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Original Paper

Ginsenoside-Rs_4, a New Type of Ginseng Saponin Concurrently Induces Apoptosis and Selectively Elevates Protein Levels of p53 and $p21^{WAF1}$ in Human Hepatoma SK-HEP-1 Cells

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In this paper, we present evidence that ginsenoside-Rs_4 (G-Rs4; an acetylated analogue of ginsenoside-Rg_5), a new ginseng saponin isolated from *Panax ginseng* C. A. Meyer, elevates protein levels of p53 and $p21^{WAF1}$, which are associated with the induction of apoptosis in SK-HEP-1 cells. Flow cytometric analyses showed that G-Rs4 initially arrested the cell cycle at the G1/S boundary, but consequently induced apoptosis as evidenced by generating an apoptotic peak. The induction of apoptosis was confirmed by the results of DNA fragmentation assays and alterations in cell morphology after treatment of the cells with G-Rs4. Immunoblot assays showed that G-Rs4 significantly elevated protein levels of p53 and $p21^{WAF1}$, concurrently with the downregulation of both cyclins E- and A-dependent kinase activities and induction of apoptosis. We suggest that G-Rs4 induces apoptosis, the effect of which is closely related to the downregulation of both cyclins E- and A-dependent kinase activity as a consequence of selectively elevating protein levels of p53 and $p21^{WAF1}$ in SK-HEP-1 cells.

Key words: ginsenoside-Rs_4, apoptosis, p53, $p21^{WAF1}$, CDK2
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INTRODUCTION

IN PREVIOUS reports, ginseng saponins, such as prosapogenins and sapogenins, isolated from the roots of *Panax ginseng* C.A. Meyer, have been shown to have cell growth suppressive activities in various cancer cell types, for example, A549, SK-Mel-2 and K562 [1]. Others have reported that ginsenoside-Rh_2 (G-Rh2), a panaxadiol type of ginseng saponin, potently suppresses cell growth of Lewis lung, Morris hepatoma, B16 and HeLa cells [2–4]. Thus, it appears that some ginsenosides are able to suppress cell growth of cancer cells. However, the underlying mechanisms are largely unknown. We have previously shown that G-Rh2 blocks the cell cycle of SK-HEP-1 cells at the G1/S boundary by selectively inducing the protein expression of $p27^{Kip1}$ [5]. We have also reported that ginsenoside-Rg_5 (G-Rg5), a panaxadiol type of ginseng saponin but differing with G-Rh2 in their side chain, downregulates cyclin E-dependent kinase activity by selectively elevating the protein levels of $p21^{WAF1/Cip1}$ in SK-HEP-1 cells [6]. In that study, we demonstrated that G-Rg5 not only suppressed DNA synthesis activity with an IC_{50} value of 0.5 μM, but also decreased cell viability with an IC_{50} value of 2 μM. In addition, the compound downregulated cyclin E-dependent kinase activity with an IC_{50} value of 0.5 μM. Thus, it appears that G-Rh2 and G-Rg5 can suppress cell growth via a different mechanism, although these compounds share a common dammarane skeleton and only differ in their side chain. Recently, we isolated a new ginsenoside from red ginseng and determined the chemical structure as an acetylated analogue of G-Rg5 on its 6′-OH of glucose; we named the ginsenoside G-Rs4 (Figure 1). The aim of this study was to examine whether G-Rs4 also downregulates the activity of cyclin-dependent protein kinases (CDKs) through the same mechanism adopted by G-Rg5 and to examine the biological consequence of the downregulated activity of CDKs in SK-HEP-1 cells.

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It has been well documented that the major transitions of the eukaryotic cell cycle are regulated by CDKs [7]. Each CDK interacts with a specific subset of cyclins [8]. For example, CDK2 associates with either cyclin E or cyclin A, while CDK4 and CDK6 associate with cyclins D1–D3 [9]. CDK activity has been shown to be regulated at several different levels; for example, by association with cyclins, phosphorylation [10, 11] and/or association with inhibitors [12–16]. Thus, protein levels of these CDK regulators have been monitored to explain the regulatory mechanisms of CDK activities in animal cells. The gene for $p21^{WAF1}$ protein, a universal CDK inhibitor [14], has been identified as a downstream target of p53 in regulating cell cycle progression through the G1 phase checkpoint [13, 17]. Furthermore, $p21^{WAF1}$ has been shown to bind and inactivate components of the DNA replication machinery; for example, the proliferating cell nuclear antigen (PCNA), a cofactor of the DNA polymerase delta [18]. $p27^{Kip1}$ has also been shown to inhibit the cyclin E–CDK2 complex [15, 16].

In this paper, we provide evidence that G-Rs4 specifically elevates the protein levels of p53 and $p21^{WAF1/Cip1}$ and thereby downregulates both cyclins A- and E-dependent kinase activities. In addition, the downregulating effect is temporally related to apoptosis in SK-HEP-1 cells induced by G-Rs4.

MATERIALS AND METHODS

Materials

All materials except where specified were purchased from Sigma. G-Rs4 was isolated from Korean red ginseng and determined by spectral and physical analyses. Red ginseng was milled and extracted in methanol at room temperature overnight, and filtered through no. 2 Whatman filter paper. The filtrate was evaporated under vacuum to give the extract, which was dissolved in water and defatted with *n*-hexane. The aqueous layer was extracted with *n*-BuOH and the combined *n*-BuOH layer was evaporated under vacuum to give the extract. The extract was applied to a silica gel column eluting with $CHCl_3$-MeOH-H_2O (15:3:1, lower phase), *n*-BuOH-EtOAc-H_2O (15:1:4, upper phase) and $CHCl_3$-MeOH-H_2O (12:3:1, lower phase), repeatedly, to give rise to G-Rs4, whose chemical structure was identified as an acetylated analogue of G-Rg5 at 6′-OH of its glucose (Figure 1) by using infra-red spectrophotometry (IR), fast atom bombardment-mass spectrometry (FAB-MS), ^{1}H-NMR (nuclear magnetic resonance spectrometry) and ^{13}C-NMR (data not shown).

Cell cultures

SK-HEP-1 human hepatoma cells obtained from the Seoul National University Cancer Center were cultured as a monolayer in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, U.S.A.) supplemented with 5% calf serum (CS, Gibco BRL) and 50 mg/l gentamicin at 37°C under humidified air with 5% CO_2. The cells were synchronised by incubation in a medium containing 1.5 mM hydroxyurea for 14 h and released from the block by washing with phosphate buffered saline (PBS), followed by incubation in 5% CS-DMEM containing G-Rs4.

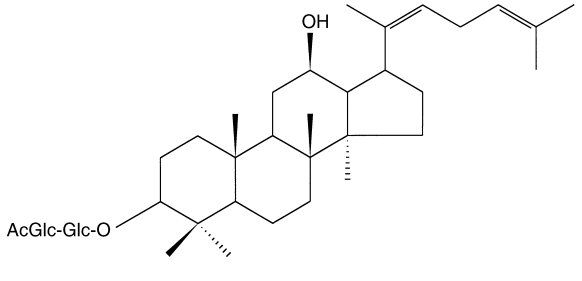

Figure 1. Chemical structure of ginsenoside-Rs_4 (G-Rs4).

MTT tetrazolium dye assay

The viability of the cells after treatment with increasing concentrations of G-Rs4 was measured using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were inoculated on to a 96-well microtitre plate at an initial density of 1×10^5 cells/ml and exposed to varying concentrations of G-Rs4 for 24 h. At the end of the exposure, the medium was removed and the monolayer was washed with PBS, and then given 2 mg/ml MTT in PBS (Sigma, U.S.A.). After 4 h incubation at 37°C, the supernatant was removed and the precipitated formazan crystals were dissolved in dimethyl sulphoxide (DMSO). The absorbance proportional to the degree of cell viability was determined by an enzyme linked immunosorbent assay (ELISA) reader.

[^{3}H]*thymidine incorporation assay*

SK-HEP-1 cells were plated at a density of 1×10^5 cells/ml on to 24-well plates in 1 ml of 5% CS medium. After synchronisation, the cells were incubated with G-Rs4 for 24 h. The cells were exposed to 1 μCi/ml [^{3}H]thymidine (Amersham, Buckinghamshire, U.K.) for the last 8 h of culture and the radioactivity in trichloroacetic acid-insoluble material was measured using a liquid scintillation counter (Wallac, Finland).

Flow cytometry

SK-HEP-1 cells incubated with or without G-Rs4 were trypsinised, harvested and washed with PBS. The cells were fixed with 75% ethanol and stored at 4°C until analysis. The cells were suspended in DAPI solution (Partec, Germany) and the cell cycle analysis was performed using a flow cytometer (Partec).

Histone H1 kinase assays

The monolayer of SK-HEP-1 cells was washed with ice-cold PBS and solubilised in lysis buffer containing 20 mM Tris, pH 7.5, 0.5% Triton X-100, 2 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 50 mM β-glycerophosphate, 25 mM NaF, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 1 μ/ml pepstatin A, 1 μg/ml antipain and 1 mM phenyl methyl sulphonyl fluoride (PMSF). After incubation on ice for 30 min, the insoluble material was removed by centrifugation at 12 000 rpm for 15 min. Two hundred micrograms of total protein from each sample were precleared with protein A–sepharose beads (Upstate Biotechnology, U.S.A.) and the supernatant was incubated overnight at 4°C with anti-cyclin A or E antibody. Immune complexes were collected after incubating for 2 h with protein A–sepharose beads and the beads were washed three times with lysis buffer and twice with kinase assay buffer containing 50 mM Tris, pH 7.4, 10 mM $MgCl_2$, 1 mM DTT, 50 mM β-glycerophosphate, 0.1 mM Na_3VO_4 25 mM NaF, 1 μg/ml antipain, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM PMSF. Kinase activity was assayed by incubating the complex for 15 min at 30°C in 50 μl of kinase assay buffer supplemented with 5 μg histone H1 (Gibco BRL), 10 μCi [γ-^{32}P]-ATP (10 μM, Amersham),

5 μM protein kinase inhibitor (PKI) and 20 mM EGTA and analysed by 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis(SDS–PAGE) followed by autoradiography.

Immunoblot analysis

Thirty micrograms of protein from each sample, prepared as above, were analysed by SDS–PAGE followed by electrotransfer on to polyvinylidene difluoride (PVDF) membranes (Millipore). The membranes were blocked with 5% non-fat milk (Carnation, U.S.A.) and probed with human anticyclin E (Santa Cruz, U.S.A.), anticyclin A (UBI), anti-CDK2 (Santa Cruz), anti-p27^{Kip1} (UBI), anti-p21$^{WAF1/Cip1}$ (Oncogene Science, U.S.A.), anti-p53 (Oncogene Science) or anti-PCNA (Calbiochem, U.S.A.) antibodies. The blots were washed and incubated with a horseradish peroxidase-coupled antirabbit IgG antibody (Pierce, U.S.A.) followed by detection with an enhanced chemiluminescence (ECL) revelation system (Amersham).

DNA fragmentation assays

Both floating and adherent cells were pelleted and lysed with 50 mM Tris-Cl, pH 8.0, 2 mM ethylene diamine tetraacetic acid (EDTA), 10 mM NaCl, 1% SDS and 500 μg/ml proteinase K at 50°C for 48 h. Samples were extracted with phenol/chloroform, precipitated with ethanol, treated with 300 μg/ml RNase for 1 h at 37°C and separated by electrophoresis on a 1.8% agarose/Tris-Acetate-EDTA (TAE) gel. Visualisation of the DNA band was performed by staining with ethidium bromide, destaining in water and viewing on an ultraviolet transilluminator. The gel was photographed under ultraviolet light with Polaroid film.

Phase-contrast microscopy

Morphological alterations of the cells were microscopically evaluated at timed intervals on an inverted phase-contrast microscope (Olympus, Japan).

RESULTS

G-Rs4 suppresses cell growth of SK-HEP-1

The results from the MTT assays using SK-HEP-1 cells showed that cell viability decreased after treatment with G-Rs4 at doses higher than 10 μM for 24 h, although it remained unaltered at doses lower than 5 μM (Figure 2a). The data showed that the IC_{50} value was approximately 20 μM. However, the IC_{50} value in terms of DNA synthesis activity was approximately 0.5 μM (Figure 2b).

G-Rs4 arrests the cell cycle at the G1/S boundary and consequently induces apoptosis

The results from flow cytometric analyses showed that the synchronised cells arrested mainly at the G1/S boundary after 10 h of treatment, but underwent apoptosis as evidenced by an apoptotic peak generated after 24 h of treatment with 25 μM G-Rs4 (Figure 3a). In support of this notion, the results from the DNA fragmentation assays (Figure 3b) and alterations in cell morphology (Figure 3c) showed that G-Rs4, at doses higher than 10 μM, induced apoptosis of the cells in a dose- and time-dependent manner.

G-Rs4 downregulates activity of both cyclin E- and cyclin A-associated kinases

The results from immune complex kinase assays using specific antibodies against cyclins E or A, showed that the kinase activity of both cyclins E- and A-associated kinases significantly decreased after treatment with 10 μM G-Rs4 as compared with those of untreated cells (Figure 4). The effective dose of 10/μM G-Rs4 was consistent with that of inducing DNA fragmentation (Figure 3b).

G-Rs4 elevates protein levels of both p53 and p21^{WAF1}

We determined protein levels of cyclin E, cyclin A, p21^{WAF1}, p27^{Kip1}, PCNA and p53 by immunoblot assays using the corresponding specific antibodies. The results showed that protein levels of CDK2 and p27^{Kip1} slightly decreased after treatment with G-Rs4 at doses higher than 15 μM, whilst those of other components such as cyclins E and A and PCNA remained unaltered even after treatment at 25 μM (Figure 5). Interestingly, G-Rs4 increased protein levels of p53 and p21^{WAF1} at doses higher than 10 μM in a dose-dependent manner, while those of PCNA again remained unaltered (Figure 6a). The same results were reproduced when the cells were treated with G-Rs4 for 12 h in doses ranging from 0.1 to 25 μM, indicating that the effective dose on the expression of p53 and p21^{WAF1} was 10 μM (Figure 6b). The effective dose was consistent with the doses that could downregulate the kinase activity of both

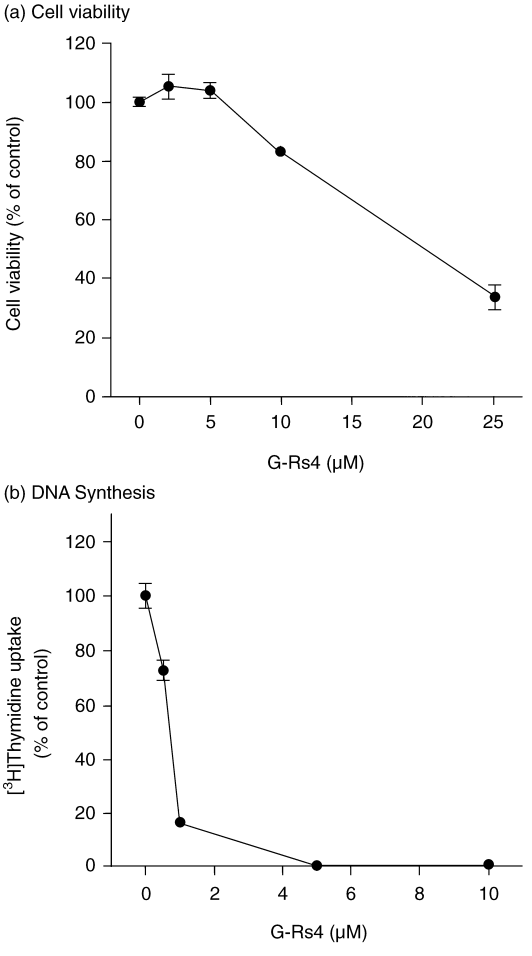

Figure 2. Inhibition of cell growth and DNA synthesis by ginsenoside-Rs$_4$ (G-Rs4). SK-HEP-1 cells were incubated with varying concentrations of G-Rs4 for 24 h. Cell viability was determined by MTT assay (a) or DNA synthesis activity was determined by [^{3}H]thymidine uptake assay (b). Data are presented as the mean ± standard deviation (S.D.) of triplicate experiments.

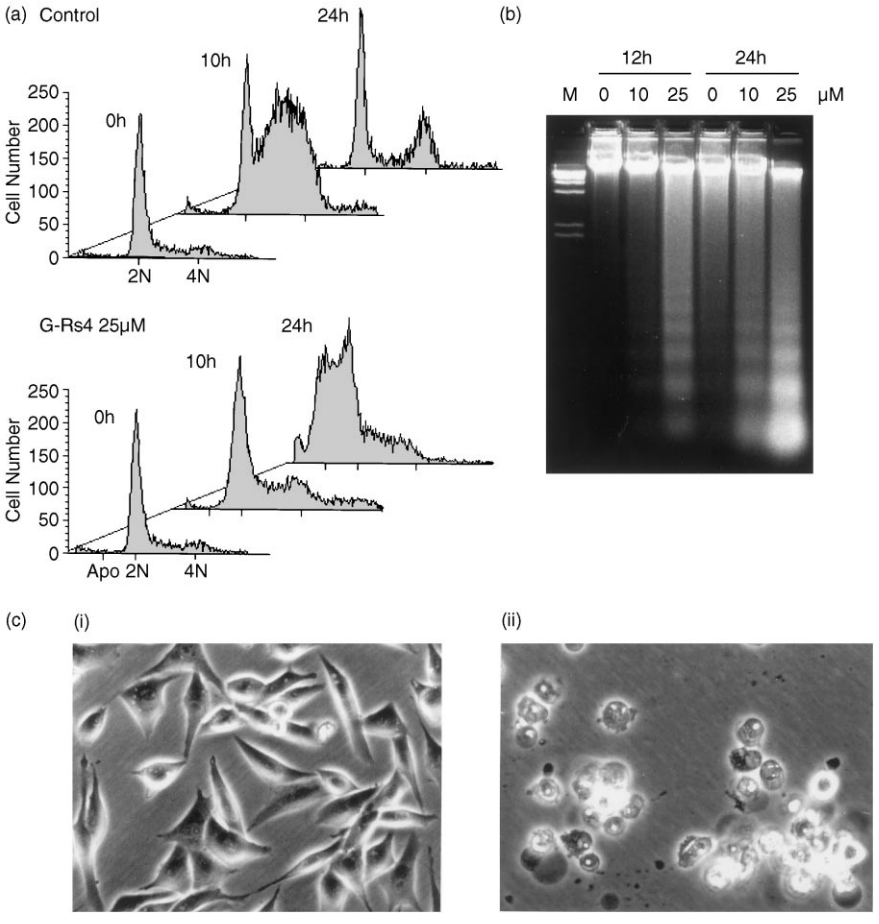

Figure 3. Induction of apoptosis by ginsenoside-Rs_4 (G-Rs4) (a) Flow cytometric analysis. The experiments were performed on synchronised cultures of SK-HEP-1 cells, upon their release from the hydroxyurea block. The cells were incubated with or without 25 μM G-Rs4 and harvested at the indicated times. Flow cytometric analysis was performed as described in Materials and Methods. *Apo* denotes the apoptotic peak. (b) Internucleosomal DNA fragmentation. DNA prepared from the cells treated for 12 or 24 h with the indicated concentrations of G-Rs4 was electrophoresed and visualised with ethidium bromide. (c) Morphological changes. Cells treated for 24 h without (i) or with (ii) 25 μM G-Rs4 were photographed under a phase-contrast microscope (150X).

cyclins E- and A-associated kinases and induce apoptosis in the cells.

DISCUSSION

The results of this study have shown that G-RS4, an analogue of the ginsenoside G-Rg5 elevates p53 and $p21^{WAF1}$ protein levels, which are associated with the induction of apoptosis in SK-HEP-1 cells. From the MTT assay, the IC_{50} value for G-Rs4 was 20 μM, 10-fold higher than that of G-Rg5 [6], but in terms of DNA synthesis, the IC_{50} value was approximately 0.5 μM which was comparable with the value of 0.5 μM for G-Rg5. The results indicate that these two

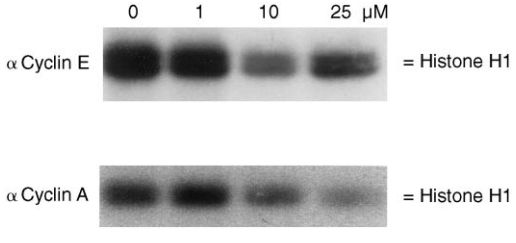

Figure 4. Inhibition of cyclin-dependent protein kinase (CDK) activity by ginsenoside-Rs_4 (G-Rs4). SK-HEP-1 cells were incubated with varying concentrations of G-Rs4 for 24 h and cell lysates were prepared. Histone H1 kinase activity associated with the immune complex precipitated by anti cyclin E or anti cyclin A antibody was measured.

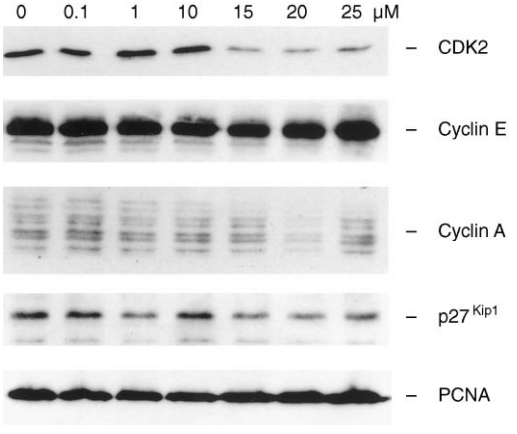

Figure 5. Effect of ginsenoside-Rs_4 (G-Rs4) on the expression of several cell cycle regulatory proteins. SK-HEP-1 cells were incubated with varying concentrations of G-Rs4 for 24 h. Cell lysates were prepared and Western blot analysis was performed against the indicated proteins.

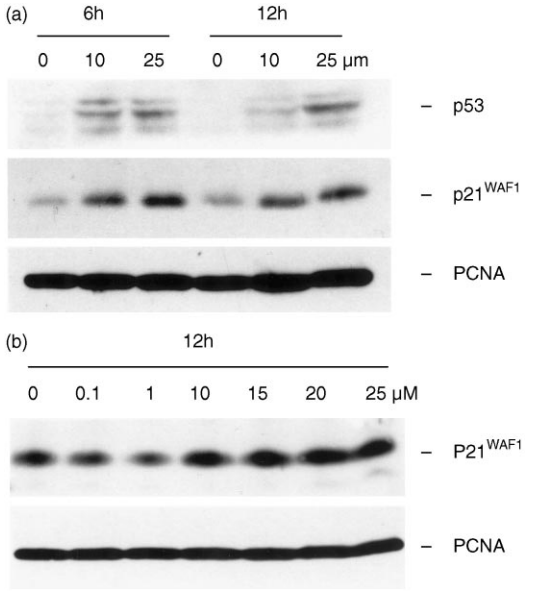

Figure 6. Effect of ginsenoside-Rs$_4$ (G-Rs4) on the expression of p53 and p21^{WAF1}. SK-HEP-1 cells were incubated with indicated concentrations of G-Rs4 and harvested at the indicated times. Cell lysates were prepared and Western blot analysis was performed against p53, p21^{WAF1} or proliferating cell nuclear antigen (PCNA).

saponin analogues, whose structures differ only at the 6′-OH of the glucose residue, display a similar inhibiting activity for DNA synthesis but not for cell viability. Nevertheless, the results suggest that G-Rs4 inhibits DNA synthesis at lower doses but induces cell death at higher doses in SK-HEP-1 cells. The inhibitory effect on DNA synthesis resulted in cell cycle arrest at the G1/S boundary, which at G-Rs4 doses above 10 μM was followed by apoptosis. Both these effects were shown to be associated with downregulation of cyclin E- and A-associated kinases (Figure 4), but this was not attributable to lower protein levels of CDK2 or p27^{Kip1}, but rather increased levels of p53 and p21^{WAF1} protein, a universal inhibitor of cyclin/CDK2.

In addition, apoptosis induced by G-Rs4 is closely associated with elevated protein levels of p53 and p21^{WAF1}, although it is not yet certain that elevated p53 and p21^{WAF1} proteins directly play roles in the apoptosis induction process. It is important to note here that mutations in the tumour suppressor *p53* are a common event in hepatocellular carcinoma [19] and the *p53* gene is rearranged in SK-HEP-1 cells [20]. Others have also reported that C6-ceramide induces apoptotic cell death in the same cells, during which process the protein level of p21^{WAF1}, but not that of p53, elevated [21]. In addition, it has been proposed that the apoptotic and transactivation activity of p53 can be dissociated [22]. Thus, it appears that abnormal p53 expressed in SK-HEP-1 cells may not directly contribute to its transactivation of the *p21*^{WAF1} gene.

Taking these data together, we suggest that G-Rs4 arrests cell proliferation possibly by downregulating the kinase activities of both cyclins E- and A-associated kinases, which is tightly associated with elevating protein levels of p53 and p21^{WAF1}. In addition, we propose that these molecular events may be functionally related to induction of apoptosis in the cells in response to G-Rs4.

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