Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical and Biophysical Research Communications 342 (2006) 101–106
BBRC
www.elsevier.com/locate/ybbrc

Aaptamine, a spongean alkaloid, activates p21 promoter in a p53-independent manner

Shunji Aoki [a], Dexin Kong [a], Hideaki Suna [a], Yoshihiro Sowa [b], Toshiyuki Sakai [b], Andi Setiawan [c], Motomasa Kobayashi [a,*]

[a] Graduate School of Pharmaceutical Sciences, Osaka University, Yamada-oka 1-6, Suita, Osaka 565-0871, Japan
[b] Department of Molecular-Targeting Cancer Prevention, Kyoto Prefectural University of Medicine, Kawaramachi-Hirokoji, Kamigyo-ku, Kyoto 602-8566, Japan
[c] Department of Chemistry, Faculty of Science, Lampung University, Jl. Prof. Dr. Sumantri Brodjonegoro No. 1, Bandar Lampung 35145, Indonesia

Received 14 January 2006
Available online 3 February 2006

Abstract

Aaptamine, a benzonaphthyridine alkaloid was isolated from a marine sponge on the guidance of a bioassay using the transfected human osteosarcoma MG63 cells (MG63luc^{+}). Aaptamine activated p21 promoter stably transfected in MG63 cells dose-dependently at the concentrations of 20–50 μM. Expression of p21 and its mRNA in the wild-type MG63 cells also increased by aaptamine-treatment. Furthermore, the cell cycle of MG63 cells was arrested at the G2/M phase within 48 h by the aaptamine-treatment. To analyze a responsive element of p21 promoter in the up-regulation of p21 by aaptamine, MG63 cells were transiently transfected with a series of the deleted or mutated promoter segments, and induction of luciferase with aaptamine treatment was examined by using these corresponding transfected cells. The activation of p21 promoter by aaptamine was led through acting Sp1 sites between −82 and −50 bp in a p53-independent manner.

Keywords: Aaptamine; p21 promoter; MG63 cells; Sp1 sites; G2/M arrest

Abnormal proliferation, which caused by the disruption of the cell cycle regulation, is a major undesirable property in tumor cells. Cell proliferation is mainly regulated by interactions between two families of proteins, cyclin-dependent kinase (CDK)–cyclin complexes and CDK inhibitors (CKIs). CDK–cyclin complexes promote the progression of cell cycle in an organized manner, while CKIs inhibit it for checking and repairing the damage on chromosome. P21 was originally identified as a target protein of p53 [1] and is known to be an inhibitor of CDK–cyclin complexes [2] to act as a negative regulator of the cell cycle progression as a brake. The p21 expression is mainly controlled by diverse mechanisms in a p53-dependent manner. Due to the accumulating evidence that p53 is mutated in many human cancer cells [3,4], mutation of p53 has been recognized as one of the major events in carcinogenesis, while mutation of p21 is rarely observed in human tumors [5,6]. Therefore, the agents that induce an increase of p21 expression in a p53-independent manner might contribute to cancer prevention or treatment. The approach using such an agent has been termed ''gene-regulating chemotherapy or prevention'' [7].

As a part of our discovery of bioactive substances from marine organisms, we have been searching for new anticancer agents by using various screening bioassay methods. To date, we have found some interesting lead compounds such as long-chain acetylene alcohol (lembehyne A) [8,9], which induces neurite outgrowth of murine neuroblastoma Neuro 2A cells, sesquiterpene aminoquinones (smenospongine) [10,11], and pentacyclic guanidine alkaloid (crambescidin 800) [12], which induces erythroid differentiation of human chronic myelogenous leukemia K562 cells. Interestingly, all

* Corresponding author. Fax: +81 6 6879 8219.
E-mail address: kobayasi@phs.osaka-u.ac.jp (M. Kobayashi).

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.01.119

of these compounds induce an increase of p21 expression in the corresponding cells [9,10,12]. These results prompted us to search for substances, which induce p21 expression, from natural sources by a more direct and simple bioassay method.

Recently, we constructed a bioassay method using the p53-mutated human osteosarcoma MG63 cells to search for substances that activate p21 promoter in a p53-independent manner [7]. Guided by this bioassay method, we isolated aaptamine, a benzonaphthyridine alkaloid, from an Indonesian marine sponge.

Materials and methods

Materials. Gel cassettes (4-20%) were purchased from Daiichi Pure Chemicals Co., Ltd. DNA-Prep Reagents Kit was purchased from Coulter Co., Ltd. Dulbecco's modified Eagle's medium (DMEM) was purchased from Nissui Pharmaceutical Co., Ltd. Polyvinylidene difluoride (PVDF) membrane was purchased from Amersham Pharmacia Biotec UK, Ltd. Other reagents were purchased from Sigma Co., Ltd or Wako Pure Chemical Industries Co., Ltd, except for those separately described in methods.

Plasmid preparation. The full length human p21 promoter-luciferase fusion plasmid (pWWP) and a series of the mutant or deleted p21 promoter-luciferase fusion plasmids (pWP124, pWP101, pWP101-mtSp1-3, pWP101-mtSp1-4, pWP101-mtSp1-5,6, and pWPdel-SmaI) were constructed as described previously [7,13].

Cell culture. Human osteosarcoma MG63 cells and the stably transfected cells (MG63luc$^+$) with p21 promoter-luciferase fusion plasmid were routinely maintained in the DMEM supplemented with 10% fetal bovine serum, 100 μg/ml of kanamycin, and 0.44 mg/ml of glutamine at 37 °C in a humidified atmosphere containing 5% CO_2.

Luciferase assay for evaluation of p21 promoter activation. The luciferase assay was performed using the luciferase assay system (E1501, Promega). The suspension of the transfected MG63 cells (5×10^4 cells/ml/well) was incubated in a 12-well plate for 24 h. The testing sample was added as the 5 μl of EtOH solution and further incubated for 24 h. The cells were washed twice with D-PBS (−) solution. After addition of the 100 μl of 1 × lysis buffer to each well, the plate was shaken at room temperature for 15 min, then centrifuged at 2000*g*, 4 °C. The 10 μl of supernatant was removed to a 96-well plate (96F untreated white microwell SH) available for luminescence measurement. The 50 μl of luciferase assay substrate was added to each well, and the light intensity was measured by using MICRO LUMAT Plus LB96V (BERTHOLD) and WING LOW software. The activation of p21 promoter was evaluated by the relative light intensity compared with that of the control (cells treated with EtOH).

Isolation and identification of aaptamine. Aaptamine was isolated from the marine sponge of *Aaptos suberitoides* (collected at Carita, the west end of Java Island, Indonesia in 2003) on the guidance of the luciferase assay described above. The dried marine sponges were cut into pieces and soaked in methanol overnight. The resulting methanol extract was subjected to solvent partition to give AcOEt, *n*-BuOH, and H_2O soluble portions. The active *n*-BuOH soluble portion was separated by repeated SiO_2 column, and high-performance liquid chromatography (HPLC) to obtain an active compound. The active compound was identified as aaptamine by comparison of the mass and NMR data with the authentic compound [14]. Aaptamine was first isolated from the marine sponge of *Aaptos aaptos* (Fig. 1) [14].

Extraction of total RNA and RT-PCR analysis. MG63 cells (1×10^6 cells/8 ml) were cultured with 30 μg/ml of aaptamine. The cells were collected after 0, 3, 12, 24, and 48 h, respectively, and total RNA was extracted by Sepasol (Nacalai Tesque, Inc.). First strand cDNA was synthesized by using 6 μg of RNA with Superscript III reverse transcriptase and oligo $(dT)_{12-18}$ primers. The reaction mixture was incubated at 50 °C for 1 h followed by treatment with RNase A at 37 °C for 15 min.

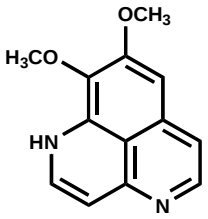

Fig. 1. Chemical structure of aaptamine.

The equivalent cDNA products were used to perform PCR with the treatment of TaKaRa PCR Amplification Kit by TaKaRa PCR Thermal Cycler MP. The primer sequences for the p21 gene (318 bp) were 5′-ATTA GCAGCGGAACAAGGAGTCAGACAT-3′ as the forward primer, and 5′-CTGTGAAAGACACAGAACAGTACAGGGT-3′ as the reverse primer. Samples were amplified with a precycling hold at 94 °C for 10 min, followed by 35 cycles of denaturation at 94 °C, annealing at 55 °C, and extension at 72 °C for 30 s each, and finally 72 °C for 2 min. The reaction mixture was electrophoresed on 2% agarose gel. After being stained with ethidium bromide, the DNA level was detected by FluoroImager (Amersham Biosciences). As for control data of RT-PCR, the DNA level of β-actin was analyzed.

Western blot analysis. The cell suspension (1×10^6 cells/8 ml) of MG63 cells was incubated with 30 μg/ml of aaptamine for the indicated times under a 5% CO_2 atmosphere at 37 °C. The cells were harvested by centrifugation (1000*g* for 3 min at 4 °C), washed with cold D-PBS (−) solution, and treated with lysis buffer (50 mM Tris–HCl, pH 7.2; 1% NP-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EDTA; 1 mM PMSF; 1% proteinase inhibitor cocktail) to furnish a cell lysate. Protein assay was carried out by Bio-Rad protein assay kit. After boiling at 95 °C for 5 min in the sample buffer (0.125 M Tris–HCl, pH 6.8; 10% 2-mercaptoethanol; 4% SDS; 10% sucrose; 5% bromophenol blue), the equal amounts of protein were subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and then transferred to PVDF membrane. The membrane was blocked with 5% milk TBS (Tween PBS), exposed to anti-Cip1/WAF-1/p21 (Upstate Biotechnology, Inc.) and then to the anti-mouse HRP-conjugated antibody (Nacalai Tesque, Inc.). The bound antibody was finally visualized by enhanced chemiluminescence (ECL) system.

Flow cytometric analysis of cell cycle. The suspension (2×10^5 cells/2 ml/well) of MG63 cells was placed in an 8-well plate and incubated for 24 h at 37 °C under a 5% CO_2 atmosphere. The testing sample (10 μl) was added as a EtOH solution and further incubated for 48 h. Then the cells were harvested and washed twice with cold D-PBS (−). The cells were then dyed by DNA-Prep Reagents Kit for 20 min. After centrifugation at 1000*g*, the supernatant was removed. Five hundred microliters of D-PBS (−) was added to the cell pellet, and the cell suspension was filtered with a 40 μm nylon mesh filter for cell cycle analysis. The analysis was carried out by flow cytometer (FACS Calibur, Beckton Dickinson, $\lambda_{ex} = 493$ nm, $\lambda_{em} = 630$ nm) and quantified by ModFit Software (Verity Software, Topsham, ME).

Transient transfection. MG63 cells were transfected by DEAE–Dextran (CellPhect Transfection Kit, Amersham Pharmacia Biotech). The cells were incubated at a density of 5×10^4 cells/ml in a 10 cm diameter dish for 24 h. The 125 ng of reporter plasmid DNA in DEAE–Dextran was added to perform transfection for 15 min. The transfected cells were further incubated for 24 h and then incubated with or without aaptamine for 24 h. Finally, the cells were collected for luciferase assay as described above.

Results

Activation of p21 promoter by aaptamine in MG63luc$^+$ cells

The activation of p21 promoter by aaptamine in MG63luc$^+$ cells was examined (Fig. 2). The relative luciferase activity of the aaptamine-treated cells indicates

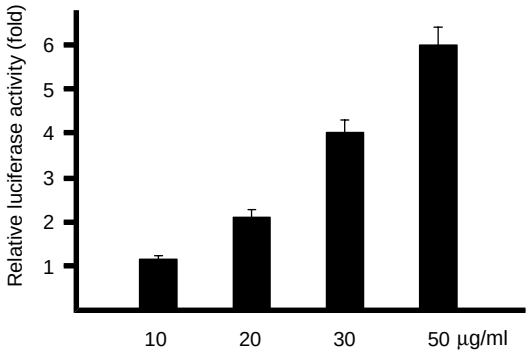

Fig. 2. Activation of p21 promoter by aaptamine in MG63luc^{+} cells. The MG63luc^{+} cells were treated with or without aaptamine for 24 h. The cells were treated with lysis buffer, and the resulting lysate was analyzed by luciferase assay. The activation of p21 promoter was evaluated by the relative light intensity compared with that of the control.

intensification of the activation of p21 promoter in comparison with that of the untreated cells. Aaptamine showed activation of p21 promoter dose-dependently and luciferase expression was increased approximately 6-fold over the untreated cells at the 50 μg/ml concentration.

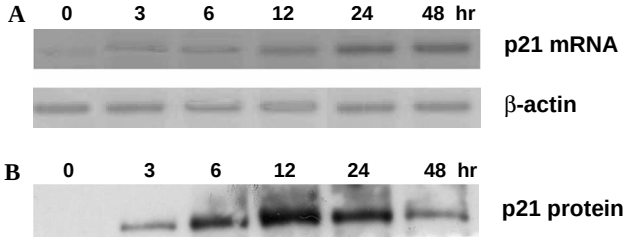

Fig. 3. Detection of p21 expression by RT-PCR and Western blot analyses. MG63 cells (1×10^{6}) were incubated with 30 μg/ml of aaptamine for the indicated times. (A) RT-PCR analysis. Total RNA was extracted from the MG63 cells treated with aaptamine for the indicated times. First strand cDNA was synthesized from the mRNA as a template. PCR reaction was performed to amplify the synthesized cDNA. The reaction mixtures were electrophoresed on 2% agarose gel. After being stained with ethidium bromide, the DNA levels were detected by FluoroImager. (B) Western blot analysis. Cell lysates were prepared and applied to 4–20% SDS PAGE. After being transferred to PVDF membrane, the blots were exposed to anti-Cip1/WAF-1/p21, and then to anti-mouse HRP conjugated IgG antibody.

The effect of aaptamine on p21 expression in the wild-type MG63 cells

The effect of aaptamine on p21 expression in the wild-type MG63 cells was examined. MG63 cells were treated with aaptamine (30 μg/ml) and incubated 0, 3, 6, 12, 24, and 48 h, respectively. The cells were harvested and analyzed by Western blot and RT-PCR. The expression of both p21 protein and its mRNA was induced within 3 h and continued throughout 48 h (Fig. 3).

The effect of aaptamine on the cell cycle in MG63 cells

P21, a member of the Cip/Kip family of CDK–cyclin inhibitors (CKIs), is known to regulate the cell cycle as a brake. Then, the effect of aaptamine on the cell cycle progression was analyzed. Randomly cultured MG63 cells were treated with 30 μg/ml of aaptamine for 48 h and then the cell cycle was analyzed by a flow cytometer. Aaptamine arrested the cell cycle of MG63 cells at the G2/M phase (Fig. 4).

Deletion and mutation analyses of p21 promoter in MG63 cells

We further investigated the sites through which aaptamine activates p21 promoter by transient transfection assay. The full-length p21 promoter (pWWP) and a series of the deleted or mutated p21 promoter plasmids as diagrammed in Fig. 5A, which were fused with luciferase reporter, were used for this analysis. PWP124, which lacks the two p53 binding sites around −2.3 and −1.4 kb, respectively [15], was fully activated by aaptamine (Fig. 5B). This indicates that aaptamine activates p21 promoter in a p53-independent manner. The pWP124 plasmid includes six Sp1 segments (Fig. 5A). Sp1-1 (−119 to −114 bp), Sp1-2 (−109 to −104 bp), Sp1-3 (−82 to −76 bp), and Sp1-4 (−70 to −64 bp) are independent, whereas Sp1-5 (−60 to −53 bp) and Sp1-6 (−56 to −50 bp) are partly overlapped [15]. To investigate whether these Sp1 segments participate in the activation by aaptamine, two deletion plasmids,

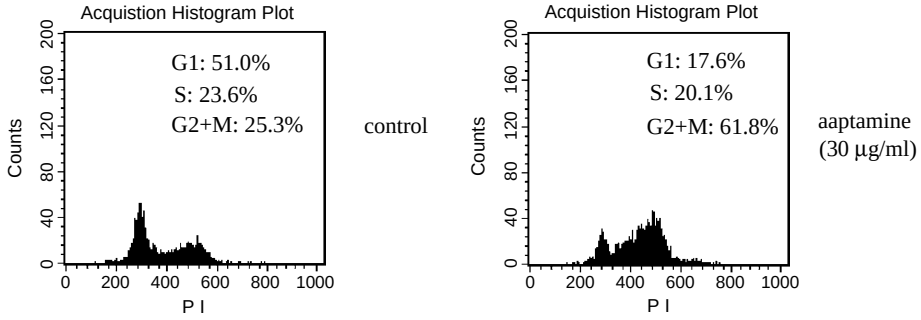

Fig. 4. Effect of aaptamine on the cell cycle of MG63 cells. A suspension of MG63 cells (2×10^{5}) was incubated in the presence or absence of aaptamine under 5% CO_2 at 37 °C for 48 h. The collected cells were dyed with DNA-Prep Reagents Kit and analyzed by flow cytometer ($\lambda_{ex} = 493$ nm, $\lambda_{em} = 630$ nm). The analysis was quantified by ModFit software.

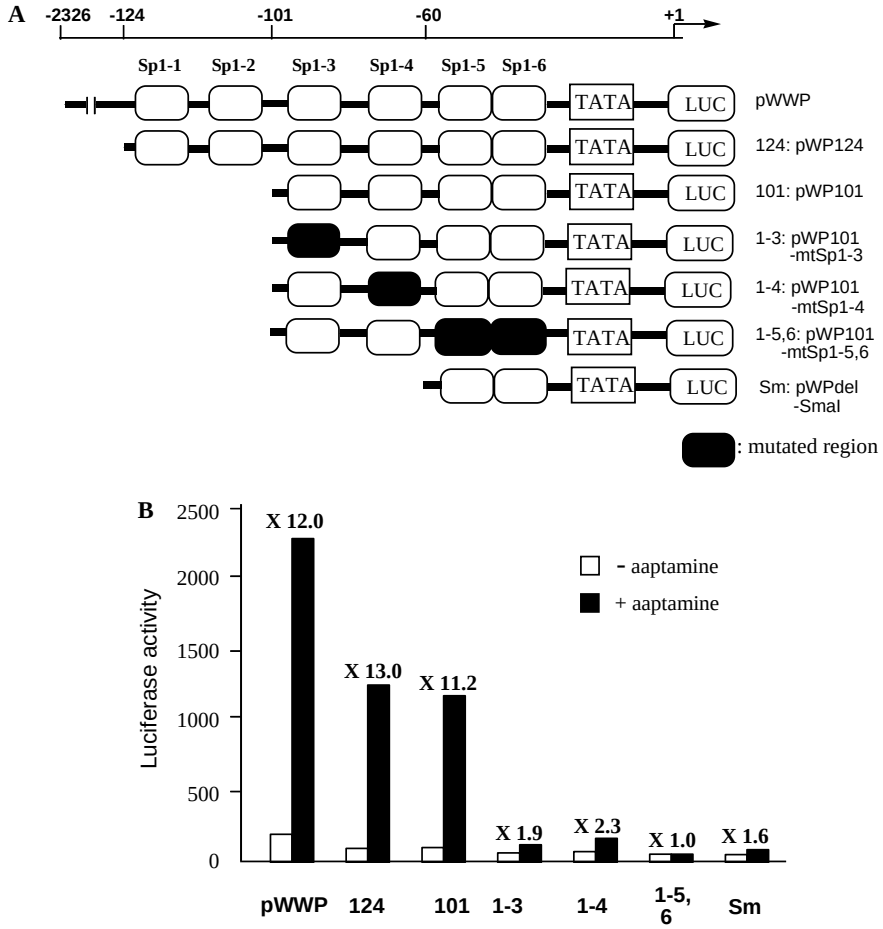

Fig. 5. Deletion and mutation analyses of p21 promoter in MG63 cells. (A) Schematic representation of p21 promoter-luciferase fusion plasmids: full size (pWWP), deletion (pWP124, pWP101, and pWPdel-SmaI), mutation (pWP101-mtSp1-3, pWP101-mtSp1-4, and pWP101-mtSp1-5,6). The open boxes represent the locations of Sp1 sites, and those in black indicate mutant Sp1 sites. LUC represents the luciferase reporter gene. The transcription start site is indicated as +1. The locations of the 5′ ends of the promoters are indicated by the negative numbers of nucleotides relative to the start site of transcription. (B) Transient transfection of MG63 cells with the constructed plasmids and luciferase assay. MG63 cells (5×10^4) were transfected with the respective fusion plasmids for 24 h and treated with or without 50 μg/ml of aaptamine for 24 h, and then analyzed by luciferase assay. Fold induction of luciferase by aaptamine was calculated in comparison with the luciferase activity of the untreated cells (open bars). The result represents three independent experiments.

pWP101 lacking Sp1-1 and Sp1-2, and pWPdel-SmaI lacking Sp1-1 to Sp1-4, were transfected into MG63 cells, and luciferase activity in the presence or absence of aaptamine was examined. Deletion of Sp1-1 and Sp1-2 sites did not affect the aaptamine-induced activation of p21 promoter. In contrast, further deletion of Sp1-3 and Sp1-4 sites caused a dramatic reduction of the aaptamine-induced activation, indicating that Sp1-3 and Sp1-4 segments were important for the activation of p21 promoter by aaptamine (Fig. 5B).

To further define the aaptamine-responsive elements, we constructed three pWP101 promoter plasmids with the respective Sp1 site mutation (Fig. 5A). In both the pWP101-mtSp1-3 and pWP101-mtSp1-4 transfected MG63 cells, luciferase expression by aaptamine-treatment was obviously reduced to about one-fifth of that of pWP101, while aaptamine did not activate luciferase expression in the pWP101-mtSp1-5,6 transfected MG63 cells at all (Fig. 5B).

Discussion

Disruption of the checkpoint of the cell cycle has been known as a major cause of carcinogenesis and correspondingly leads to abnormal proliferation of tumor cells [16]. Therefore, agents that arrest the cell cycle and repair the checkpoint are expected to be novel anticancer drugs with cytostatic growth inhibition of tumor cells. The cell cycle inhibitors such as flavopiridol, UCN-01, and FK228 have proceeded to the clinical investigation phase as promising anticancer candidates [17].

Cell cycle progression is promoted by CDK–cyclin complexes and is inhibited by an inhibitor of cyclin-dependent kinase (CKIs), which suppresses kinase activity of CDK–cyclin complexes. CKIs are divided into the INK4 family (p15, p16, p18, and p19) and the Cip/Kip family (p21, p27, and p57) based on the primary structure and the specificity against their target. The INK4 family specifically inhibits CDK4- or CDK6–cyclin D complexes and arrests

the cell cycle at the G1 phase in an Rb-dependent manner [18]. On the other hand, the Cip/Kip family has broader specificity for CDK–cyclin complexes [19]. Among the Cip/Kip family members, p21 is well studied. Accumulating evidence has shown that p21 expression is closely related to differentiation or apoptosis of cancer cells, indicating its high potential as a target for cancer chemotherapy.

The p21 expression is controlled by diverse mechanisms in a p53-dependent or independent manner. The mutation of p53 has been recognized as one of the major events in carcinogenesis, because the mutation of p53 is found in more than 50% of human cancer cells [3,4], while the mutation of p21 in human cancers is rare [5,6]. Therefore, the agents that induce p21 expression through a p53-independent pathway might contribute to chemotherapy or prevention of cancer as molecular-targeted drug candidates.

Based on this background, we started a project to search for agents from natural sources that can induce p21 expression in a p53-independent manner. We established a stably transfected human osteosarcoma MG63 cell (MG63luc$^+$) with p21 promoter fused with the luciferase reporter gene. The MG63 cell is known to be p53 negative. We started the screening assay using MG63luc$^+$ cells to evaluate activity of the testing sample to activate p21 promoter. The screening of up to 500 extracts from natural sources led to the discovery of the marine sponge of *Aaptos suberitoids* and aaptamine was isolated as the major active component in line with this bioassay. Aaptamine was first isolated from the marine sponge of *Aaptos aaptos* as an α-adrenoceptor blocking active compound [14]. As shown in Fig. 2, aaptamine induced luciferase expression in MG63luc$^+$ cells dose-dependently, and the relative luciferase activity by aaptamine-treatment at the 50 μg/ml concentration was 6-fold stronger than that of the control. Moreover, the effect of aaptamine on p21 expression was investigated by using the wild-type MG63 cells. The p21 protein and its mRNA of the wild-type MG63 cells were not detectable by Western blot and RT-PCR analysis. On the other hand, the wild-type MG63 cells treated with aaptamine induced an expression of both p21 protein and its mRNA within 3 h and remained elevated for 48 h. Since MG63 cells are p53 negative, this result indicated that aaptamine induced p21 expression through a p53-independent pathway.

Next, we examined the effect of aaptamine on the cell cycle of MG63 cells. As shown in Fig. 4, aaptamine arrested the cell cycle mainly at the G2/M phase after 48 h treatment. Although p21 was initially suggested to be involved in the G1 checkpoint, it has been later shown to also play a crucial role in the G2/M checkpoint [20]. Recently, Choi et al. [21] and Sato et al. [22] reported that the induction of p21 led to the G2/M arrest of the cell cycle through a p53-independent pathway.

To analyze a responsive element of p21 promoter in the up-regulation of p21 by aaptamine, MG63 cells were transiently transfected with a series of the deleted or mutated promoter segments, and induction of luciferase with aaptamine-treatment was examined by using these corresponding transfected cells. The relative luciferase activity for the pWP124-transfected MG63 cells, which lacks a p53 binding site, was similar to that of the MG63 cells transfected by the pWWP plasmid. This evidence supported that the p21 induction by aaptamine in MG63 cells was p53-independent. Furthermore, the relative luciferase activity for the pWP101-transfected MG63 cells was also similar to that of the pWWP transfected MG63 cells. This indicates that the deletion of Sp1-1 and Sp1-2 sites did not affect the aaptamine-induced activation of p21 promoter. On the other hand, the MG63 cells transfected with the pWP101-mtSp1-5,6 plasmid showed no activity at all. Moreover, when either Sp1-3 or Sp1-4 site was mutated, aaptamine showed much weaker activity compared with pWP101. This evidence implies that Sp1-3, Sp1-4, Sp1-5,6 each plays an important role in the activation of p21 promoter by aaptamine.

Several small molecules have been reported as activators of p21 promoter through a p53-independent pathway. We previously reported that butyrate and trichostatin A activate p21 promoter through Sp1-3 to 6 sites (−82 to −50 bp) [7,13]. Biggs et al. reported that the region between −122 and −61 bp (including Sp1-1 to Sp1-4 sites) is required for the activation of p21 promoter by phorbol esters and okadaic acid [23]. Lovastatin, an HMG-CoA reductase inhibitor, was also reported to activate p21 promoter through Sp1-3 site [24]. Interestingly, all the compounds activate p21 promoter through Sp1 sites.

To investigate the specificity of the activation of p21 promoter by aaptamine, we transfected human breast adenocarcinoma MCF-7 cells with plasmids containing p16 or p19 promoter fused with luciferase reporter, respectively, and evaluated the activation of p16 or p19 promoter by aaptamine. Luciferase activity was not increased by aaptamine-treatment in either p16 or p19 promoter-luciferase transfected cells (data not shown). This evidence indicates that aaptamine activates p21 promoter of MG63 cells selectively.

In summary, we isolated aaptamine as an activator of p21 promoter from the marine sponge of *Aaptos suberitoides* under the guidance of bioassay. Aaptamine activated transcription of the p21 gene of MG63 cells in a p53-independent manner and arrested the cell cycle at the G2/M phase. The Sp1 sites between −82 and −50 bp in p21 promoter were clarified to mediate the induction of p21 by aaptamine. Further study of the mechanism is under way.

Acknowledgments

This work was supported by the Takeda Science Foundation and the Ministry of Education, Culture, Sports, Science, and Technology of Japan. The authors are grateful to Prof. R.W.M. Soest, Zoologisch Museum, University of Amsterdam, for identification of the sponge. The authors are grateful to Prof. Keiichi Tanaka, Dr. Tsuyoshi

Nakanishi, and Dr. Ximei Wu in our graduate school for the technical advice regarding RT-PCR analysis.

References

[1] W.S. El-Deiry, T. Tokino, V.E. Velculescu, D.B. Levy, R. Parsons, J.M. Trent, D. Lin, W.E. Mercer, K.W. Kinzler, B. Vogelstein, WAF1, a potential mediator of p53 tumor suppression, Cell 75 (1993) 817–825.

[2] J.W. Harper, G.R. Adami, N. Wei, K. Keyomarsi, S.J. Elledge, The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases, Cell 75 (1993) 805–816.

[3] M. Hollstein, D. Sidransky, B. Vogelstein, C.C. Harris, P53 mutations in human cancers, Science 253 (1991) 49–53.

[4] B. Vogelstein, K.W. Kinzler, P53 function and dysfunction, Cell 70 (1992) 523–526.

[5] M. Chedid, P. Michieli, C. Lengel, K. Huppi, D. Givol, A single nucleotide substitution at codon 31 (Ser/Arg) defines a polymorphism in a highly conserved region of the p53-inducible gene WAF1/CIP1, Oncogene 9 (1994) 3021–3024.

[6] Y.J. Li, P. Laurent-Puig, R.J. Salmon, G. Thomas, R. Hamelin, Polymorphisms and probable lack of mutation in the WAF1-CIP1 gene in colorectal cancer, Oncogene 10 (1995) 599–601.

[7] K. Nakano, T. Mizuno, Y. Sowa, T. Orita, T. Yoshino, Y. Okuyama, T. Fujita, N. Ohtani-Fujita, Y. Matsukawa, T. Tokino, H. Yamagishi, T. Oka, H. Nomura, T. Sakai, Butyrate activates the WAF1/Cip1 gene promoter through Sp1 sites in a p53-negative human colon cancer cell line, J. Biol. Chem. 272 (1997) 22199–22206.

[8] S. Aoki, K. Matsui, T. Takata, H. Wei, M. Kobayashi, Lembehyne A, a spongean polyacetylene, induces neuronal differentiation in neuroblastoma cell, Biochem. Biophys. Res. Commun. 289 (2001) 558–563.

[9] S. Aoki, K. Matsui, T. Takata, M. Kobayashi, In situ photoaffinity labeling of the target protein for lembehyne A, a neuronal differentiation inducer, FEBS Lett. 544 (2003) 223–227.

[10] S. Aoki, D. Kong, K. Matsui, M. Kobayashi, Smenospongine, a spongean sesquiterpene aminoquinone, induces erythroid differentiation in K562 cells, Anti-Cancer Drugs 15 (2004) 363–369.

[11] S. Aoki, D. Kong, K. Matsui, R. Rachmat, M. Kobayashi, Sesquiterpene aminoquinones, from a marine sponge, induce erythroid differentiation in human chronic myelogenous leukemia, K562 cells, Chem. Pharm. Bull. 52 (2004) 935–937.

[12] S. Aoki, D. Kong, K. Matsui, M. Kobayashi, Erythroid differentiation in K562 chronic myelogenous cells induced by crambescidin 800, a pentacyclic guanidine alkaloid, Anticancer Res. 24 (2004) 2325–2330.

[13] Y. Sowa, T. Orita, S. Minamikawa, K. Nakano, T. Mizuno, H. Nomura, T. Sakai, Histone deacetylase inhibitor activates the WAF1/CIP1 gene promoter through the Sp1 sites, Biochem. Biophys. Res. Commun. 241 (1997) 142–150.

[14] H. Nakamura, J. Kobayashi, Y. Ohizumi, Isolation and structure of aaptamine, a novel heteroaromatic substance possessing α-blocking activity from the sea sponge *Aaptos aaptos*, Tetrahedron Lett. 23 (1982) 5555–5558.

[15] A.L. Gartel, A.L. Tyner, Transcriptional regulation of the $p21^{(WAF1/CIP1)}$ gene, Exp. Cell Res. 246 (1999) 280–289.

[16] C.J. Sherr, Cancer cell cycles, Science 274 (1996) 1672–1677.

[17] T. Owa, H. Yoshino, K. Yoshimatsu, T. Nagasu, Cell cycle regulation in the G1 phase: a promising target for the development of new chemotherapeutic anticancer agents, Curr. Med. Chem. 8 (2001) 1487–1503.

[18] J. Lukas, D. Parry, L. Aagaard, D.J. Mann, J. Bartkova, M. Strauss, G. Peters, J. Bartek, Retinoblastoma-protein-dependent cell-cycle inhibition by the tumour suppressor p16, Nature 375 (1995) 503–506.

[19] K. Nakayama, K. Nakayama, Cip/Kip cyclin-dependent kinase inhibitors: brakes of the cell cycle engine during development, BioEssays 20 (1998) 1020–1029.

[20] F. Bunz, A. Dutriaux, C. Lengauer, T. Waldman, S. Zhou, J.P. Brown, J.M. Sedivy, K.W. Kinzler, B. Vogelstein, Requirement for p53 and p21 to sustain G2 arrest after DNA damage, Science 282 (1998) 1497–1501.

[21] Y.H. Choi, W.H. Lee, K.Y. Park, L. Zhang, p53-Independent induction of p21(WAF1/CIP1), reduction of Cyclin B1 and G2/M arrest by the isoflavone Genistein in human prostate carcinoma cells, Jpn. J. Cancer Res. 91 (2000) 164–173.

[22] T. Sato, T. Koseki, K. Yamato, K. Saiki, K. Konishi, M. Yoshikawa, I. Ishikawa, T. Nishihara, p53-Independent expression of $p21^{CIP1/WAF1}$ in plasmacytic cells during G_2 cell cycle arrest induced by *Actinobacillus actinomycetemcomitans* cytolethal distending toxin, Infect. Immun. 70 (2002) 528–534.

[23] J.R. Biggs, J.E. Kudlow, A.S. Kraft, The role of the transcription factor Sp1 in regulating the expression of the WAF1/CIP1 gene in U937 leukemia cells, J. Biol. Chem. 271 (1996) 901–906.

[24] S.J. Lee, M.J. Ha, J. Lee, P.M. Nguyen, Y.H. Choi, F. Pirnia, W.K. Kang, X.F. Wang, S.J. Kim, J.B. Trepel, Inhibition of the 3-Hydroxy-3-methylglutaryl-coenzyme A reductase pathway induces p53-independent transcriptional regulation of $p21^{WAF1/CIP1}$ in human prostate carcinoma cells, J. Biol. Chem. 273 (1998) 10618–10623.