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Induction of G1 phase arrest in MCF human breast cancer cells by pentagalloylglucose through the down-regulation of CDK4 and CDK2 activities and up-regulation of the CDK inhibitors $p27^{Kip}$ and $p21^{Cip}$

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Abstract

Pentagalloylglucose (5GG) is a potent and specific inhibitor of NADPH dehydrogenase or xanthine oxidase. In our previous study, we showed that 5GG was able to induce apoptosis in HL-60 cells in a time- and concentration-dependent manner via the activation of caspase-3. Recently, we found that 5GG was capable of perturbing the cell cycle of the human breast cancer cell line MCF-7. DNA flow cytometric analysis showed that 5GG exhibited the ability of blocking MCF-7 cell cycle progression at the G1 phase. The level of several G1 phase-related cyclins and cyclin-dependent kinases did not change in these cells during a 24-hr exposure to 5GG. However, the activity of cyclin E/CDK2 was decreased in a concentration- and time-dependent manner and the activity of cyclin D/CDK4 was inhibited when serum-starved synchronized cells were released from synchronization. $p27^{Kip}$ and $p21^{Cip}$, inhibitors of cyclin/CDK complexes in G1-phase, were gradually increased after 5GG treatment in a time-dependent manner and the induction of $p21^{Cip}$ was correlated with an increase in p53 levels. These results suggest that the suppression of cell-cycle progression in the G1 phase by 5GG was mediated in MCF-7 cells, at least in part, by either the inhibition of cyclin D/CDK4 and cyclin E/CDK2 activity or the induction of the CDK inhibitors $p27^{Kip}$ and $p21^{Cip}$.

Keywords: 5GG; G1 phase; $p27^{Kip}$; $p21^{Cip}$; p53; Cyclin D/CDK4; Cyclin E/CDK2

1. Introduction

Pentagalloylglucose (1,2,3,4,6-penta-*O*-galloyl-β-D-glucose/5GG) (Fig. 1), originally purified from tannin contained in Chinese and Turkish gall, broadly exists in the Moutan cortex (the root cortex of *Paeonia suffruicosa* ANDREXS) and in Paeoniae Radix (the root of *Paeonia lactiflora* PAlls var. *trichocarpa* BUNGE). 5GG is an important component in crude traditional Chinese drugs, and is used for the treatment of gastric and peptic ulcers. Previous studies indicated that pure 5GG had profound biological activities such as the inhibition of xanthine oxidase [1], succinate dehydrogenase, ubiquinone-1 oxidase, the respiratory control ratio in rat liver [2], NADH dehydrogenase I/II (type I and II) [2–5], and H^+, K^+-ATPase [6]. It has been demonstrated that 5GG is synthesized from gallic acid and tetragalloylglucose (the product from the sequential addition of gallic acid units to glucose) and is catalyzed by acyltransferase in the young leaves of the peduneulate oak [7].

The essential molecules that regulate cell cycle progression are cyclin-dependent kinases (CDKs) and cyclins. In the early G1 phase, cyclin D is expressed in response to a growth-promoting mitogen and subsequently binds to CDK4/6. The cyclin D/CDK4/6 complex is then activated by Cdk-activating kinase (CAK) which leads to the phosphorylation of the Rb protein. Phosphorylation of the Rb protein disrupts its association with the E2F family of

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Abbreviations: 5GG, pentagalloylglucose; CDK, cyclin-dependent kinase; IP, immunoprecipitation; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; RT-PCR, reverse transcription-polymerase chain reaction.

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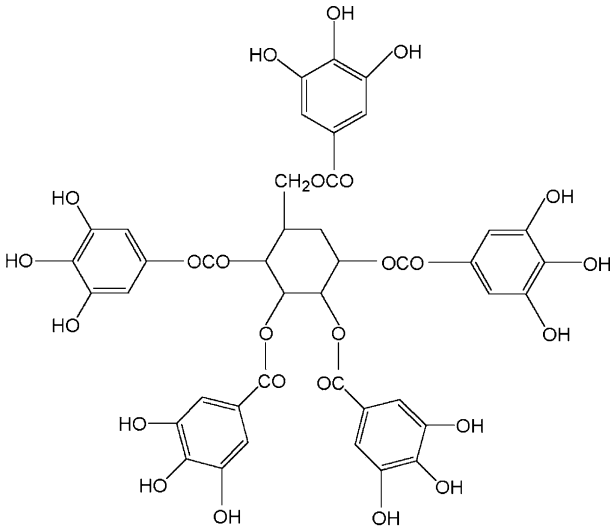

Fig. 1. Chemical structure of penta-*O*-galloyl-β-D-glucose (5GG).

transcription factors. The released E2F triggers the expression of several essential proteins for cell cycle progression, such as cyclin E, cyclin A, and thymidine kinase [8,9]. Cyclin E forms a complex with CDK2 and the activated cyclin E/CDK2 complex then completes the process by phosphorylating the Rb protein on additional sites. Cyclin/CDK complexes regulate progression through the G1 phase and the initiation of DNA synthesis or entry into the S phase. Cyclin/CDK complex activity is constrained by CDK inhibitors (CKIs). The CKIs that govern these events are classified into two families based on their structures and CDK targets, i.e. the INK4 and Cip/Kip family's [10]. Cip/Kip family members: (1) include $p21^{Cip}$, $p27^{Kip1}$, and $p57^{Kip2}$, (2) affect the activities of cyclin D-, E-, and A-dependent kinases and (3) bind both components of the cyclin/CDK complex [11–14]. Previous studies have shown that $p21^{Cip}$ and $p27^{Kip}$ can bind to cyclin/CDK complexes and physically prevent the complexes from phosphorylating their target proteins [15–18]. Recent evidence indicates that $p21^{Cip}$ can facilitate and stabilize the formation of the cyclin D/CDK4 complex via a weak interaction with both components during the early G1 phase [19]. Titration of unbound $p21^{Cip}$ and $p27^{Kip}$ into the cyclinD/CDK4-assembled complex relieves cylcin E/CDK2 from the constraints imposed by Cip/Kip, thereby facilitating cyclin E/CDK2 activation later in the G1 phase [20].

In our previous report [21], we indicated that 5GG induced apoptosis in the HL-60 leukemia cell line. In addition, 5GG suppressed the LPS-induced activation of NF-κB [22]. Here, we demonstrate that 5GG is a potent compound for the induction of G1 phase arrest in MCF-7 cells. The mechanism behind this G1 arrest principally involves a decrease in cyclin/CDK activity and an induction of $p27^{Kip}$ and $p21^{Cip}$ proteins.

2. Materials and methods

2.1. Reagents

Pentagalloylglucose was provided by Dr. J.H. Lin *et al.* [23]. The enhanced chemiluminescence detection kit and [γ-^{32}P]ATP were purchased from Pharmacia. Protein A/G beads and the Rb peptide (769-921) were purchased from Santa Cruz. Histone H1 was purchased from Calbiochem.

2.2. Cell culture

The human breast cancer cell line, MCF-7, used in this study, was provided by Dr. M.T. Lee (Taiwan University). Cells were cultured in Dulbecco's minimal essential medium (DMEM) supplemented with 10% FBS. Cultures were incubated in 5% CO_2 at 37° in a humidified environment. For MCF-7 cell cycle experiments, 1×10^5 cells/mL were seeded into culture plates. After 18 hr the cultures were transferred to DMEM containing 5% FBS and exposed to the chemical.

2.3. Cell growth and viability assay

Cells were cultured in 96-well dishes under the growth conditions described in Section 2.2 and were harvested after exposure to various concentrations of 5GG for various times. Cell viability was determined by the ATP-Lite™-M method according to the specification of the manufacturer (Luminescent ATP detection assay kit, Packard).

2.4. Cell cycle analysis

Cells were treated with various concentrations of 5GG and harvested after 24 hr of exposure. For flow cytometry analysis, cells were trypsinized, washed twice with PBS, and fixed in 80% ethanol for at least 18 hr at −20°. Fixed cells were washed twice with PBS and incubated with RNase A for 30 min at 37° before staining with 10 μg/mL of propidium iodide. The cell cycle was analyzed using a FACs laser flow cytometer (Becton Dickinson).

2.5. Western blot analysis

Cells growing on 10-cm dishes were washed twice with 5 mL of ice-cold PBS and lysed by scraping in gold lysis buffer (10% glycerol, 1% Triton X-100, 1 mM sodium orthovanadate, 1 mM EGTA, 5 mM EDTA, 10 mM sodium pyrophosphate, 20 mM Tris–HCl pH 7.9, 100 μM β-glycerophosphate, 137 mM NaCl, 1 mM PMSF, 10 μg/mL of aprotinin and 10 μg/mL of leupeptin) for 30 min at 4°. The cell lysates were clarified by centrifugation at 12,000 *g* for 15 min at 4°. Equal amounts of protein (50 μg) were separated by SDS-PAGE (8% gels for the Rb protein and its phosphorylated form; 10% gels for p53, CDK2, CDK4, cyclin D1, cyclin D3, cyclin E, Rb (769-921) and

13% gels for p16, p21, and p27) and transferred to PVDF membranes. The membranes were probed with primary antibodies (antibodies against CDK2, CDK4, cyclin D1, cyclin E, p27, p16, p53, and the Rb protein were purchased from Santa Cruz; anti-cyclin D3, and -p21 from Transduction Laboratories; anti-phosphorylated Rb from Cell Signaling (New England); and anti-α-tubulin from Zymed) followed by horseradish peroxidase-conjugated secondary antibodies. Antibody detection was performed using an enhanced chemiluminescence detection kit (Amersham).

2.6. Immunoprecipitation and kinase assay

For CDK kinase assay, exponentially growing MCF-7 cells were washed with cold PBS and lysed with gold lysis buffer for 30 min at 4°. The cell lysates were clarified by centrifugation at 12,000 *g* for 10 min at 4°. One hundred fifty μg of protein was incubated with either a CDK2- or CDK4-specific antibody (Santa Cruz) and protein A/G plus agarose in immunoprecipitation buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris–HCl pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.2 mM sodium orthovanadate, 0.2 mM PMSF, 0.5% NP-40) at 4°. The immunoprecipitates were washed with immunoprecipitation buffer three times.

For the IP-western analysis, the CDK2 and CDK4 immunoprecipitates were resuspended in 30 μL of lysis buffer, mixed with 5× Laemmli's loading buffer and separated by SDS-PAGE.

For the kinase assay, the CDK2 and CDK4 immunoprecipitates were washed with one of two kinase buffers (50 mM HEPES pH 7.4, 10 mM $MgCl_2$, 2.5 mM EDTA, 1 mM DTT for CDK2; the same buffer but with 10 mM β-glycerophosphate and 1 mM NaF for CDK4) three times. The kinase assay was carried out by incubating the suspension of immunocomplexes with 2 μg histone H1 (for CDK2) or 1 μg Rb (769-921) (for CDK4), 1 mM cold (non-radioactive) ATP, and 0.5 μCi [γ-^{32}P]ATP for 20 min at 25°. The reaction mixture was combined with 5× Laemmli's loading buffer, boiled, and subjected to SDS-PAGE. The radioactivity of the dried gels was visualized by autoradiography.

2.7. RT-PCR

Total RNA of each sample was extracted with ISOGEN (Nippon Gene Company, LTD) according to the specification of the manufacturer. For reverse transcription, the template mixture containing 2 μg RNA and 0.5 mg oligo-dT in a total volume of 5 μL of water was heated to 70° for 5 min (to relax the secondary structure of the template) and then was cooled immediately on ice. The reverse transcription was performed in a volume of 20 μL, containing the chilled template mixture, 50 mM Tris–HCl (pH 8.3), 135 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 5 mM dNTP, 25 units RNasin and 2 units M-MLV RT at 37° for 60 min. One-half of the RT reaction was used for PCR, performed in a volume of 100 μL by adding 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 0.01% gelatin, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 2 mM dNTP, 25 pM sense and antisense primers, and 2.5 units Pro *Taq* DNA polymerase. The sequences used in PCR are as follows for $p21^{Cip}$: sense: 5′-AGGAGGCCCGTGAGCGAGCGATGGAAC-3′, antisense: 5′-ACAAGTGGGGAGGAAGTAGC-3′; and for $p27^{Kip}$: sense: 5′-CAGCTTGCCCGAGTTTA-3′, antisense: 5′-TGGGGAACGTCTGAAAC-3′. The thermal cycle conditions used were as follows: 1 cycle at 94° for 5 min; 30 cycles at 94° for 1 min; 55° (p21) or 53° (p27) for 1 min (p21) or 30 s (p27); 72° for 1 min (p21) or 2 min (p27); and 1 cycle at 72° for 10 min.

2.8. Electrophoretic mobility shift assay (EMSA)

5GG-treated cultures were harvested and washed twice with ice-cold PBS for nuclear protein extraction. The harvested cells were suspended in 200 μL of buffer A (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, and 1 mM leupeptin) containing 12.5 μL of 10% NP-40, and were incubated on ice for 10 min. Nuclear pellets were obtained by centrifugation at 12,000 *g* for 2 min at 4°. The pellets were resuspended in 25 μL buffer C (20 mM HEPES, 1 mM DTT, 1 mM EDTA, 0.4 M NaCl, 1 mM PMSF). Nuclear lysates were incubated on ice for 15 min and vortexed at least three times. The nuclear extract for EMSA was clarified by centrifugation at 12,000 *g* for 20 min at 4°. The reaction mixture for the gel-shift assay (10 μL final volume) contained 10 mM Tris–HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 5% glycerol, 2 pg salmon sperm DNA, probe DNA (approximately 2 ng), and 5 μg nuclear extract. The binding reaction was allowed to proceed at room temperature for 20 min. The products then were resolved by electrophoresis on 6% TBE non-denaturing gels. The sequence of the SP-1 primer used in these studies is as follows: 5′-ATTCGATCGGGGCGGGGCGAGC-3′.

3. Results

3.1. Growth inhibition and cytotoxicity of 5GG in MCF-7 cells

In our previous studies [21], we found that 5GG could block cell cycle progression in the HL-60 leukemia cell line. Here, we used the human breast cancer cell line, MCF-7, to investigate the mechanism by which 5GG perturbs the cell cycle. Proliferation of exponentially growing MCF-7 cells was inhibited in a concentration-dependent manner. After 24 hr of exposure to various doses of 5GG (from 1 to 100 μM), the number of 5GG-treated cells were compared with control cells in the logarithmic phase of growth. This analysis revealed that cell growth was inhibited by about 10% with 10 μM 5GG but reached

42% with 50 μM 5GG (Fig. 2A). Compared with exponentially growing control cells, treatment with both 30 and 50 μM 5GG resulted in a marked decrease in cell number after 24 hr (Fig. 2B). The trypan blue exclusion assay was used to determine whether the drop in cell number was due to 5GG toxicity. After 24 hr of 5GG (50 μM) treatment, less than 5% of the total cells were dead (data not shown). From this it seemed that 5GG was not toxic but was inhibiting cell growth.

3.2. Effects of 5GG on DNA content distribution in MCF-7 cells

After a 24 hr of exposure to 15 μM 5GG flow cytometry analysis of the cellular DNA content revealed that there was a slight decrease in the percentage of cells in the G2 phase as cells accumulated in G1 (62.45%). In contrast, at 50 μM 5GG there was a marked decrease in the percentage of cells in G2 as cells in the G1 phase increased dramatically to 80.8% (Fig. 2C). Based on the apparent ability of 50 μM 5GG, a non-toxic concentration, to arrest these cells in the G1 phase we used this concentration in our subsequent experiments.

3.3. Cyclin D, cyclin E and associated CDK expression in 5GG-treated MCF-7

It is well known that cyclin D, cyclin E, CDK2, and CDK4/6 cooperate to promote G1 phase progression. We first determined whether 5GG affected the expression of these G1-related proteins. Cells were treated with 50 μM 5GG for 3, 6, 9, 12, 18, and 24 hr and then harvested for western blotting. The protein levels of cyclin D1, cyclin D3, and CDK4 (components of the cyclin-CDK complex that are active in early G1 phase) were found to be unaltered after 5GG treatment (Fig. 3A). In addition, the protein levels of cyclin E and CDK2 were relatively unaffected by 5GG under the same conditions (Fig. 4A), although there was a very slightly decrease in the level of cyclin E at 18 hr. Since the protein levels did not explain the inhibition of S phase entry we examined the kinase activities of these cyclin-CDK complexes.

3.4. Kinase activities of cyclin-CDK complexes in 5GG-treated MCF-7 cells

To determine whether a modification in cyclin D/CDK4 kinase activity contributes to G1 phase arrest, the effects of 5GG on cyclin D/CDK4 kinase activity were examined in synchronized cells. Cell lysates were immunoprecipitated with an anti-CDK4 antibody, and CDK4 kinase activity was measured using Rb (769-921) as the substrate, as described in Section 2. In control cells, cyclin D/CDK4 kinase activity increased following release from synchronization with 5% FBS; maximal activity was achieved

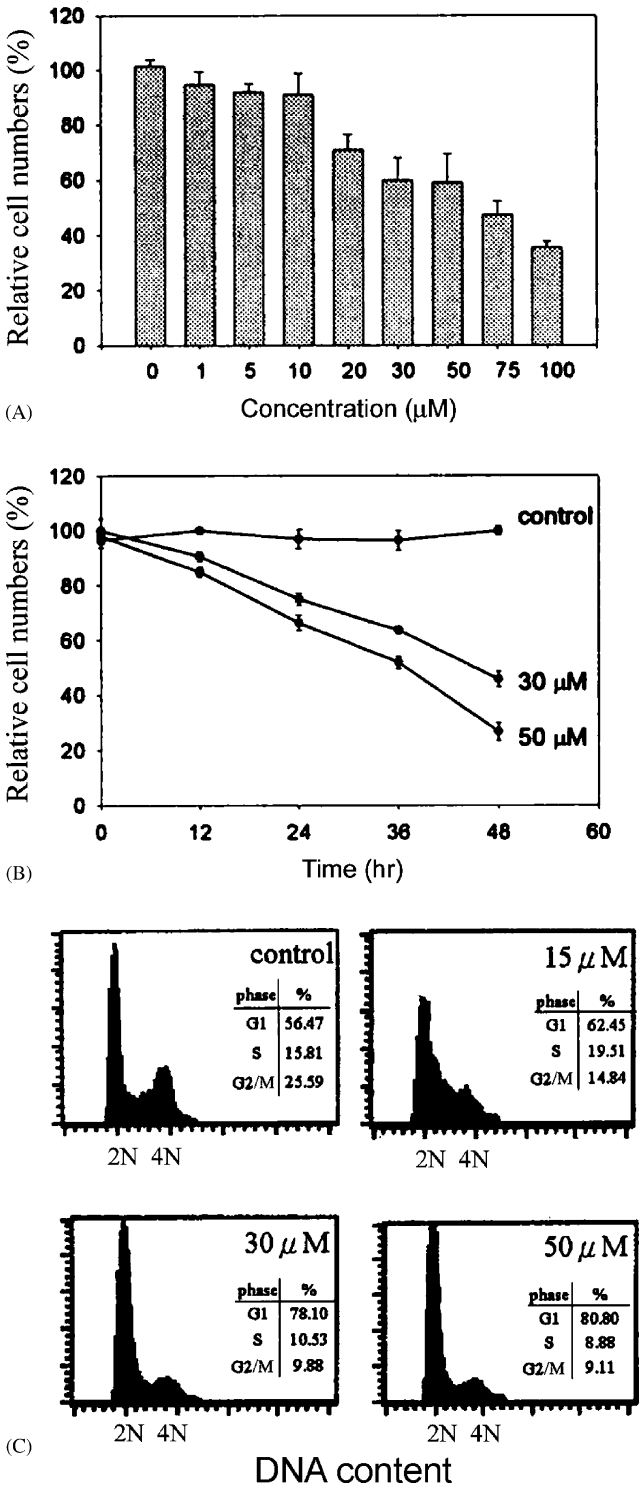

Fig. 2. Effect of 5GG on growth and viability of MCF-7 cells. (A) Effect of 5GG concentration on the relative number of MCF-7 cells. Cells were cultured in 96-well dishes for 24 hr and then treated with various concentrations of 5GG for 24 hr. (B) Time course effects of 5GG on the growth of MCF-7 cells. Cells were cultured and exposed to 0, 30, or 50 μM 5GG for the indicated times. Relative cell number was determined by the ATP-Lite™ method as described in Section 2. Data are expressed as the percentage of control. Each value was the mean ± SEM of triplicate samples from five different experiments. (C) Effect of 5GG on DNA content in MCF-7 cells. Cells harvested after a 24-hr treatment with 5GG (0, 15, 30, or 50 μM) were stained with propidium iodide. Cell cycle distribution was determined by FACs analysis.

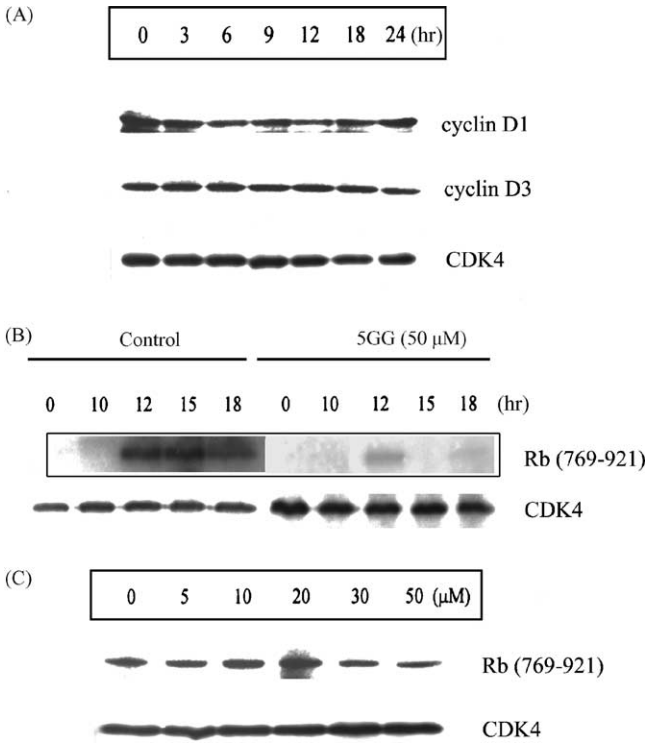

Fig. 3. Effect of 5GG on cyclin D/CDK4 protein expression and CDK4 kinase activity. (A) Effect of 5GG on the protein levels of cyclin D and CDK4 in the presence of 5GG at the indicated times. Cells were treated with 50 μM 5GG and harvested at the times indicated. The relative protein levels of cyclin D and CDK4 were determined by western blot analysis. (B) Effect of 5GG on cyclin D/CDK4 kinase activity. Cells were synchronized in serum-free medium for 24 hr and then were released from synchronization by re-feeding with fresh medium containing 5% FBS with or without 5GG. Kinase activities were determined using Rb (769-921) as the substrate (see Section 2). CDK4 levels were determined by IP-western analysis and used as loading controls. (C) Effect of 5GG on the *in vitro* cyclin D/CDK4 kinase activity. Immunoprecipitated MCF-7 CDK4 was incubated with various concentrations of 5GG to determine kinase activity. CDK4 levels were used as loading controls.

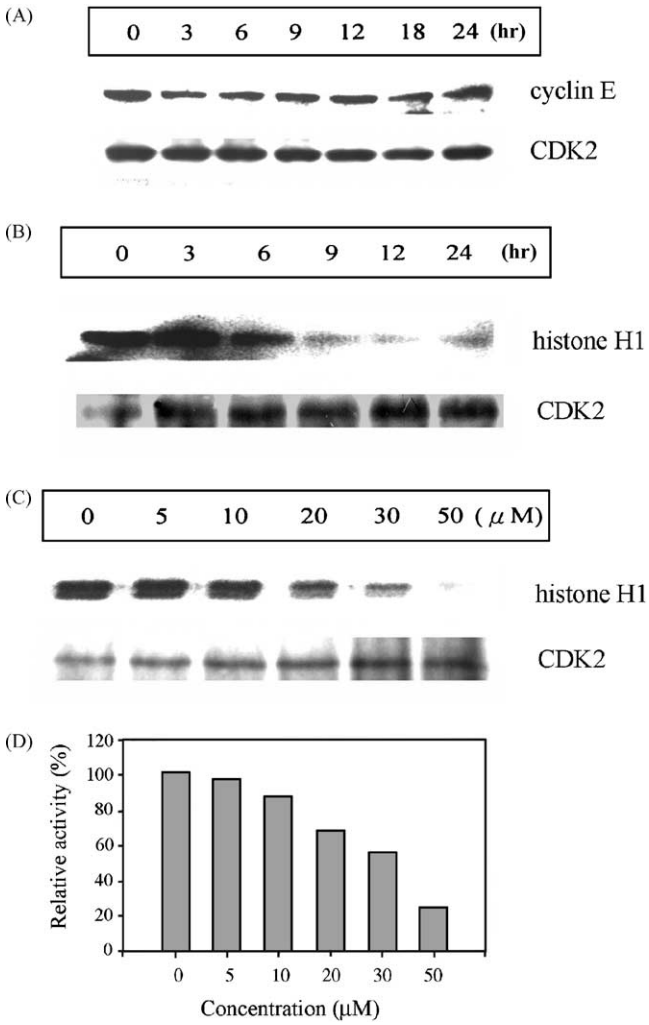

Fig. 4. Effect of 5GG on the protein level of cyclin E/CDK2 and the kinase activity of CDK2. (A) Cyclin E and CDK2 protein levels after 24 hr of treatment with 5GG. (B) Effect of 5GG on CDK2 kinase activity over time. The conditions for 5GG-treatment were as described in Section 2. CDK4 levels were determined by IP-western analysis and used as loading controls. (C) Effect of 5GG concentration on cyclin E/CDK2 kinase activity in an *in vitro* assay system. CDK2 levels were used as loading controls. (D) Densitometric quantification of the 5GG concentration effect on CDK2 activity.

12–15 hr after stimulation with the FBS (Fig. 3B). In contrast to the control, cyclin D/CDK4 kinase activity was suppressed in 5GG-treated cells (Fig. 3B). Immunoprecipitated CDK2 from 5GG-treated cells also was examined for kinase activity using histone H1 as the substrate. The activity of CDK2 decreased in a time-dependent manner following exposure to 5GG and was essentially abrogated after 6–9 hr of treatment with 5GG (Fig. 4B). After 24 hr of exposure to 5GG, CDK2 kinase activity was inhibited by about 80% compared with the untreated control cells. This caused us to question whether 5GG could directly affect CDK2 kinase activity in an *in vitro* assay system. The kinase activity of the CDK4 immunocomplex, prepared from exponentially growing MCF-7 cells, was barely interfered with by the presence of a high concentration of 5GG (30 μM) (Fig. 3C). However, the kinase activity of the CDK2 immunocomplex was decreased in a concentration-dependent manner in the *in vitro* assay system (Fig. 4C). The IC_{50} of the CDK2 kinase activity in the *in vitro* assay system was about 30 μM (Fig. 4D).

3.5. Effect of 5GG on the phosphorylation status of the Rb protein

The phosphorylation of the Rb protein is mediated by both cyclinD/CDK4 and cyclinE/CDK2 and is required for cells to progress from G1 into the S phase. In order to confirm that 5GG inhibits the kinase activities of CDK4 and CDK2 within cells, the phosphorylation status of the Rb protein was investigated following exposure of exponentially growing MCF-7 cells to 5GG. It was found that the degree of phosphorylation of the Rb protein was decreased after 6 hr of 5GG treatment (Fig. 5A).

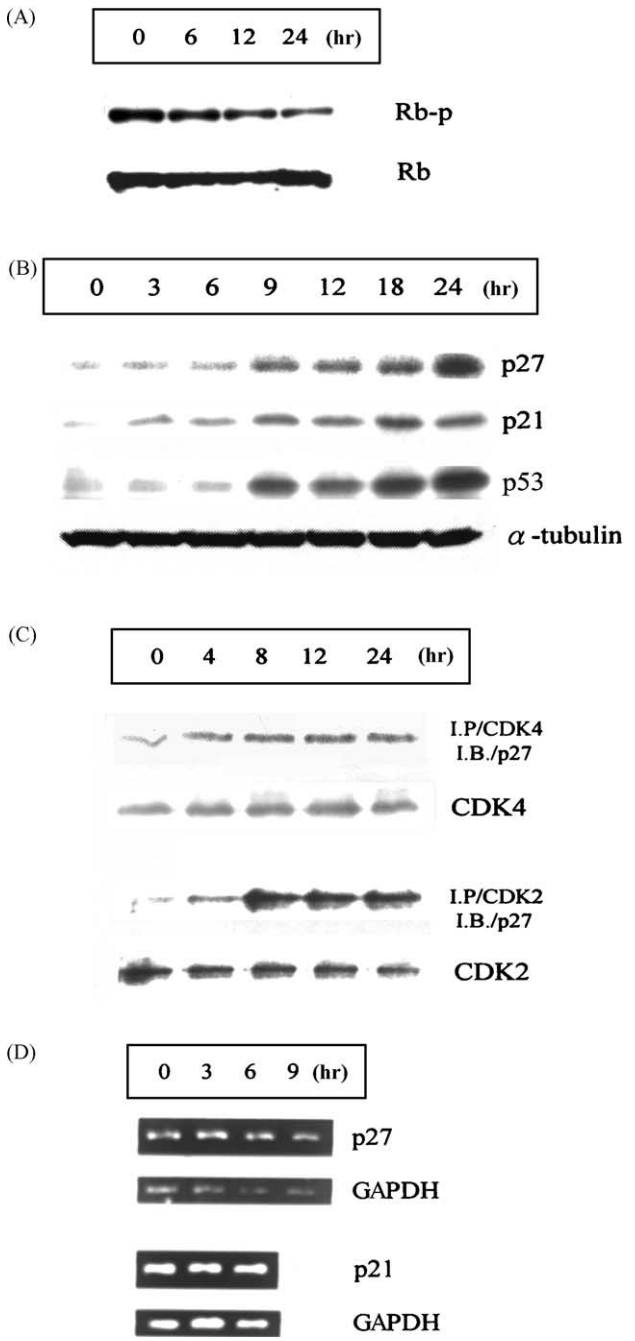

Fig. 5. Effect of 5GG on proteins involved in G1 phase progression. (A) Effect of 5GG-treatment on the phosphorylation state of the Rb protein. Total and phosphorylated forms of the Rb (Rb-p) protein were detected with specific antibodies for each. (B) Alterations in $p27^{Kip}$, $p21^{Cip}$, and p53 protein levels by 5GG. The internal control was α-tubulin. (C) Effect of 5GG on CDK4- and CDK2-bound $p27^{Kip}$. CDK4/$p27^{Kip}$ and CDK2/$p27^{Kip}$ complexes were immunoprecipitated with antibodies against CDK4 and CDK2, respectively. The $p27^{Kip}$ protein was then detected by western blotting. (D) Effect of 5GG on $p21^{Cip}$ and $p27^{Kip}$ mRNA levels. Total RNA was harvested from 5GG-treated MCF-7 cells at the indicated times, and the relative amounts of target mRNA were assessed by RT-PCR.

3.6. CKI levels in MCF-7 cells following 5GG treatment

CKIs are well known to interfere with cell cycle progression to cause phase-specific cycle arrest [24,25]. These kinase inhibitors perturb the phosphorylation process by directly interacting with their target proteins, i.e. cyclins or CDKs. The protein levels of certain CKI family members, crucial in the regulation of G1-phase progression, were determined by western blot analysis. Levels of $p27^{Kip}$, a member of the Cip/Kip subfamily, increased gradually over time following treatment of MCF-7 cells with 5GG; after a 24 hr treatment the level was 6.5-fold over the 0 hr control (Fig. 5B). As with $p27^{Kip}$, $p21^{Cip}$ increased markedly under the same conditions (Fig. 5B).

The expression of $p21^{Cip}$ has been reported to be regulated by either a p53-dependent or p53-independent mechanism [26–31]. To investigate whether the growth-inhibitory response to 5GG was dependent on the status of p53, we examined the level of p53 in 5GG-treated MCF-7 cells. Consistent with the increase in $p21^{Cip}$, p53 also accumulated in a time-dependent manner after 5GG treatment (Fig. 5B).

3.7. Effect of 5GG on cyclin/CDK-associated CKIs

Although we showed that treatment of MCF-7 cells with 5GG can directly inhibit the activity of CDK2 (Fig. 4B) and increase the level of $p27^{Kip}$ (Fig. 5B), we were not certain whether the mechanism of inhibition was through a direct effect of 5GG on CDK2. Additionally, we questioned whether or not there was a correlation between these phenomena. To determine this, 5GG-treated cells were harvested and extracted for protein. An equal amount of protein was immunoprecipitated and subjected to SDS-PAGE as described in Section 2. When co-immunoprecipitated with CDK2, CDK2-associated $p27^{Kip}$ showed an increase at 4 and 8 hr and then remained at the same level up to 24 hr (Fig. 5C). Similarly, following co-immunoprecipitation with CDK4, it was observed that CDK4-associated $p27^{Kip}$ also increased, reaching maximal levels after 12 hr of 5GG-treatment. Under the same conditions, $p21^{Cip}$ and p16 were not detected in these immunocomplexes (data not shown).

3.8. Effect of 5GG on the level of $p21^{Cip}$ and $p27^{Kip}$ transcripts

According to our previous results, 5GG could elevate $p21^{Cip}$ and $p27^{Kip}$ after 6–9 hr of 5GG treatment (Fig. 5B). We were now interested in determining whether the level of the mRNAs encoding these proteins changed and whether these changes were reflected at the protein level. To determine this, RT-PCR was conducted as described in Section 2. Our data showed that although $p27^{Kip}$ and $p21^{Cip}$ proteins increased in a time-dependent manner, expression of their mRNAs did not change after 9 hr of 5GG treatment (Fig. 5D).

3.9. Upstream mechanism altered in the transcription of $p21^{Cip}$ and $p27^{Kip}$

There are many regulatory factors that can affect the promoter regions of the $p21^{Cip}$ and $p27^{Kip}$ genes. SP-1 is a

common regulatory sequence that exists in the 5′-flanking region of both the *p27*^{Kip} and *p21*^{Cip} gene promoter [32–35]. Over a 24 hr exposure period to 5GG, there was no obvious difference in the protein level of SP-1, in either the total cell lysate or the nuclear fraction (data not shown). In addition, the DNA-binding activity of the SP-1 protein, as examined by EMSA, was not altered during the period of 5GG treatment (data not shown).

4. Discussion

This study confirms that 5GG has a potent growth-inhibitory effect on certain types of cancer cells. Our investigation showed that the growth of human breast cancer cells, i.e. the MCF-7 cell line, is arrested in the G1 phase of the cell cycle by treatment with 5GG. The growth arrest was associated with an inhibition in the activities of both CDK4 and CDK2. These results are in agreement with the current model of cell-cycle regulation, i.e. that the activation of CDK4 and CDK2 by cyclin D and cyclin E, respectively, plays a key role in the progression from the G1 to the S phase [36,37]. In our studies, the decrease in the activities of CDK4 and CDK2 in 5GG-treated MCF-7 cells is not due to changes in the levels of these proteins (Figs. 3A and 4A). Nevertheless, we could not rule out the possibility that 5GG might interfere with cyclin binding to CDK, thereby inhibiting the kinase activities of CDKs.

The interaction between the cyclin/CDK complex and CKIs is another important mechanism for the regulation of cyclin/CDK activity [24]. In the progression of the cell cycle, the CKIs p21^{Cip} and p27^{Kip} often play important roles in the suppression of CDK activity. Overexpression of either p21^{Cip} or p27^{Kip} causes cell cycle arrest in the G1 phase. *In vivo* targets of p21^{Cip} and p27^{Kip} are cyclin D/CDK4 and cyclin E/CDK2, which physically associate with p21^{Cip} and p27^{Kip} [20]. In our study 5GG-induced an increase in the level of p27^{Kip} which, in turn, bound to CDK4 and CDK2. This binding interfered with and down-regulated the kinase activities of both CDK4 and CDK2.

Comparing the time course of the inhibition of the cyclin/CDK2 complexes in MCF-7 cells and in an *in vitro* assay demonstrated that in the former kinase activities decreased gradually after 6 hr of 5GG-treatment whereas in the latter there was an immediate inhibition of kinase activity. The mechanism by which CDK2 activity is reduced in 5GG-treated MCF-7 cells might not be fully explained through direct inhibition by 5GG.

It recently has been found that all of the cyclin D/CDK kinase activity in proliferating cells is found in complexes containing Cip/Kip proteins [38–40]. LaBaer *et al.* [39] first demonstrated that the activation of cyclin D/CDK complexes actually is facilitated by their interaction with CKIs. Their studies demonstrated that both p21^{Cip} and p27^{Kip} promoted interactions between D-type cyclins and their CDK partners *in vitro*, primarily by stabilizing the complexes. *In vivo*, p21^{Cip} stimulates the assembly of enzymatically active CDKs by entering into higher order complexes with cyclin D and CDK4. The incorporation of p21^{Cip} into cyclin/CDK4 complexes causes the dissociation of INK4 from CDK4, and results in the formation of active p21^{Cip}/cyclin D/CDK4 ternary complexes [19]. Primary mouse embryonic fibroblasts that lack both *p21*^{Cip} and *p27*^{Kip} genes fail to assemble detectable amounts of cyclin D/CDK complexes [40]. There are at least two ways for Cip/Kip to promote the activation of cyclin D/CDK complexes: (1) by directing the heteromeric complexes to the cell nucleus and (2) by increasing the stability of the cyclin D/CDK complex [25]. It seems that p21^{Cip} and p27^{Kip} differentially regulate various cyclin/CDK complexes from the observation that cyclin E/CDK2 and cyclin A/CDK2 are much more susceptible to inhibition by these CKIs than are cyclin D/CDK4 *in vivo* [38,39,41]. In our experiment, p21^{Cip} could not be detected in CDK4- and CDK2-immunocomplexes (data not shown) although 5GG induced p21^{Cip} expression. It might be possible that 5GG blocks the formation of the p21^{Cip}/cyclin D/CDK4 complex resulting in unstable ternary cyclin D/CDK4 complexes, which could lower the activity of these holoenzymes.

Phosphorylation of the Rb protein in the G1 phase of the cell cycle causes the release of active E2F that, in turn, drives the expression of S phase genes [42]. Our results show that in the presence of 5GG there is a decrease in the activity of CDK4 and CDK2 as well as an accompanying decrease in the phosphorylation state of the Rb protein. This implies that the resultant increase in the interaction between E2F and the hypophosphorylated Rb protein is the cause of the G1 arrest observed in the 5GG-treated MCF-7 cells. The phosphorylation sites of the Rb protein in MCF-7 cells that were released from sychronization by the re-addition of serum were at Ser^{807} and Ser^{811} [43].

It has been reported that the regulation of p21^{Cip} can be either p53-dependent or p53-independent [26–31]. Our data showed that in response to 5GG treatment there was a time-dependent increase in the level of both p53 and p21^{Cip}. It might be possible that the induction of p21^{Cip} by 5GG is related to the regulation of p53.

Previous studies have indicated that an SP-1 site exists in the 5′-flanking region of *p27*^{Kip} gene [32,44]. In our study, there was no difference in the level of SP-1protein in either the total lysate or the nuclear fraction from control and 5GG-treated cells over a 24 hr period. Additionally, based on EMSA analysis there was no difference in the DNA binding activity of SP-1 in 5GG-treated cells compared to control cells. It is possible that the up-regulation of p27^{Kip} involves a post-transcriptional mechanism, such as ubiquitination [45]. It also has been suggested that tannic acid (gallotannin) may inhibit the chemotrypsin-like activity of the proteasome and result in an increase of p27^{Kip} [46]. Based on the structural similarities between 5GG and gallotannin it might be possible that the increase in

$p27^{Kip}$ observed in this study may be the result of an analogous mechanism.

The study presented here demonstrates that 5GG strongly inhibits cell growth *in vitro* by inhibiting progression through the G1 phase of the cell cycle, i.e. by inducing G1 arrest. The growth inhibitory action of 5GG appears to involve the up-regulation of $p27^{Kip}$ and $p21^{Cip}$ and appears to be accompanied by reduction of the kinase activities of two G1 phase-related cyclin/CDK complexes.

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