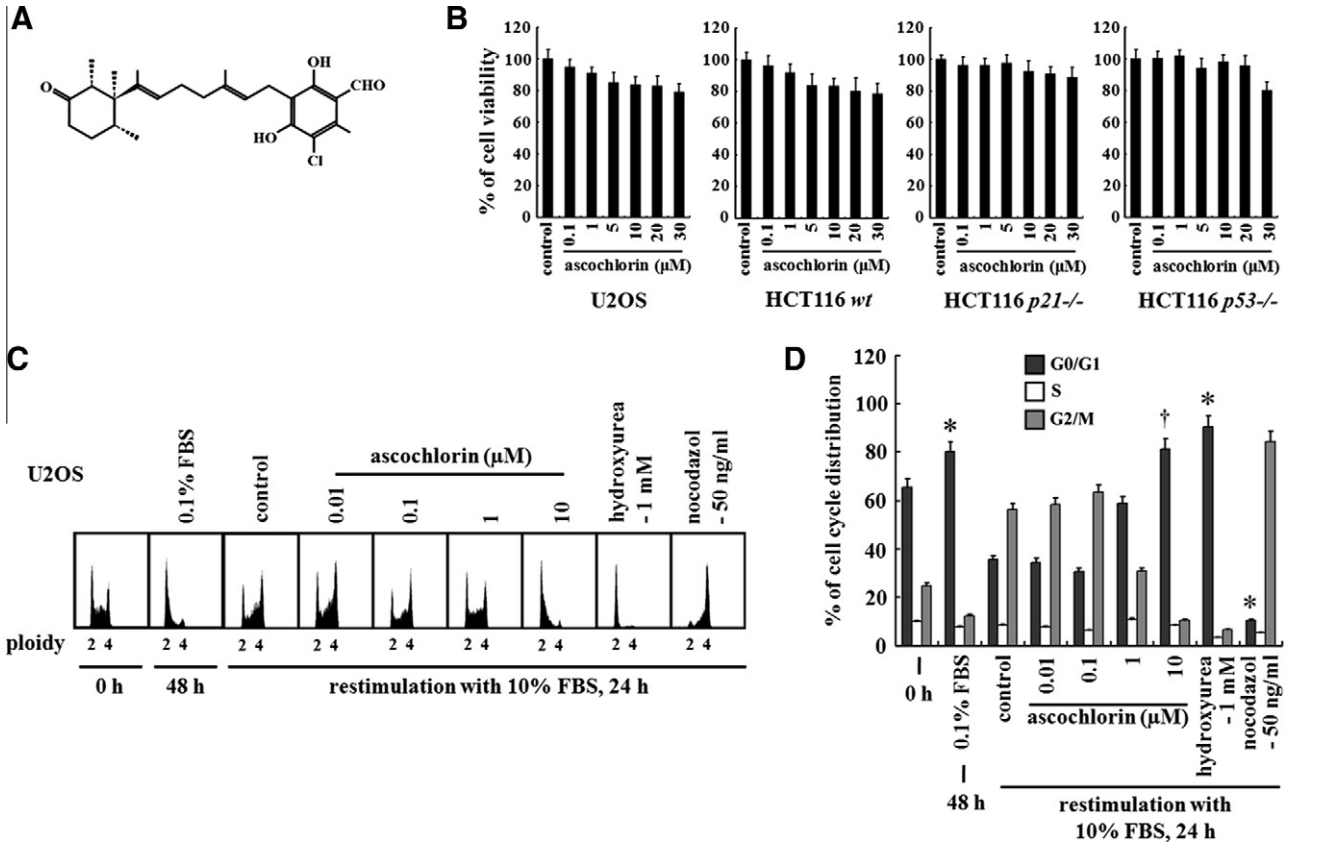

Fig. 1. G1 arrest induced by ascochlorin. (A) Chemical structure of ascochlorin. (B) Dose-dependent effect of ascochlorin on the viability of several human cancer cell lines. Cells were treated with various concentrations of ascochlorin for 24 h. Viability was determined using the MTT assay. Values represent the mean ± SD of triplicate experiments. (C) Cell cycle distributions of U2OS cells treated with ascochlorin for 24 h, as determined by flow cytometry. (D) Cell cycle distribution was quantified using a densitometer, and the data are presented as the mean ± SD of three independent experiments. $^{*}P < 0.01$, statistically significant compared to the untreated control.

humidified atmosphere of 5% CO_2. Ascochlorin was supplied by Chugai Pharmaceutical (Tokyo, Japan).

2.2. Cytotoxicity assays

Cell viability was determined using a Cell Proliferation Kit I (MTT) (Roche Applied Science, Mannheim, Germany), which measures mitochondrial respiratory function. Cells (1×10^4 cells/well) were cultured in serum-containing medium in the presence of 10 μM ascochlorin for 24 h and then incubated with 100 μg/well MTT dye for 4 h. The resultant formazan deposits were solubilized with 20 μl of 10% SDS, and the absorbance was measured at 590 nm. All data were calculated and expressed as a percentage of the A_{590} measured for the control (untreated) cells, which was set at 100%.

2.3. Cell cycle analysis

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

2.4. Immunoblot analysis

Immunoblotting was performed as described previously [12], using first antibodies and the corresponding second antibodies for whole immunoglobulins from mouse or rabbit (Amersham Biosciences, Buckinghamshire, UK). Specific proteins were detected using a BM Chemiluminescence Western Blotting Kit (Roche Diagnosis, Mannheim, Germany), 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), and pRb (C-15) were purchased from Santa Cruz Biotechnology Inc. (California, CA, USA). An anti-β-actin (C4) antibody was obtained from Abcam Ltd. (Cambridgeshire, UK).

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA), and reverse-transcriptase reactions were performed with a Superscript™ II RNase H-reverse transcriptase (Invitrogen Corp., Carlsbad, CA, USA) using total RNA (1 μg) from wild-type HCT116 cells and HCT116 sub-lines 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′-CTTTTTGGACTTCAGGTGGC-3′ (antisense); for *c-Myc*, 5′-TCGGGGCTTTATCTAACTCG-3′ (sense) and 5′-GCTGCTATGGGCAAAGTTTC-3′ (antisense); for *p21*, 5′-ATGTCAGAACCGGCTGGGGA-3′ (sense) and 5′-GCCGTTTTCGACCCTGAGAG-3′ (antisense); and for *β-actin*, 5′-GCCATCGTCACCAACTGGGAC-3′ (sense) and 5′-CGATTTCCCGCTCGGCCGTGG-3′ (antisense). PCR products were resolved electrophoretically on a 1.0% (w/v) agarose gel and visualized by staining with ethidium bromide.

2.6. Chromatin immunoprecipitation (ChIP) assay

A ChIP assay was performed as outlined by the Chromatin Immunoprecipitation (ChIP) Assay Kit (Upstate Biotechnology, Lake Placid, NY, USA). DNA-binding proteins were crosslinked to DNA and lysed in SDS lysis buffer containing 1× protease inhibitor. DNA was sheared to 200–500 bp fragments by sonication for 30 s in a VC100 sonicator (Sonics & Materials Inc., Danbury, CT, USA).

The chromatin solution was precleared with salmon DNA/protein A agarose 50% slurry for 30 min 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., California, CA, USA) 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 [13]: sense, 5′-ACCGGCTGGCCTGCTGGAACT-3′ and antisense, 5′-TCTGCCGCCGCT CTCTCACCT-3′. PCR products were separated on a 2% agarose gel.

2.7. RNA interference

HCT116 cells, at 50% confluency, were transfected with either a negative control siRNA, a c-Myc-specific siRNA duplex (Dharmacon Inc., Chicago, IL, USA), or a p53-specific siRNA duplex (Santa Cruz Biotechnology Inc., California, CA, USA) at 50 nM each, using Trans IT-TKO (Mirus Bio Corp., Madison, WI, USA), according to the manufacturer's instructions.

2.8. Luciferase assay

The human $p21^{WAF1/CIP1}$ promoter construct, p21P-luc (p21P), has been previously described [14]. For E2F1 promoter assays, E2F1-luc, E2F1-(DE2F)-luc, and [E2F]x4-luc reporter constructs were used [15]. The [E2F]x4 luciferase reporter, which contains four E2F sites bearing the sequence TTTCGCGC and the TATA box from the adenovirus E1B gene, was generated by subcloning the promoter from an [E2F]x4 chloramphenicol acetyltransferase reporter plasmid into pGL3-Basic. The expression plasmid for β-galactosidase (pCMV-β-gal) used for normalization was the kind gift of N. H. Heintz (University of Vermont, Burlington, VT, USA). Transient transfection was carried out with the Lipofactamine reagent (Invitrogen, Carlsbad, CA, USA). Cells were washed twice with PBS and lysed with 200 μl of 1× Reporter Lysis Buffer (Promega, Madison, WI, USA). Each lysate (50 μl) was examined for luciferase activity. Relative luciferase activity was determined after normalization with β-galactosidase activity.

2.9. Statistical analysis

All results are representative of at least three independent experiments performed in triplicate. Statistical significance between experimental and control values was calculated using the Newman–Keuls multicomparison test.

3. Results

3.1. Effect of ascochlorin on cell cycle progression of cancer cells

To investigate the inhibitory effect of ascochlorin (Fig. 1A) on the proliferation of U2OS and isogenic HCT116 variant ($p53^{WT}$, $p53^{-/-}$, and $p21^{WAF1-/-}$) cancer cell lines, we treated the cells with ascochlorin (0–30 μM) for 24 h and measured cell viability using the MTT assay. The reduction in cell viability of U2OS cells ranged from 6 to 20% ($P < 0.05$); in HCT116 $p53^{WT}$ cells, from 4 to 21% ($P < 0.05$); in HCT116 $p53^{-/-}$ cells, from 3 to 12% ($P < 0.05$); and in HCT116 $p21^{WAF1-/-}$ cells, from 0 to 20% ($P < 0.05$) after 24 h of ascochlorin treatment, as shown in Fig. 1B.

Since we previously demonstrated that ascochlorin upregulated $p21^{WAF1/CIP1}$ [12], we investigated whether ascochlorin might target cell cycle regulation in cancer cells, using flow cytometry. U2OS cells were starved in low-serum media containing 0.1% FBS for 48 h and were restimulated with complete media containing 10% FBS in the absence or presence of various concentrations (0.01–10 μM) of ascochlorin for 24 h. Upon starvation, 80.2% of U2OS cells were arrested in the G1 phase within 48 h, and then the cells drove into G2/M phase (55.4%) by treatment with 10% FBS to medium for 24 h, as shown in Fig. 1C. U2OS cells failed to progress to the G2/M phase following ascochlorin treatment, resulting in a dose-dependent increase in the number of cells in the G1 phase. The efficacious dose of ascochlorin was determined to be 10 μM. Cell cycle distribution data of ascochlorin-treated U2OS cells are summarized in Fig. 1D.

3.2. Transcriptional activation of $p21^{WAF1/CIP1}$ by ascochlorin

U2OS cells and isogenic HCT116 variant cells ($p53^{WT}$, $p53^{-/-}$, and $p21^{WAF1-/-}$) were treated with various concentrations (0.1–10 μM) of ascochlorin for 24 h. Whereas ascochlorin treatment resulted in a marked increase in the expression of p53 and $p21^{WAF1/CIP1}$ in p53 wild-type cells or p53-deficient cells (Fig. 2A), it did not enhance the expression of $p27^{KIP1}$. A significant increase in p21 mRNA in these cell lines was verified by RT-PCR, as described previously [12]. Moreover, this result was confirmed by reporter

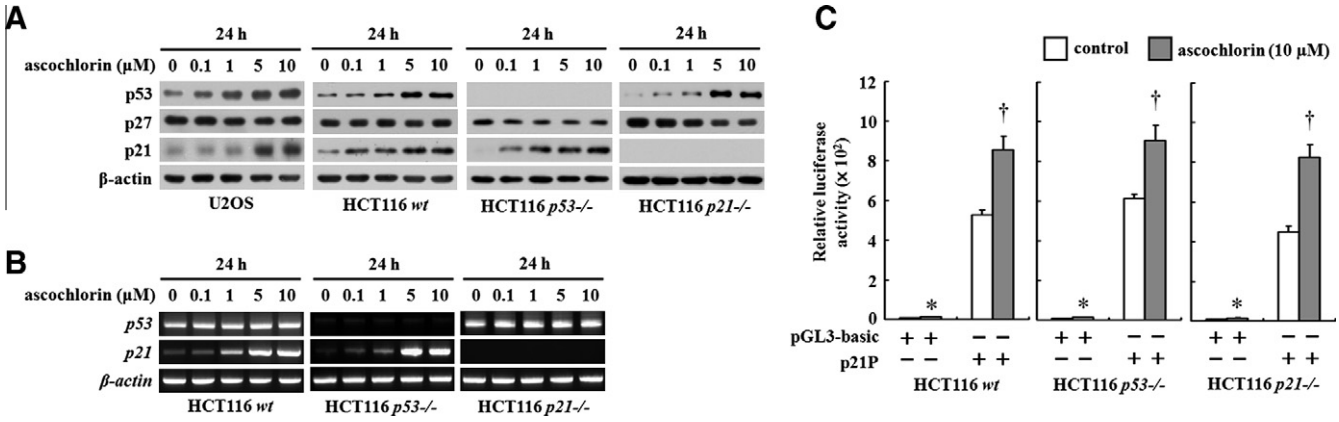

Fig. 2. Induction of $p21^{WAF1/CIP1}$ by ascochlorin. (A) Protein expression of ascochlorin-treated cells. Cells were treated with various doses of ascochlorin for 24 h, and the expression of p53, $p21^{WAF1/CIP1}$, and $p27^{KIP1}$ proteins was determined by immunoblot analysis. β-Actin was used to verify equal loading of samples. (B) RT-PCR analysis of mRNA expression of p53 and $p21^{WAF1/CIP1}$ was performed using total RNA prepared from HCT116 wild-type, HCT116 $p53^{-/-}$, and HCT116 $p21^{-/-}$ cells treated with various doses of ascochlorin for 24 h. (C) Suppression of $p21^{WAF1/CIP1}$ promoter activity of HCT116 and its sub-lines deficient in p53 or $p21^{WAF1/CIP1}$ following ascochlorin treatment. Cells were transfected with $p21^{WAF1/CIP1}$ reporter plasmid (p21P) or control plasmid (pGL3-basic) together with pCMV-β-gal. Relative luciferase activity after normalization against β-galactosidase activity is shown. The values represent the means ± SD of five independent experiments. Statistical significance was determined compared to untreated control (*$P < 0.05$, †$P < 0.01$).

experiments for the p21$^{WAF1/CIP1}$ promoter, which proved a significant increase in luciferase gene expression by ascochlorin treatment (Fig. 2C). These results indicate that the ascochlorin-induced increase in p21$^{WAF1/CIP1}$ expression is due to transcriptional activation.

3.3. Correlation of p21$^{WAF1/CIP1}$ expression with c-Myc downregulation following ascochlorin treatment

Because the protein level, mRNA level, and reporter gene activity of p21$^{WAF1/CIP1}$ in p53-deficient cells were enhanced by ascochlorin treatment (Fig. 2A–C), we hypothesized that c-Myc was another regulator of p21$^{WAF1/CIP1}$ related to ascochlorin-induced G1 arrest. Thus, we used immunoblot analysis to examine the effect of ascochlorin on the expression of c-Myc. We found that c-Myc protein levels were significantly suppressed by ascochlorin in a dose-dependent manner in p53 wild-type or p53-deficient cells (Fig. 3A), whereas c-Myc mRNA levels were not affected (Fig. 3B). These results suggest that post-transcriptional regulation is the main means of ascochlorin-induced downregulation of c-Myc.

Next, we used small interfering RNA (siRNA) with a c-Myc-specific or p53-specific siRNA duplex. We used immunoblot analysis to examine the expression of p53, c-Myc, and p21$^{WAF1/CIP1}$ in HCT116 cells transfected with siRNAs targeted against c-Myc or p53, or with non-specific siRNAs (negative control). We found that the ascochlorin-induced increase in p21$^{WAF1/CIP1}$ was suppressed by c-Myc knockdown and not by p53 knockdown, and that its basal expression level was also increased by c-Myc knockdown (Fig. 3C). These results suggest that c-Myc, but not p53, plays a central role in ascochlorin-induced p21$^{WAF1/CIP1}$ induction related to G1 arrest.

3.4. Ascochlorin-induced release of c-Myc from the endogenous p21$^{WAF1/CIP1}$ promoter

Previous studies reported that c-Myc is directly recruited to the p21$^{WAF1/CIP1}$ promoter by Miz-1, a DNA-binding protein, and that this association suppresses p21$^{WAF1/CIP1}$ expression [15,16]. To examine whether c-Myc binds the promoter region of p21$^{WAF1/CIP1}$ in ascochlorin-treated isogenic HCT116 variant cells (p53^{WT}, p53$^{-/-}$, and p21$^{WAF1-/-}$), we analyzed these cells with ChIP assays using antibodies against Miz-1, Sp1, and c-Myc. After immunoprecipitation with these antibodies, the promoter region of p21$^{WAF1/CIP1}$ was amplified by PCR using specific primer sets. The strong PCR product amplified from DNA isolated using the c-Myc antibody was completely eliminated by ascochlorin treatment, whereas the PCR products amplified from DNA isolated using antibodies against Miz-1 and Sp1 were not affected by ascochlorin treatment (Fig. 3D). These results suggest that ascochlorin dissociates c-Myc, an upstream repressor of p21$^{WAF1/CIP1}$, from the p21$^{WAF1/CIP1}$ promoter regardless of the presence or absence of p53, and specifically reduces c-Myc-mediated repression of the p21$^{WAF1/CIP1}$ promoter via the downregulation of c-Myc expression.

3.5. Effect of ascochlorin on phosphorylation of pRb protein and on E2F promoter activity

To elucidate the role of pRb in ascochlorin-induced G1 arrest, we assessed the phosphorylation status of pRb in the absence or presence of 10 μM ascochlorin, using immunoblot analysis and an antibody against pRb that detects a shift in the migration rate of pRb in serum-stimulated HCT116 cells. Stimulation by serum led to a

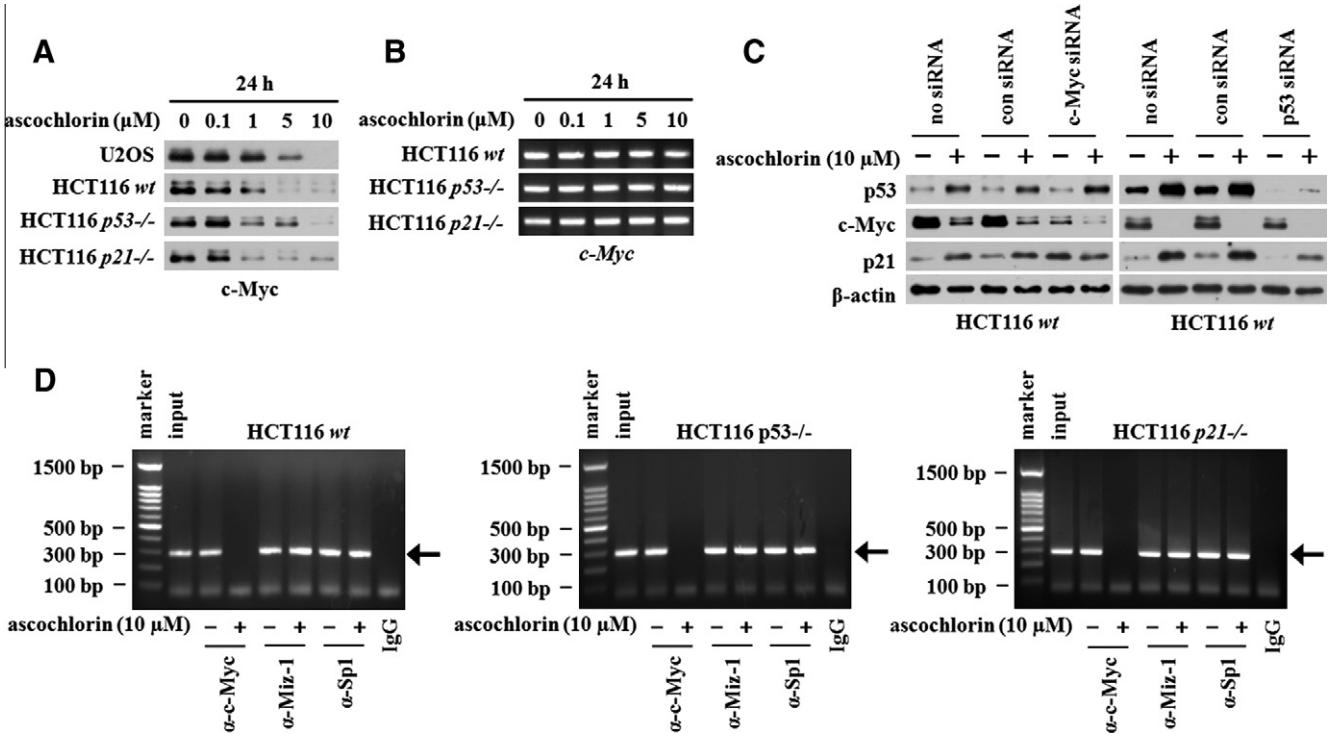

Fig. 3. Ascochlorin-induced G1 arrest is dependent on c-Myc, but not on p53. (A) c-Myc expression of U2OS, HCT116 wild-type, HCT116 p53$^{-/-}$, and HCT116 p21$^{-/-}$ cells treated with various doses of ascochlorin for 24 h, as determined by immunoblot analysis. (B) RT-PCR analysis of mRNA expression of c-Myc was performed using total RNA prepared from U2OS, HCT116 wild-type, HCT116 p53$^{-/-}$, and HCT116 p21$^{-/-}$ cells treated with various doses of ascochlorin for 24 h. (C) Cells were transfected with 50 nM of negative control (con) siRNA, or with siRNA duplex specific for c-Myc and p53. Ascochlorin was added 24 h after transfection, and the cells were cultured for another 24 h. Protein expression in c-Myc knockdown or p53 knockdown cells was analyzed by immunoblot. (D) HCT116 wild-type, HCT116 p53$^{-/-}$, and HCT116 p21$^{-/-}$ cells were treated with ascochlorin for 24 h, and c-Myc, Miz-1, and Sp1 bound to the p21 promoter were detected using the ChIP assay. Input: DNA from cell lysates prior to immunoprecipitation. Arrow indicates the target PCR product. Molecular weights (base pairs) of marker DNA are indicated to the left of each blot.

steady shift from the hypophosphorylation (pRb) to hyperphosphorylation (p-pRb) forms, 12–24 h after stimulation. In contrast, ascochlorin treatment effectively abolished this shift in phosphorylation status of pRb 12 h after stimulation, as shown in Fig. 4A.

Since E2F is regulated by its association with pRb [17], we examined the effects of ascochlorin on E2F transcriptional activity in serum-stimulated HCT116 cells. For E2F1 promoter assays, E2F1-luc, E2F1-(ΔE2F)-luc, and [E2F]x4 reporter constructs (Fig. 4B) were transfected alone or co-transfected with DP1 into HCT116 cells for 24 h, and luciferase activity was measured after exposure to ascochlorin over a 24 h period. As shown in Fig. 4D, E2F1-luc gene expression was activated up to ~2.7-fold in HCT116 cells transfected with pCMV-E2F1, and ~3.9-fold in HCT116 cells co-transfected with pCMV-E2F1 and pCMV-DP1, compared with pCMV-vector transfected cells. However, luciferase activity decreased ~1.6-fold following a 24-h treatment with 10 μM ascochlorin in HCT116 cells co-transfected with pCMV-E2F1 and pCMV-DP1. As expected, luciferase activity was not affected in HCT116 cells transfected with E2F1-(ΔE2F)-luc or co-transfected with pCMV-E2F1 and pCMV-DP1. For the [E2F]x4 luciferase reporter, luciferase activity was activated ~17.5-fold in HCT116 cells transfected with pCMV-E2F1, and ~22.9-fold in HCT116 cells co-transfected with pCMV-E2F1 and pCMV-DP1, compared with that in cells transfected with the pCMV vector. Similar to the results with E2F1-luc, [E2F]x4 luciferase activity was significantly decreased (~6.9-fold) by a 24-h treatment with 10 μM ascochlorin.

4. Discussion

Most anti-cancer agents tested in clinical trials activate p53 to promote the repair of DNA damage caused by genotoxins. The mechanisms of action of these agents include cross-linking of intra- or inter-strand DNA (interference with DNA replication and transcription), and inhibition of the activities of thymidilate synthase, topoisomerase I and II, and dihydrofolate reductase (disruption of DNA replication and protein synthesis) [18]. Since the agents do not specifically recognize tumor tissues, they can also damage healthy tissues in patients. These deleterious side-effects limit the usefulness of these agents as carcinostatic substances. In this regard, recent studies have focused on identifying chemotherapeutic agents that can increase the potency of genotoxins specifically in cancer cells or can function as tumor suppressors that do not cause DNA damage. Therefore, we examined the anticancer effects using ascochlorin, a non-DNA damaging agent.

We previously reported that ascochlorin and ascofuranone activate p53 by a distinctive mechanism involving mitochondrial respiration [12]. p53 activation by ascochlorin was accompanied by phosphorylation of p53 serine 392 with a negligible effect on the phosphorylation serine 15, which is a distinct pattern of phosphorylation to that seen after p53 activation by genotoxins, such as doxorubicin or etoposide. Ascochlorin has been shown to activate p53 against various tumors, but the precise molecular mechanism by which it inhibits cell cycle progression of cancer cells remains unclear. Hence, we investigated the molecular mechanisms by which ascochlorin exerts its effect on G1 arrest.

p53 is known to play a central role in the response to DNA damage; however, the induction of G1 arrest by ascochlorin did not involve p53, as shown in Figs. 2A–C and 3C–D. Therefore, we screened for the involvement of another cell cycle regulator in the G1 phase. Previous studies suggest that the choice of cell fate between cytostasis and apoptosis is determined by transcription factor Myc [19]. Furthermore, c-Myc acts as a transcription activator or a

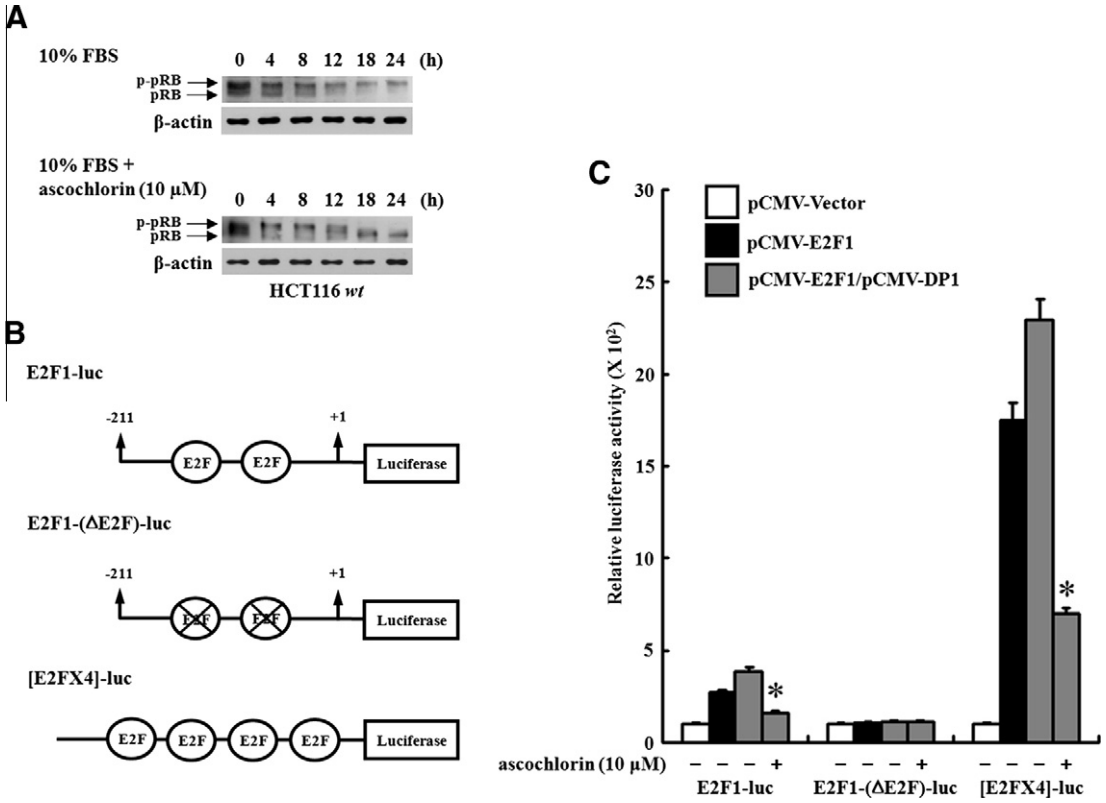

Fig. 4. Ascochlorin-induced inhibition of biochemical events in the G1-S transition. (A) HCT116 cells starved in low-serum medium containing 0.1% FBS for 72 h and restimulated with complete medium containing 10% FBS in the absence or presence of 10 μM ascochlorin for various periods of time. p-pRb denotes hyperphosphorylated pRb, and pRb denotes hypophosphorylated pRb. (B) Diagram of the reporter plasmids used in these experiments. (C) Suppression of E2F transcription activation by ascochlorin. HCT116 cells were transfected with the reporter plasmids for E2F together with expression plasmids for E2F1, DP1, and β-galactosidase. The values represent the means ± SD of five independent experiments after normalization for β-galactosidase activity. Asterisk (*), statistically significant compared to untreated control ectopically expressing E2F1 and DP1 ($P < 0.05$).

transcription repressor that regulates proliferation, mitogenesis, differentiation, and programmed cell death [20]. c-Myc represses differentiation-related transcription of $p21^{WAF1/CIP1}$ by interacting with Miz-1, a zinc-finger transcription factor. To further explore the mechanism by which ascochlorin facilitates $p21^{WAF1/CIP1}$ transcription, we performed a ChIP assay. A recent study reported that the c-Myc interaction partner, Miz-1, binds to and activates the transcription of the $p21^{WAF1/CIP1}$ promoter and inhibits cell cycle progression [21]. It has also been reported that Sp1 plays an essential role in $p21^{WAF1/CIP1}$ transcription through its binding with the $p21^{WAF1/CIP1}$ promoter [22]. We set out to determine whether Miz-1, Myc, and Sp1 bound to the $p21^{WAF1/CIP1}$ promoter using a ChIP assay, and observed that ascochlorin decreased the c-Myc level and relieved the repressive activity of c-Myc on $p21^{WAF1/CIP1}$, leading to activation of the $p21^{WAF1/CIP1}$ promoter by Miz-1 and induction of G1 arrest. As shown in Fig. 3D, $p21^{WAF1/CIP1}$ promoter DNA was precipitated with both antibodies against Miz-1 and Sp1, indicating that these proteins bound to the $p21^{WAF1/CIP1}$ promoter and regulated $p21^{WAF1/CIP1}$ expression as transcription factors. However, the antibody against c-Myc was released from the $p21^{WAF1/CIP1}$ promoter by ascochlorin, which resulted in activation of $p21^{WAF1/CIP1}$ transcription. Taken together, these results suggest that ascochlorin-induced G1 arrest by modulating the activity of selective proteins, and that the ascochlorin-induced enhancement of $p21^{WAF1/CIP1}$ expression was dependent on c-Myc, and not p53.

The CDK2/Cyclin E complex plays a central role in the G1-S transition and has been shown to phosphorylate Rb family members in the G1 phase of the cell cycle [23]. The Rb family includes pRb/p105, p107, and Rb2/p130, referred to as 'pocket proteins', which bind viral oncoproteins and cellular factors such as the E2F family of transcription factors [24]. The family of E2F transcription factors includes eight E2F proteins (E2F1–E2F8) and two DP proteins (DP1 and DP2) [24,25]. Hyperphosphorylated pRb is released from the E2F/DP complex. The free E2F/DP heterodimers bind to E2F sites to activate transcription, resulting in DNA synthesis and cell cycle progression. In the present study, hyperphosphorylation of the pRb family was inhibited by ascochlorin (Fig. 4A), which increased the binding of E2F1 to pRb, and thereby prevented E2F promoter activity (Fig. 4D). Our data indicate that ascochlorin inhibits the phosphorylation of pRb, and that this inhibition represses E2F1 transcriptional activity by promoting the binding of pRB with E2F1.

In summary, our results indicate that ascochlorin treatment causes cancer cells to accumulate at the G1 phase by inducing the activity of $p21^{WAF1/CIP1}$. The accumulation of $p21^{WAF1/CIP1}$ was caused by a release of c-Myc from the $p21^{WAF1/CIP1}$ promoter, resulting in $p21^{WAF1/CIP1}$ activation and loss of CDK2 activity following $p21^{WAF1/CIP1}$ association with CDK2. Moreover, induction of $p21^{WAF1/CIP1}$ by ascochlorin was p53-independent, as demonstrated by our RNA interference analysis and induction of $p21^{WAF1/CIP1}$ in HCT116 $p53^{-/-}$ cells. Finally, $p21^{WAF1/CIP1}$ activated by ascochlorin inhibited the hyperphosphorylation of pRb and increased the amount of pRb-bound E2F1 transcription factor, thereby decreasing E2F promoter activity. Based on its novel effects on the cell cycle, ascochlorin could affect multiple cellular c-Myc-related activities without causing DNA damage, and thus represents a potentially useful strategy for the prevention and/or treatment of various cancers.

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