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Biochemical Pharmacology 66 (2003) 541–550
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Analysis of cell cycle regulation by 1-mono-*O*-acyl-3-*O*-(α-D-sulfoquinovosyl)-glyceride (SQMG), an inhibitor of eukaryotic DNA polymerases

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Received 22 November 2002; accepted 18 March 2003

Abstract

One of the sulfo-lipids, 1-mono-*O*-acyl-3-*O*-(α-D-sulfoquinovosyl)-glyceride (SQMG), potently and selectively inhibited the activity of mammalian DNA polymerases. SQMG was also a potent apoptosis inducer and the SQMG effect occurred through the induction of G1 arrest with a reduction in the proportion of cells in the S phase. SQMG clearly increased the levels of p53 and p21 proteins, but did not induce the expression of p27 and p16 proteins. SQMG markedly reduced the pRb protein level and inhibited pRb phosphorylation after 48 hr. These results suggested that SQMG activates the G1 checkpoint as a result of the DNA polymerase inhibition, and then promotes a p53-dependent apoptotic response. Since aphidicolin, a well-known replicative DNA polymerase inhibitor, did not promote these protein expressions, the apoptosis-inducing pathway by SQMG differs from that by aphidicolin.

Keywords: Sulfoquinovosyl monoacylglycerol (SQMG); Aphidicolin; DNA polymerase; Enzyme inhibitor; p53; pRb phosphorylation

1. Introduction

We previously reported that some sulfo-glycolipids were potent inhibitors of DNA polymerase α (pol α) and β (pol β), and sulfoquinovosyl diacylglycerol (SQDG) was one of the strongest [1–3]. However, since SQDG was unable to penetrate into cells, the cell viability and cell cycle did not change compared with the control cells [4]. The class of sulfoquinovosyl monoacylglycerol (SQMG), which is enzymatically synthesized in animals [5], was also a potent inhibitor of pol α and β to the same extent as SQDG [4,6,7]. SQMG could penetrate into the cells and decreased the cell viability in a dose-dependent manner [4].

In this paper, we investigated the action of SQMG on cells in more detail. In the eukaryotic cells, DNA replication is performed by the concerted action of a number of DNA polymerases with accessory proteins. Pol α is the only enzyme capable of initiation of DNA synthesis *de novo* by first synthesizing a RNA primer and then extending it by polymerization to produce a short DNA extension. Then, the DNA, after being initiated and having entered into the S phase, is elongated by pol δ and pol ε. Therefore, the replicative DNA polymerases are highly conserved in eukaryotes, and the inhibition of replicative DNA polymerases could induce the cell cycle arrest at the G1 and S phases, and subsequently, apoptosis. SQMG may disrupt the cell cycle regulation. The action of SQMG was studied from this viewpoint.

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Abbreviations: SQMG, sulfoquinovosyl monoacylglycerol; pol, DNA polymerase (EC 2.7.7.7); dTTP, 2′-deoxythymidine 5′-triphosphate; CDK, cyclin-dependent kinase.

0006-2952/$ – see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/S0006-2952(03)00345-9

In this paper, we examined the involvement of SQMG in cell cycle regulation and apoptosis. We demonstrated that SQMG induces G1 arrest and apoptosis mediated by a p53-dependent pathway and dephosphorylation of pRb and suggested that the cell cycle is tightly regulated by the mutual relationships of numerous proteins including pRb.

2. Materials and methods

2.1. Materials

Nucleotides and chemically synthesized DNA template-primers such as [^{3}H]-2′-deoxythymidine 5′-triphosphate (dTTP, 37 MBq/ml), poly(dA) and oligo(dT)$_{12–18}$ were purchased from Amersham Biosciences Inc. p53 antibody was purchased from Sigma. Antibodies of p21, p27, p16, and pRb were purchased from NeoMarkers. SQMG (1-mono-*O*-acyl-3-*O*-(α-D-sulfoquinovosyl)-glyceride with one stearic acid molecule) was synthesized according to the procedure described previously [6,7], and the synthesized lot was completely pure. The chemical structure of SQMG is shown in Fig. 1. SQMG is a sulfonic analog of D-glucose bound to a glycero-bearing C_{18}-saturated fatty acid (stearic acid). All other reagents were of analytical grade and were purchased from Wako Chemical Co. Ltd.

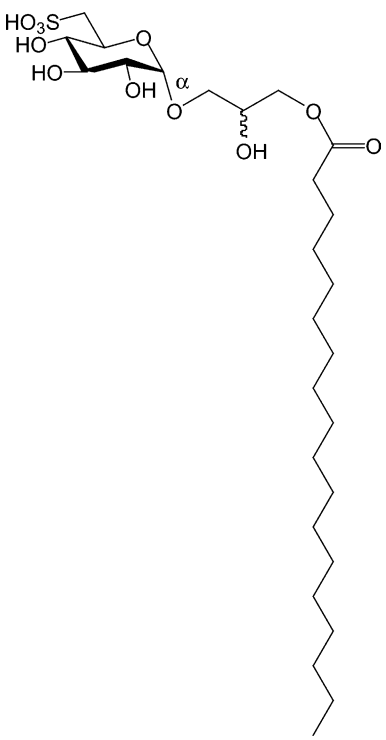

Fig. 1. Chemical structure of synthesized SQMG (1-mono-*O*-acyl-3-*O*-(α-D-sulfoquinovosyl)-glyceride with a stearic acid).

2.2. DNA polymerase assays

Pol α was purified from calf thymus by immuno-affinity column chromatography as described previously [8]. DNA polymerase β was purified from a recombinant plasmid expressing rat pol β [9]. Pol δ was purified from calf thymus [10], and pol ε was purified from HeLa cells as described previously [11]. The activities of the DNA polymerases were measured by the methods described previously [12,13]. For the DNA polymerases, poly(dA)/oligo(dT)$_{12–18}$ (A/T = 2/1) and dTTP were used as the DNA template-primer and nucleotide substrate, respectively. SQMG was dissolved in dimethyl sulfoxide at various concentrations and sonicated for 30 s. Four microliters of the sonicated samples were mixed with 16 μL of each enzyme (final 0.05 units) in 50 mM Tris–HCl (pH 7.5) containing 1 mM dithiothreitol, 50% glycerol and 0.1 mM EDTA, and kept at 0° for 10 min. These inhibitor–enzyme mixtures (8 μL) were added to 16 μL of each of the enzyme standard reaction mixtures, and incubation was carried out at 37° for 60 min. One unit of each DNA polymerase activity was defined as the amount of enzyme that catalyzed the incorporation of 1 nmol deoxyribonucleotide triphosphates into synthetic DNA template-primers at 37° for 60 min [12,13].

2.3. Cell culture and cell viability assay

Two human gastric cancer cell lines, NUGC-3, which has the p53 gene, and KATO-III, which is a p53-null cell line, were obtained from Health Science Research Bank. The cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 units/mL), and streptomycin (100 μg/mL) at 37° in a humid atmosphere of 5% CO_2/95% air. For the cell viability assay, cells were plated at 5×10^3 cells into each well of a 96-well microplate, and then SQMG or aphidicolin which was dissolved in dimethyl sulfoxide at various concentrations and sonicated for 30 s was added. The cell viability was determined at 48 hr by MTT assay [14]. The LD_{50} value of SQMG or aphidicolin was calculated from the dose–response curve of each compound for every lot.

2.4. DNA fragmentation

DNA fragmentation was determined by electrophoresis in 1.5% agarose gels. The cells (6×10^5 cells in a 60 mm dish) were lysed with 10 mM Tris–HCl (pH 7.4) containing 10 mM EDTA, 0.5% Triton X-100 and RNase A (0.2 mg/mL) and incubated at 37° for 1 hr followed by digestion with proteinase K (0.5 mg/mL) at 50° for 30 min. After the addition of 0.5 vol. 10 M ammonium acetate, the DNA was precipitated with 2.5 vol. ethanol, dissolved in gel loading buffer (40 mM Tris–5 mM sodium acetate–1 mM EDTA, pH 7.8) and separated by electrophoresis in a 1.5% agarose gel. The gel was stained with ethidium bromide, and then the DNA bands were visualized under UV light.

2.5. Measurement of caspase-3 activity

The enzymatic activity of caspase-3 was measured by fluorometoric assay. The NUGC-3 cells (6×10^5 cells in a 60 mm dish) were lysed with RIPA buffer (50 mM Tris–HCl (pH 7.2), 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.05% SDS, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM leupeptin) and the lysate was centrifuged at 12,000 *g* for 15 min. The supernatant was added to the assay buffer (50 mM Hepes (pH 7.4), 100 mM NaCl, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, and 5 mM dithiothreitol), along with the caspase substrate, Ac-DEVD-MAC and the enzyme reaction was carried out at 30° for 30 min. The cleavage of the substrates was in terms of amino-4-methylcoumarin (AMC) liberation using a microplate reader with a 380/460 nm filter.

2.6. Cell cycle analysis

The cells (3×10^5 cells in a 35 mm dish) were collected by trypsinization and washed with ice-cold PBS by centrifugation. The cells were suspended in PBS, fixed with 70% ethanol (v/v), and stored at −20°. The cells were collected by centrifugation and stained with DAPI (2 μg/mL) for at least 20 min at room temperature in the dark. The DNA content was analyzed using a cell counter analyzer (Partec, CCA model) with Multicycle 3.11 software (Phoenix Flow Systems). The cell debris and fixation artifacts were gated out.

2.7. Western blot analysis

The cells were lysed in RIPA buffer (50 mM Tris–HCl (pH 7.2), 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.05% SDS, 1 mM PMSF, and 1 μM leupeptin). The lysate was fractionated on SDS–PAGE, then blotted on a PVDF membrane. The blots were subsequently incubated with the desired primary antibodies, p53, p21, p27, p16, and pRb. After rinsing, the membranes were incubated with horseradish peroxidase-linked secondary antibody. The proteins were then detected with an enhanced chemiluminescence detection system (Perkin-Elmer Life Sciences, Inc.). Zero-D scan (version 1.0, M & S Instruments Trading Inc.) was used for densitometric quantitation.

3. Results

3.1. Effect of SQMG on the activities of mammalian DNA polymerases

Table 1 shows the inhibition of the chemically synthesized SQMG (Fig. 1) against mammalian DNA polymerases. SQMG selectively inhibited the activity of replicative DNA polymerases such as pol α, pol δ, and pol ε, and the IC_{50} values were 2.2, 4.8, and 3.6 μM, respectively. Pol β, which is a repair-related polymerase, was also inhibited by SQMG with an IC_{50} value of 26 μM. Moreover, SQMG was more effective than aphidicolin, which is a well-known replicative DNA polymerase inhibitor [15]. The IC_{50} value in Table 1 did not change when the DNA template-primer was activated DNA. It was determined that SQMG was one of the effective replicative DNA polymerase inhibitor.

Table 1
IC_{50} values of SQMG and aphidicolin on the activities of mammalian DNA polymerases

Enzyme	IC_{50} values (μM)	
	SQMG	Aphidicolin[a]
Calf DNA polymerase α	2.2	20
Rat DNA polymerase β	26	>1000
Calf DNA polymerase δ	4.8	13
Human DNA polymerase ε	3.6	16

SQMG or aphidicolin was incubated with each enzyme (0.05 units). The enzymatic activity was measured as described in Section 2. Enzyme activity in the absence of the compounds was taken as 100%.

[a] From Mizushina *et al.* [15].

3.2. Effect of SQMG on apoptosis induction

At first, the effect of SQMG on the cell viability using a human gastric cancer cell line, NUGC-3, was determined. The LD_{50} values of SQMG (150 μM) efficiently decreased in a number of cells in a time-dependent manner (Fig. 2). The LD_{50} values of aphidicolin (5 μM) also inhibited the cell growth with the same effect as SQMG. The possibility that this cell death might occur through apoptosis was evaluated based on DNA fragmentation utilizing agarose gel electrophoresis. When the cells were incubated with

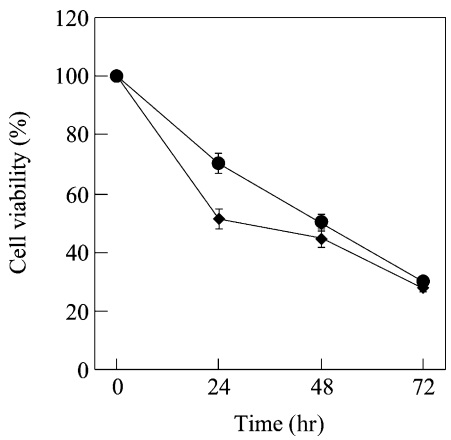

Fig. 2. Effect of SQMG on the proliferation of NUGC-3 cells. Time-dependent growth inhibition of NUGC-3 cells incubated with (●) 150 μM SQMG and 5 μM aphidicolin (◆). Viability of 100% was established from the measurements taken at 24, 48, and 72 hr with the control cells. Cell proliferation was determined by MTT assay [14]. Data are shown as means ± SEM of four independent experiments.

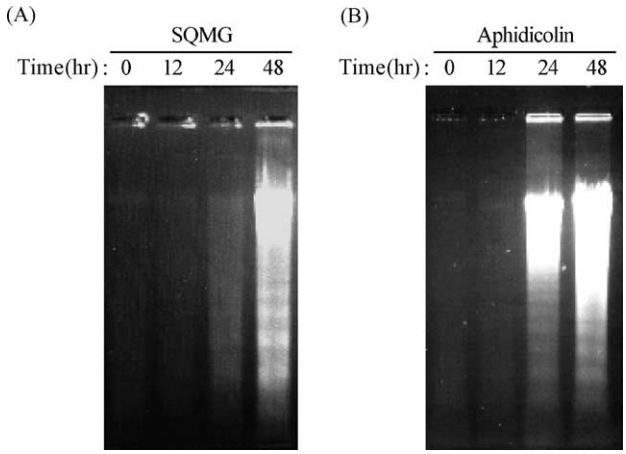

Fig. 3. Apoptosis induction by SQMG and aphidicolin. NUGC-3 cells were incubated with 150 μM SQMG or 5 μM aphidicolin. DNA fragments were analyzed by 1.5% agarose gel electrophoresis.

150 μM of SQMG for the indicated times, the fragmented DNA was detected in 48-hr treated cells (Fig. 3A). However, when the cells were treated with lower than the LD_{50} value of SQMG, the formation of fragmented DNA was not detected (data not shown). We also investigated the effect of SQMG on the caspase-3 activity, which is a key enzyme activity in the execution of apoptosis mediated by various anti-tumor agents [16]. Caspase-3 activity was measured in cells treated with 150 μM SQMG for various periods. The enzyme activity of caspase-3 increased in a time-dependent manner, and especially, after 48 hr, the caspase-3 activity in the SQMG-treated cells increased by about 10-fold (Table 2). On the other hand, the cells treated with aphidicolin (5 μM) induced apoptosis in a time-dependent manner (Fig. 3B). In comparison with the cell numbers in Fig. 2, it would appear that the decreases in cells by SQMG and aphidicolin were the result of apoptotic cell death.

3.3. Effect of SQMG on cell cycle regulation

To examine the mechanism of the NUGC-3 cell growth suppression by SQMG, the cells were treated with 150 μM of SQMG for the indicated times and the cell cycle was analyzed by flow cytometry (Fig. 4). The cell cycle analysis showed that the population in the G1 phase started to increase after SQMG treatment for 24 hr (from 38.5 to 47.3%) and reached approximately 60% by 48 hr.

Table 2
Effect of SQMG on caspase-3 activity in NUGC-3 cells

Time (hr)	Caspase activity (mAU/mg protein)
0	0.045 ± 0.003
12	0.075 ± 0.006
24	0.103 ± 0.010
36	0.380 ± 0.035
48	0.463 ± 0.030

NUGC-3 cells were incubated with 150 μM SQMG for 0, 12, 24, 36, and 48 hr. Data are shown as means ± SEM of four independent experiments.

This increase was mirrored by a decrease in the S phase population. The proportion of cells in the S phase decreased from 44.1 to 26.7%. On the other hand, the cells treated with 5 μM of the aphidicolin already started to undergo arrest in the G1 phase after 12 hr incubation (from 41.9 to 51.2%). After 24 hr, the proportion of cells in the G1 phase increased to approximately 58.3%, and then the sub-G1 phase cells were increased at 48 hr. These results suggest that SQMG and aphidicolin can induce G1 cell cycle arrest resulting in apoptosis.

3.4. Influence of SQMG on the levels of p53, p21, and p27 protein

Cell cycle control is the major regulatory mechanism of cell growth [17]. The cell cycle is regulated by the coordinated action of cyclin-dependent kinases (CDKs) in association with their specific regulatory cyclin proteins. CDK inhibitors, including p21, p27, and p16, also contribute to the regulation of cell cycle progression by controlling CDK activity. p21, as well as p27, inhibits a wide variety of cyclin-CDK complexes *in vitro*, including CDK4 and CDK2 complexes which are activated early in G1 [18,19], and overexpression of these proteins blocks the progression of cells through G1 [20]. In addition to p21 and p27, a distinct family of 15- to 20-kDa inhibitors called p16 is also associated with CDK4 and specifically inhibit CDK activity. p53 plays the central role in G1 arrest because p53 is known to be an upstream regulator of p21, a CDK inhibitor.

To explore the mechanisms of SQMG-induced cell cycle arrest, the expression of p53 and CDK inhibitors including p27, p21, and p16 proteins was examined by Western blotting (Fig. 5A) because these inhibitory proteins play a negative regulatory role in the G1 phase, and disappearance of these inhibitory activities is associated with G1/S progression induced by a number of mitogens [21,22]. When the NUGC-3 cells were incubated with 150 μM SQMG, the p53 protein expression markedly increased time-dependently. The p21 protein expression also increased in parallel to the p53 protein expression, while the levels of the p27 and p16 proteins did not change and hardly changed by SQMG treatment, respectively. Since the SQMG-induced cell cycle arrest showed time dependence, these results suggest that the p53 and p21 proteins may be held in a state of active repression in cells that have stalled DNA replication forks, and were expressed with the cell cycle arrest and induction of apoptosis. Since p53 protein was induced by DNA-damaging agents such as methyl methane sulfonate (MMS; Fig. 5C), we examined whether the p53 protein expression by SQMG is involved in the inhibition process of replication or not. In the presence of hydroxyurea (HU), which inhibits DNA synthesis, the cells are unable to progress through the S phase, and the accumulation of p53 and cyclin E protein was observed [25]. SQMG could also induce the cyclin E

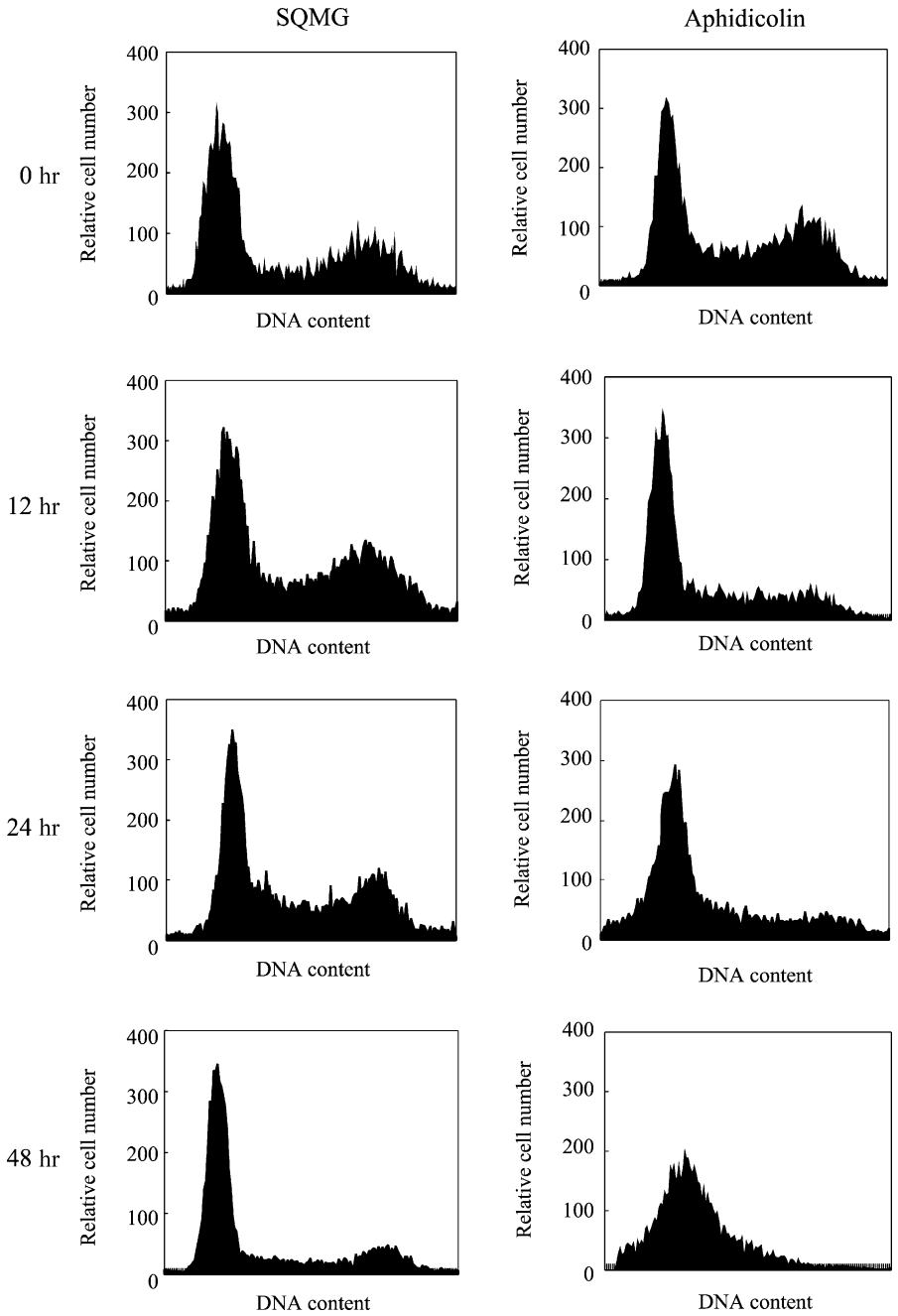

Fig. 4. Effect of cell cycle distribution by SQMG and aphidicolin. NUGC-3 cells were incubated with 150 μM SQMG or 5 μM aphidicolin for 0, 12, 24, and 48 hr. DNA content was analyzed by flow cytometry with DAPI staining.

protein expression level (Fig. 5B). This result suggested that G1 arrest by SQMG was at least involved in the inhibition process of replication. Moreover, we determined the p53 protein expression in the aphidicolin-treated cells at 24 hr, because the 24-hr exposure to aphidicolin induced G1 arrest and apoptosis (Figs. 3B and 4). The p53 protein expression did not change by the aphidicolin treatment, and the levels of the p21, p27, and p16 proteins also did not change when compared to the initiation level (Fig. 5A).

KATO-III, which has a p53 gene deletion, is also a human gastric cancer cell line [23]. We investigated the inhibitory effect on the cell growth by SQMG and aphidicolin. The dose–response curves of the KATO-III cell growth inhibition by SQMG and aphidicolin were the same as those of NUGC-3 cells, which have the p53 gene (data not shown). The p53 protein was not detected in KATO-III cells by the treatment with SQMG or aphidicolin (Fig. 6A). When the KATO-III cells were treated with SQMG, the distribution during the cell cycle was different from that of NUGC-3 cells. The cells in the G1 phase increased from 35.6 to 42.9% in KATO-III (Fig. 6B-b), but this accumulation was reduced partially compared to NUGC-3 (35.6–42.9% and 38.5–61.9%, respectively), and cells in the G2/M phase also increased from 13.3 to 33.8% in KATO-III,

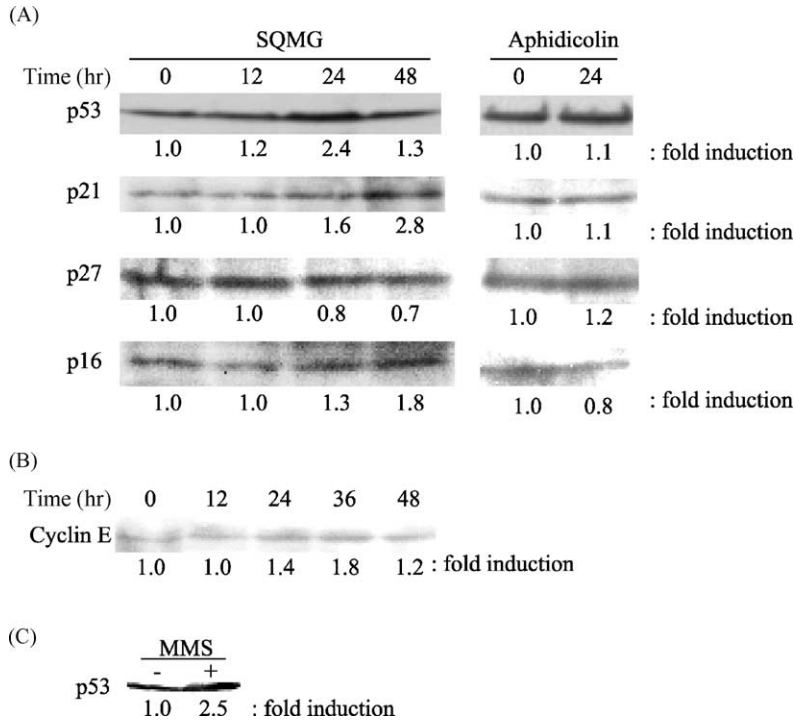

Fig. 5. Effect of SQMG and aphidicolin on the expression of p53 and CDK inhibitors. (A) Cell lysates from NUGC-3 cells incubated with 150 μM SQMG or 5 μM aphidicolin were analyzed by SDS–PAGE, and Western blots were detected with specific antibodies to p53, p21, p27, and p16. (B) Cyclin E expression in NUGC-3 cells incubated with 150 μM SQMG for the indicated times. (C) p53 expression in NUGC-3 cells incubated with 100 μg/mL MMS for 6 hr. Densitometric analysis of the proteins was performed and fold induction was calculated.

but in NUGC-3, G2/M phase did not change (17.4–11.5%) after 48 hr (Figs. 4 and 6B-b). On the other hand, the cell cycle inhibitory pattern by aphidicolin was the same as that of NUGC-3 cells. The G1 phase increased from 35.6 to 53.9% in KATO-III and from 41.9 to 51.2% in NUGC-3 (Figs. 4 and 6B-c). In a human colorectal carcinoma cell line (RKO), the p53 protein was reportedly expressed by aphidicolin [24,25], but as described here, not in the NUGC-3 cells. The other cell lines, HeLa (human carcinoma of cervix cell line), MOLT-4 (human acute lymphoblastic leukemia cell line), and BALL-1 (human peripheral blood cell line), also showed no stabilization of p53 by treatment with aphidicolin (data not shown). Apoptosis was not induced on the KATO-III cells by SQMG or aphidicolin (Fig. 6C). These results using the p53-null cells suggested that cell cycle checkpoint activation and apoptosis were related to the p53 protein induction. SQMG was suggested to induce G1 arrest and apoptosis through a p53-dependent pathway, and the aphidicolin-induced G1 arrest is mediated through a different signal transduction pathway.

3.5. pRb phosphorylation by SQMG

One of tumor suppressor proteins, pRb, which encodes the 110-kDa phosphoprotein, regulates cell cycle progression from the G1 to S phase [26,27]. pRb is maintained in an underphosphorylated active state through much of the G1 phase and becomes inactivated by further phosphorylation in the late G1 phase, releasing sequestered transcription regulators which are members of the E2F family transcription factors and enable cells to progress to the S phase [28,29]. Another tumor suppressor protein, p53, is activated in response to DNA damage. This activation leads to activation of one of the CDK inhibitors, p21, and inhibits pRb phosphorylation [30]. The functional status of p53 and pRb phosphorylation affect the regulation of entry into the S phase [31].

We described above that SQMG induced apoptosis and G1 arrest through p53. Since pRb phosphorylation promotes entry into the S phase and apoptosis induction, we further studied pRb expression and its phosphorylation in the NUGC-3 cells. The expression of pRb was monitored by Western blot analysis (Fig. 7). Treatment with 150 μM SQMG resulted in a decrease of the pRb protein expression and the pRb phosphorylation in a time-dependent manner (Fig. 7A). Additionally, the expression of pRb phosphorylated at Ser-780 was determined after 48-hr exposure, since the phosphorylation of Ser-780 on pRb resulted in the loss of the ability of pRb to bind to E2F. The Ser-780 expression was significantly decreased by treatment with SQMG (Fig. 7B). These results suggest that the pRb function is related to the SQMG-induced G1 cell cycle arrest, and dephosphorylation of the Ser-780 phosphorylation site on pRb was particularly important in the SQMG-induced G1 arrest. On the other hand, no dephosphorylation in pRb in

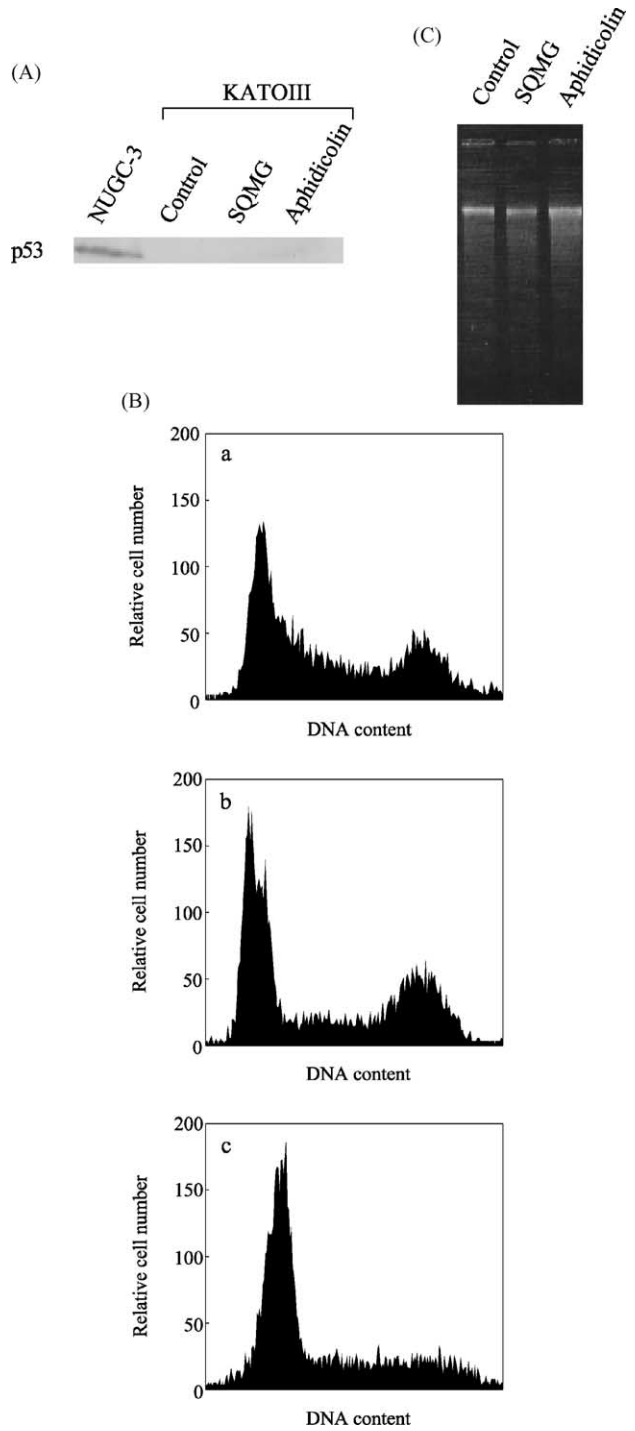

Fig. 6. Effect of SQMG in KATO-III cells. (A) Cell lysates from KATO-III cells incubated with 150 μM SQMG or 5 μM aphidicolin were analyzed by SDS–PAGE, and Western blots were detected with specific antibodies to p53. (B) KATO-III cells were incubated with 150 μM SQMG (b), 5 μM aphidicolin (c), or without (control, a) for 24 hr. DNA content was analyzed by flow cytometry with DAPI staining. (C) Apoptosis induction by SQMG and aphidicolin. KATO-III cells were incubated with 150 μM SQMG or 5 μM aphidicolin for 48 hr. DNA fragments were analyzed by 1.5% agarose gel electrophoresis.

the aphidicolin-treated cells was detected. These results suggest that the cell cycle abnormality and apoptosis in the SQMG-treated cells were induced through systems which required numerous factors.

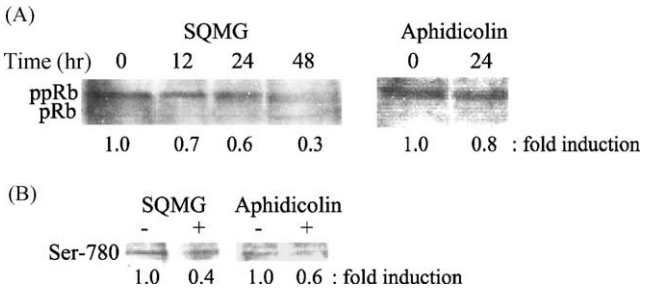

Fig. 7. Effect of SQMG and aphidicolin on pRb and its phosphorylation. Cell lysates from NUGC-3 cells incubated with 150 μM SQMG or 5 μM aphidicolin were analyzed by Western blots. (A) pRb phosphorylation was detected with anti-pRb antibody. The upper band, ppRb, represents the hyperphosphorylated form of pRb, and the lower band, pRb, represents the hypophophorylated form. (B) Phosphorylated pRb (Ser-780) was detected by Western blotting with an antibody that reacts specifically to Ser-780. Densitometric analysis of the proteins was performed and fold induction was calculated.

4. Discussion

A sulfo-glycolipid, SQMG, was a potent inhibitor of DNA polymerase α and β, and of the growth of NUGC-3 cancer cells [4]. SQMG arrested the cell cycle at the G1 phase, and subsequently induced severe apoptosis. In this report, we investigated SQMG in the cell cycle regulation of signal transduction in more detail.

The checkpoint that arises after DNA damage can be active during either G1 or G2. The ability of cells to arrest in G1 response to DNA damage depends on the accumulation of the tumor suppressor p53. This p53-dependent G1 arrest is largely mediated through the induction of the CDK inhibitor p21 [32,33], which appears to be induced in a p53-dependent manner. The apoptosis-inducing pathway by SQMG and aphidicolin is shown in Fig. 8. In this

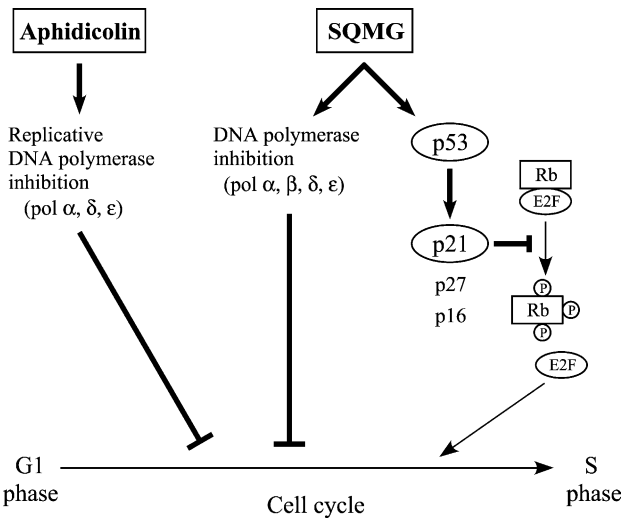

Fig. 8. Proposed model of cell cycle arrest by SQMG. DNA polymerase inhibition by SQMG induces G1 arrest directly to the same extent as aphidicolin. Moreover, SQMG induces p53-dependent p21 expression and leads to loss in pRb activity, resulting in the G1 arrest.

experiment, we showed that DNA polymerase inhibitors, SQMG and aphidicolin, induced G1 arrest in a human gastric cancer cell line, NUGC-3, in a time-dependent manner (Table 1; Fig. 4). Although both SQMG and aphidicolin led to G1 arrest and apoptosis, the sensitivity to SQMG and aphidicolin were different (Figs. 3 and 4). Aphidicolin-treated cells underwent G1 arrest and apoptosis 12 hr before SQMG. In addition, induction mechanisms were different: aphidicolin-induced apoptosis occurred in a p53-independent manner. In a human colorectal carcinoma cell line (RKO), the p53 expression was reportedly induced by aphidicolin [24,25]. However, in our experimental conditions, p53 was not induced in NUGC-3, HeLa, MOLT-4, or BALL-1 cells by aphidicolin (data not shown). Aphidicolin effectively and rapidly reduced cell growth to about 60% of control without apoptosis, then the inhibition became moderate. Indeed, aphidicolin induced G1 arrest at a lower concentration than LD_{50} value without apoptosis (data not shown). On the other hand, G1 arrest and apoptosis by SQMG was moderately induced. Taking these observations together, if the G1 arrest is moderately induced by aphidicolin at lower concentrations, it might be mediated in a p53-dependent manner. Furthermore, the cell distribution of aphidicolin-treated KATO-III cells, which have a deleted p53 gene gastric cancer cell line, did not change compare to NUGC-3 cells (Fig. 6B). On the other hand, it was observed that SQMG-treated cells increased in the G1 and G2M phases although apoptosis was inhibited (Fig. 6B and C). SQMG inhibited not only replicative type of DNA polymerases, pol α, pol δ, and pol ε, but also repair type, pol β (Table 1). The difference in sensitivity between SQMG and aphidicolin may result, therefore, from differences in the inhibitory actions of polymerases and the induction pathway of G1 arrest. Our findings suggested that G1 arrest by SQMG occurred even in the absence of p53 (Fig. 6B), but p53 was required for the induction of apoptosis (Fig. 6C). Therefore, we considered that this G1 arrest and apoptosis caused by SQMG were induced by DNA polymerase inhibition and resulted from G1 checkpoint, because when we examined the G1 arrest induced by SQMG and aphidicolin, only SQMG increased p53 and p21 expression (Fig. 5A). The p21 induction by SQMG was parallel to p53 expression. However, in our experimental conditions, aphidicolin induced G1 arrest in a p53-independent manner like ara-C [34].

From these results, SQMG appears to be a new type of mammalian DNA polymerase inhibitor. SQMG might activate the checkpoint pathway following the inhibition of DNA replication by the pol α, pol δ, and pol ε inhibition. From this viewpoint, we also tested the influence of SQMG on pRb phosphorylation (Fig. 7), as pRb is known to regulate the expression and activity of a number of proteins that play central roles in cell cycle control, and as pRb has the potential to affect the G1 arrest pathway through multiple mechanisms. pRb is phosphorylated or dephosphorylated at different stages of the cell cycle [27]. pRb phosphorylation induces conformational change of pRb and releases E2F transcription factors [35–37]. Without this phosphorylation, pRb binds to E2F and prevents cell cycle progression into the S phase and finally induces G1 arrest [30]. In addition, p53-dependent induction of p21 leads to pRb dephosphorylation and consequent G1 arrest [31,38]. SQMG induced G1 arrest, up-regulated p53 and p21, and promoted the dephosphorylation of pRb (Figs. 4 and 5). These results are consistent with evidence that demonstrates a requirement for pRb in G1 arrest induced by a variety of DNA-damaging agents [39]. On the other hand, aphidicolin did not induce the up-regulation of p53 and p21, and did not promote dephosphorylation of pRb, especially Ser-780 (Figs. 5 and 7). The G1 arrest induced by aphidicolin may be explained by another pathway, because recent studies suggest that pRb is phosphorylated in a two-step process during the normal cell cycle [37,40,41]. Cyclin D/cdk4/6 specifically phosphorylates pRb at a subset of its phosphorylation sites. Complete phosphorylation of pRb requires cyclin E/cdk2 to specifically target the remaining phosphorylation sites. These findings further indicate that a checkpoint pathway from p53 to p21 is able to impose a G1 block by specifically inhibiting CDK activity and thereby the second step of pRb phosphorylation.

Cell cycle checkpoints are signal transduction pathways that ensure the time, sequence, and fidelity of critical cell cycle events and orchestrate cellular responses to environmentally induced genotoxic stress. DNA damage induced by ionizing radiation or chemotherapeutic agents triggers checkpoint action and delays cell cycle progression [42]. Such a delay in the cell cycle would presumably allow time to repair damaged DNA, to complete DNA replication before entry to mitosis [43] or to drive apoptosis in the cells. SQMG might block the entry into the S phase from G1 by inhibition of replicative DNA polymerases, and finally to induce apoptosis through a different pathway from those disrupted by aphidicolin or ara-C. Therefore, SQMG represents a new category of DNA polymerase inhibitors, and could be an anti-tumor agent. Indeed, we reported elsewhere that some SQMGs are agents that inhibit the growth of solid tumors derived from human lung cancer *in vivo*, adenocarcinoma A-549, in nude mice [44].

Acknowledgments

We deeply thank Dr. S. Yoshida of Nagoya University for his valuable discussions about the inhibitor. We are grateful for the donation of rat DNA polymerase β and human DNA polymerase ε from Dr. A. Matsukage of the Japan Women's University and Dr. S. Linn and Dr. H. Asahara of the University of California, Berkeley. This work was partly supported by Sasakawa Scientific Research Grant (C.M.) from the Japan Science Society,

and by Grant-in-Aid for Kobe Gakuin University Joint Research (B) (C.M., H.Y., and Y.M.). Y.M. acknowledges Grant-in-Aid from the Tokyo Biochemical Research Foundation and Grant-in-Aid 14780466 for Scientific Research, The Ministry of Education, Culture, Sports, Science, and Technology, Japan.

References

[1] Mizushina Y, Watanabe I, Ohta K, Takemura M, Sahara H, Takahashi N, Gasa S, Sugawara F, Matsukage A, Yoshida S, Sakaguchi K. Studies on inhibitors of mammalian DNA polymerase α and β: sulfolipids from a pteridophyte, *Athyrium niponicum*. Biochem Pharmacol 1998;55:537–41.

[2] Ohta K, Mizushina Y, Hirata N, Takemura M, Sugawara F, Matsukage A, Yoshida S, Sakaguchi K. Sulfoquinovosyldiacylglycerol, KM043, a new potent inhibitor of uekaryotic DNA polymerases and HIV-reverse transcriptase type I from a marine red alga, *Gigartina tenella*. Chem Pharm Bull 1998;46:684–6.

[3] Ohta K, Mizushina Y, Hirata N, Takemura M, Sugawara F, Matsukage A, Yoshida S, Sakaguchi K. Action of a new mammalian DNA polymerase inhibitor, sulfoquinovosyldiacylglycerol. Biol Pharm Bull 1999;22:111–6.

[4] Hanashima S, Mizushina Y, Ohta K, Yamazaki T, Sugawara F, Sakaguchi K. Structure-activity relationship of a novel group of mammalian DNA polymerase inhibitors, synthetic sulfoquinovosyldiacylglycerols. Jpn J Cancer Res 2000;91:1073–83.

[5] Roughan PG, Batt RD. The glycerolipid composition of leaves. Phytochemistry 1969;8:363–9.

[6] Hanashima S, Mizushina Y, Yamazaki T, Ohta K, Takahashi S, Koshino H, Sahara H, Sakaguchi K, Sugawara F. Structural determination of sulfoquinovosyldiacylglycerol by chiral syntheses. Tetrahedron Lett 2000;41:4403–7.

[7] Hanashima S, Mizushina Y, Yamazaki T, Ohta K, Takahashi S, Sahara H, Sakaguchi K, Sugawara F. Synthesis of sulfoquinovosylacylglycerols, inhibitors of eukaryotic DNA polymerase α and β. Bioorg Med Chem 2001;9:367–76.

[8] Tamai K, Kojima K, Hanaichi T, Masaki S, Suzuki M, Umekawa H, Yoshida E. Structural study of immunoaffinity-purified DNA polymerase α-DNA primase complex from calf thymus. Biochim Biophys Acta 1988;950:263–73.

[9] Date T, Yamaguchi M, Hirose F, Nishimoto Y, Tanihara K, Matsukage A. Expression of active rat DNA polymerase β in *Escherichia coli*. Biochemistry 1988;27:2983–90.

[10] Shoji-Kawaguchi M, Izuta S, Tamiya-Koizumi K, Suzuki M, Yoshida S. Selective inhibition of DNA polymerase ε by phosphatidylinositol. J Biochem (Tokyo) 1995;117:1095–9.

[11] Chui GS, Linn S. Purified of mammalian polymerases: DNA polymerase ε. Methods Enzymol 1995;262:93–8.

[12] Mizushina Y, Tanaka N, Yagi H, Kurosawa T, Onoue M, Seto H, Horie T, Aoyagi N, Yamaoka M, Matsukage A, Yoshida S, Sakaguchi K. Fatty acids selectively inhibit eukaryotic DNA polymerase activities *in vitro*. Biochim Biophys Acta 1996;1308:256–62.

[13] Mizushina Y, Yoshida S, Matsukage A, Sakaguchi K. The inhibitory action of fatty acids on DNA polymerase β. Biochim Biophys Acta 1997;1336:509–21.

[14] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytltlxicity assays. J Immunol Methods 1983;65:55–63.

[15] Mizushina Y, Kamisuki S, Mizuno T, Takemura M, Asahara H, Linn S, Yamaguchi T, Matsukage A, Hanaoka F, Yoshida S, Saneyoshi M, Sugawara F, Sakaguchi K. Dehydroaltenusin, a mammalian DNA polymerase a inhibitor. J Biol Chem 2000;275:33957–61.

[16] Kaufmann SH. Cell death induced by topoisomerase-targeted drugs: more questions than answers. Biochim Biophys Acta 1998;1400:195–211.

[17] Rittling SR, Brooks KM, Cristofalo VJ, Baserga R. Expression of cell cycle-dependent genes in young and senescent WI-38 fibroblasts. Proc Natl Acad Sci USA 1986;83:3316–20.

[18] Matsushime H, Ewen ME, Strom DK, Kato JY, Hanks SK, Roussel MF, Sherr CJ. Identification and properties of a typecal catalytic subunit (p34PSK-J3/cdk4) for mammalian D type G1 cyclins. Cell 1992;71:323–34.

[19] Meyerson M, Harlow E. Identification of G1 kinase activity for cdk6, a novel cyclin D partner. Mol Cell Biol 1994;14:2077–86.

[20] Hunter T, Pines J. Cyclins and cancer. II: cyclin D and CDK inhibitors come of age. Cell 1994;79:573–82.

[21] Toyoshima H, Hunter T. p27, a novel inhibitor of G1 cyclin-Cdk protein kinase activity, is related to p21. Cell 1994;78:67–74.

[22] Xiong Y, Hannon GJ, Zhang H, Casso D, Kobayashi R, Beach D. p21 is a universal inhibitor of cyclin kinases. Nature 1993;366:701–4.

[23] Yamada Y, Yoshida T, Hayashi K, Sekiya T, Yokota J, Hirohashi S, Nakatani K, Nakano H, Sugimura T, Terada M. p53 gene mutations in gastric cancer metastases and in gastric cancer cell lines derived from metastases. Cancer Res 1991;51:5800–5.

[24] Nayak BK, Das GM. Stabilization of p53 and transactivation of its target genes in response to replication blockade. Oncogene 2002;21:7226–9.

[25] Gottifredi V, Shieh S, Taya Y, Prives C. p53 accumulates but is functionally impaired when DNA synthesis is blocked. Proc Natl Acad Sci USA 2001;98:1036–41.

[26] Buchovichi K, Duffy LA, Harlow E. The retinoblastoma protein is phosphorylated during specific phases of the cell cycle. Cell 1989;58:1097–105.

[27] Chen PL, Scully P, Shew JY, Wang JY, Lee WH. Phosphorylation of the retinoblastoma gene product is modulated during the cell cycle and cellular differentiation. Cell 1989;8:1193–8.

[28] Weinberg RA. The retinoblastoma protein and cell cycle control. Cell 1995;81:323–30.

[29] Riley DJ, Lee EY, Lee WH. The retinoblastoma protein: more than a tumor suppressor. Annu Rev Cell Biol 1994;10:1–29.

[30] Hartwell LH, Kastan MB. Cell cycle control and cancer. Science 1994;266:1821–8.

[31] SlebosR JC, Lee MH, Plunkett BS, Kessis TD, Williams BO, Jacks T, Hedrick L, Kastan MB, Cho KR. p53-dependent G1 arrest involves pRB-related proteins and is disrupted by human papillomavirus 16E7 oncoprotein. Proc Natl Acad Sci USA 1994;91:5320–4.

[32] Brugarolas J, Chandrasenkaran C, Gordon JI, Beach D, Jacks T, Hannon GJ. Radiation-induced cell cycle arrest compromised by p21 deficiency. Nature 1995;377:552–7.

[33] Deng C, Zhang P, Harper JW, Elledge SJ, Leder P. Mice lacking $p21^{CIP/WAF1}$ undergo normal development, but are defectove in G1 checkpoint control. Cell 1995;82:675–84.

[34] Dou QP, An B, Will PL. Induction of a retinoblastoma phosphatase activiry by anticancer drugs accompanies p53-independent G1 arrest and apoptosis. Proc Natl Acad Sci USA 1995;92:9019–23.

[35] Connell-Crowley L, Harper JW, Goodrich DW. Cyclin D1/Cdk4 regulates retinoblastoma protein-mediated cell cycle arrest by site-specific phosphorylation. Mol Biol Cell 1997;8:287–301.

[36] Knudsen ES, Wang JY. Differential regulation of retinoblastoma protein function by specific Cdk phosphorylation sites. J Biol Chem 1996;271:8313–20.

[37] Kitagawa M, Higashi H, Jung HK, Suzuki-Takahashi I, Ikeda M, Tamai K, Kato J, Segawa K, Yoshida E, Nishimura S, Taya Y. The consensus motif for phosphorylation by cyclin D1-Cdk4 is different from that for phosphorylation by cyclin A/E-Cdk2. EMBO J 1996;15:7060–9.

[38] Demers GW, Foster SA, Halbert CL, Galloway DA. Growth arrest by induction of p53 in DNA damaged keratinocytes is bypassed by human papillomavirus 16E7. Proc Natl Acad Sci USA 1994;91:4382–6.

[39] Harrington EA, Bruce JL, Harlow E, Dyson N. pRB plays an essential role in cell cycle arrest induced by DNA damage. Proc Natl Acad Sci USA 1998;95:11945–50.

[40] Ezhevsky SA, Nagahara H, Vocero-Akbani AM, Gius DR, Wei MC, Dowdy SF. Hypo-phosphorylation of the retinoblastoma protein (pRb) by cyclin D: cdk4/6 complexes results in active pRb. Proc Natl Acad Sci USA 1997;94:10699–704.

[41] Lundberg AS, Weinberg RA. Functional inactivation of the retinoblastoma protein requires sequential modification by at least two distinct cyclin-cdk complexes. Mol Cell Biol 1998;18:753–61.

[42] Elledge SJ. Cell cycle checkpoints: preventing an identity crisis. Science 1996;274:1664–72.

[43] Lane DP. Cancer. p53, guardian of the genome. Nature 1992;358: 15–6.

[44] Sahara H, Hanashima S, Yamazaki T, Takahashi S, Sugawara F, Ohtani S, Ishikawa M, Mizushina Y, Ohta K, Shimozawa K, Gasa S, Jimbow K, Sakaguchi K, Sato N, Takahashi N. Anti-tumor effect of chemically synthesized sulfolipids based on sea urchin's natural sulfoquinovosylmonoacylglycerols. Jpn J Cancer Res 2002; 93:85–92.