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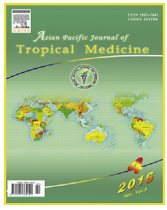

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Ethanol extract of *Kalopanax septemlobus* leaf inhibits HepG2 human hepatocellular carcinoma cell proliferation *via* inducing cell cycle arrest at G_1 phase

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ABSTRACT

Objective: To investigate the effects of an ethanol extract of *Kalopanax septemlobus* (Thunb.) Koidz. leaf (EEKS) on cell proliferation in human hepatocellular carcinoma cells and its mechanisms of action.

Methods: Cells were treated with EEKS and subsequently analyzed for cell proliferation and flow cytometry analysis. Expressions of cell cycle regulators were determined by reverse transcriptase polymerase chain reaction analysis and Western blotting, and activation of cyclin-associated kinases studied using kinase assays.

Results: The EEKS suppressed cell proliferation in both HepG2 and Hep3B cells, but showed a more sensitive anti-proliferative activity in HepG2 cells. Flow cytometry analysis revealed an association between the growth inhibitory effect of EEKS and with G_1 phase cell cycle arrest in HepG2 cells, along with the dephosphorylation of retinoblastoma protein (pRB) and enhanced binding of pRB with the E2F transcription factor family proteins. Treatment with EEKS also increased the expression of cyclin-dependent kinase (CDK) inhibitors, such as p21WAF1/CIP1 and p27KIP1, without any noticeable changes in G_1 cyclins and CDKs (except for a slight decrease in CDK4). Treatment of HepG2 cells with EEKS also increased the binding of p21 and p27 with CDK4 and CDK6, which was paralleled by a marked decrease in the cyclin D- and cyclin E-associated kinase activities.

Conclusions: Overall, our findings suggest that EEKS may be an effective treatment for liver cancer through suppression of cancer cell proliferation *via* G_1 cell cycle arrest. Further studies are required to identify the active compounds in EEKS.

1. Introduction

Hepatocellular carcinoma (HCC) is currently ranked third among the most common causes of cancer deaths, and is particularly prevalent in Asia [1,2]. One well-established factor for liver cancer is persistent hepatitis B virus infection, but the causes of HCC involve a complex interaction between numerous factors [3]. Natural or acquired resistance imposes further limits on the efficacy of currently available treatments, resulting in continued poor outcomes inpatients with advanced HCC, despite recent advances in cancer treatments [4,5]. New

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anticancer drugs, with increased selectivity and reduced toxicity against HCC, are therefore needed.

The current consensus is that the loss of normal cell cycle control plays an important role in the genesis of most cancers. Cell cycle progression is a highly ordered and tightly regulated process that involves multiple checkpoints [6,7]. In eukaryotic cells, each phase of the cell division cycle is controlled by several protein kinases, the cyclin-dependent kinases (CDKs), which act in conjunction with their corresponding regulatory cyclins and CDK inhibitors. CDK inhibitors, which bind to cyclin-CDK complexes and render these complexes inactive, are grouped into two distinct families, the INK4 and CIP/KIP families, based on sequence homology and targets of inhibition [8]. The INK4 family, including p15INK4B, p16INK4A, p18INK4C, and p19INK4Dd, interact specifically with CDK4 and CDK6; while the CIP/KIP family, including p21WAF1/CIP1, p27KIP1, and p57KIP2, inhibit a broader spectrum of CDKs [9]. In particular, p21 and p27 bind to all the G_1 and G_1 to S CDK complexes, but preferentially inhibit the ones containing CDK2, *via* p53-dependent or p53-independent mechanisms [8,10,11].

In addition to CDK inhibitors, the protein product of the retinoblastoma tumor-suppressor gene (*pRB*), a member of the pocket protein family, also serves as a negative regulator of cell proliferation through its regulation of the E2F transcription factors [12,13]. The cyclin-CDK complexes phosphorylate pRB and related family proteins, thereby preventing their growth-repressive functions. The phosphorylation of pRB in mid-G_1 negates the ability of pRB to repress transcription, thereby resulting in increased expression of cyclin E, the regulatory partner for CDK2 [12,14]. The resulting cyclin E/CDK2 complexes phosphorylate pRB, causing the release of free E2Fs and the activation of transcription of several genes involved in the regulation of S phase [13,15]. Therefore, strategies that could halt cell cycle progression by targeting CDK inhibitors or pRB-related proteins could be useful as potential molecular targets of cancer therapy [16–18].

For thousands of years, herbal medicines have been used in many countries, with apparent safety and efficacy, to alleviate and treat various diseases, including cancer [19]. Interest is now growing in the pharmacological activity of traditional herbs that are widely used in traditional medicine, and numerous studies support the potential clinical benefit of these medicines for treatment of diseases recalcitrant diseases, like cancer [20,21]. Among these are medicines derived from *Kalopanax septemlobus* (Thunb.) Koidz. (common name: prickly castor oil tree) (*K. septemlobus*), a deciduous tree in the family Araliaceae. This tree, native to northeastern Asia, has long been valued for its ethnopharmacology, being used for the treatment of rheumatic arthritis, nephritis edema, cholera, dysentery, and neurotic pain. Recent evidence shows that the extracts and/or components of *K. septemlobus* show diverse pharmacological activities, including anti-oxidant, anti-inflammatory, hypoglycemic, and neurite outgrowth functions [22–25]. However, the actual mode of the anti-cancer effects and the underlying cellular mechanisms remain to be established. In this study, as a part of our search for novel biologically active substances from traditional medicinal resources for the prevention and treatment of cancers, we investigated the potential of an ethanol extract of *K. septemlobus* leaves (EEKS) to modulate the growth and proliferation of human HCC cells. We demonstrated that EEKS treatment results in growth arrest at the G_1 to S transition of the cell cycle in HepG2 cells. We further investigated the molecular mechanisms associated with the effects of EEKS by analyzing the influence of EEKS on the proteins involved in the G_1 to S transition.

2. Materials and methods

2.1. Preparation of EEKS

The leaves of *K. septemlobus* were obtained from Gurye Wild Flower Institute (Gurye, Republic of Korea) and authenticated by Professor S.H. Hong, Department of Biochemistry, Dongeui University College of Korean Medicine (Busan, Republic of Korea). The dried leaves (50 ìg) were cut into small pieces, ground into a fine powder, and then soaked with 500 mL 70% ethanol (500 mL) for 2 d. The extracted liquid was filtered twice through Whatman No. 3 filter paper to remove any insoluble materials and was then concentrated using a rotary evaporator (EYELA N1000; Rikakikai Co., Ltd., Tokyo, Japan). The extract (EEKS) was dissolved in dimethylsulfoxide (DMSO; Sigma–Aldrich Co., St. Louis, MO, USA) to a final concentration of 200 mg/mL (extract stock solution) and was subsequently diluted with medium to the desired concentration prior to use.

2.2. Cell culture

HCC HepG2 and Hep3B cell lines were purchased from American Type Culture Collection (Manassas, VA, USA). The cells were cultured at 37 °C in humidified air with 5% CO_2 in RPMI-1640 medium (GIBCO-BRL, Gaithersburg, MD, USA), supplemented with 10% fetal bovine serum (FBS), 100 units/mL of penicillin, and 100 mg/mL of streptomycin, with or without added EEKS.

2.3. Cell proliferation assay

Cells were grown in 6-well multi-well plates (2×10^4 cells/well) in media supplemented with EEKS at the indicated concentrations. After 24 h treatment, the cells were trypsinized and viable cells were counted under an inverted microscope by measuring the total cell number using the trypan blue dye exclusion assay (Carl Zeiss, Jena, Germany). Data were reported as the number of cells per well.

2.4. Cell cycle analysis by flow cytometry

After treatment with various concentrations of EEKS for 24 h, the cells were harvested with trypsin, washed once with phosphate-buffered saline, and fixed in 70% ethanol overnight at 4 °C. The cells were treated with 1 mg/mL RNase for 30 min at 37 °C, and then stained with 10 ìg/mL propidium iodide (PI, Sigma–Aldrich Co.) for 30 min. Cells (10 000 per sample) were analyzed using a flow cytometer (Becton Dickinson, San Jose, CA, USA). The distribution of DNA in the cell cycle was determined using Modfit LT software (Verity Software House, Topsham, ME, USA).

2.5. RNA isolation and reverse transcriptase polymerase chain reaction (RT-PCR) analysis

Total RNA from the cultured cells was isolated using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA)

according to the manufacturer's instructions. cDNA was synthesized from 1 ìg of total RNA, using AccuPower® RT PreMix (Bioneer, Daejeon, Republic of Korea) containing Moloney murine leukemia virus reverse transcriptase. RT-generated cDNAs were amplified by PCR using selective primers (Table 1), which were purchased from Bioneer. After amplification, the PCR reactions were electrophoresed in 1% agarose gels and visualized with ethidium bromide (EtBr, Sigma–Aldrich Co.) staining. In a parallel experiment, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as an internal control.

2.6. Total protein extraction, immunoprecipitation, and Western blot analysis

After treatment with different concentrations of EEKS, the cells were lysed in buffer containing 40 mM Tris (pH 8.0), 120 mM NaCl, 0.5% Nonidet P-40, 0.1 mM sodium orthovanadate, 2 ìg/mL aprotinin, 2 ìg/mL leupeptin, and 100 ìg/mL phenymethylsulfonyl fluoride (Sigma–Aldrich Co.). Supernatants were collected and protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). For immunoprecipitation, cell extracts (500 ìg) were incubated with immunoprecipitating antibodies in extraction buffer for 2 h at 4 °C, and then the immunocomplexes were precipitated with protein A-Sepharose beads (Sigma–Aldrich Co.). For Western blot assays, equal amounts of protein (30–50 ìg/lane) and immunoprecipitated proteins were subjected to electrophoresis on 8%–12% sodium dodecyl sulfate (SDS)-polyacrylamide gels, and then transferred onto nitrocellulose membranes (Schleicher and Schuell, Keene, NH, USA). The membranes were blocked with Tris-buffered saline (10 mM Tris-Cl, pH 7.4) containing 0.5% Tween 20 and 5% nonfat dry milk for 1 h at room temperature and then incubated with the desired primary antibodies overnight. Membranes were then washed with phosphate-buffered saline and incubated with the secondary antibody conjugated to horseradish peroxidase (Amersham Corp., Arlington Heights, IL, USA) for 1 h at room temperature. Immunoreactivity was detected by using the enhanced chemiluminescence (ECL) Western blotting detection system (Amersham Corp.) according to the manufacturer's instructions. Primary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and Abcam (Cambridge, UK).

Table 1
Gene-specific primers for RT-PCR.

Gene name		Primer sequences
Cyclin D1	Sense	5′-TGG ATG CTG GAG GTC TGC GAG GAA-3′
	Antisense	5′-GGC TTC GAT CTG CTC CTG GCA GGC-3′
Cyclin E	Sense	5′-AGT TCT CGG CTC GCT CCA GGA AGA-3′
	Antisense	5′-TCT TGT GTC GCC ATA TAC CGG TCA-3′
CDK2	Sense	5′-GCT TTC TGC CAT TCT CAT CG-3′
	Antisense	5′-GTC CCC AGA GTC CGA AAG AT-3′
CDK4	Sense	5′-ACG GGT GTA AGT GCC ATC TG-3′
	Antisense	5′-TGG TGT CGG TGC CTA TGG GA-3′
CDK6	Sense	5′-CGA ATG CGT GGC GGA GAT C-3′
	Antisense	5′-CCA CTG AGG TTA GAG CCA TC-3′
p53	Sense	5′-GCT CTG ACT GTA CCA CCA TCC-3′
	Antisense	5′-CTC TCG GAA CAT CTC GAA GCG-3′
p21	Sense	5′-CTC AGA GGA GGC GCC ATG-3′
	Antisense	5′-GGG CGG ATT AGG GCT TCC-3′
p27	Sense	5′-AAG CAC TGC CGG GAT ATG GA-3′
	Antisense	5′-AAC CCA GCC TGA TTG TCT GAC-3′
GAPDH	Sense	5′-CGG AGT CAA CGG ATT TGG TCG TAT-3′
	Antisense	5′-AGC CTT CTC CAT GGT GGT GAA GAC-3′

2.7. Cyclin D1-and E-associated kinase activity assay

Cell lysates from untreated and EEKS-treated cells were incubated for 1 h at 4 °C with anti-cyclin D1 and anti-cyclin E antibodies. Immune-complexes were collected on protein A-Sepharose beads (Sigma–Aldrich Co.) and resuspended in a kinase assay mixture containing [ë-^{32}P]ATP (ICN Biochemicals, Irvine, CA, USA) and histone H1 (Sigma–Aldrich Co.) as substrate. After incubation at 37 °C for 30 min, the reaction was stopped by the addition of the same amount of 2 × SDS sample buffer. After boiling and spinning, the samples were separated on 10% SDS-polyacrylamide gels and dried, and the bands were detected by autoradiography.

2.8. Statistical analysis of data

The experiments were repeated three times, and the results are expressed as means ± standard deviation (SD). A one-way analysis of variance (ANOVA), followed by Dunnett's *t*-test, was applied to assess the statistical significance of the difference among the study groups. A value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. EEKS treatment suppresses cell proliferation of HCC cells

The effect of EEKS treatment on HCC (HepG2 and Hep3B) proliferation was determined by measuring the cell numbers after treatment with EEKS for 24 h. As shown in Figure 1, EEKS caused significant concentration-dependent decreases in cell numbers in both HepG2 and Hep3B cell lines; however, cell proliferation was more significantly suppressed by EEKS in HepG2 cells than in Hep3B cells. Therefore, the HepG2 cell line was selected for subsequent experiments.

3.2. EEKS treatment arrests cell cycle at G_1 phase in HepG2 cells

The possible mechanisms involved in the inhibition of cell growth observed with EEKS treatment were analyzed by fluorescence-activated cell sorting to determine the distribution of cells in various stages of the cell cycle. The percentages of G_1

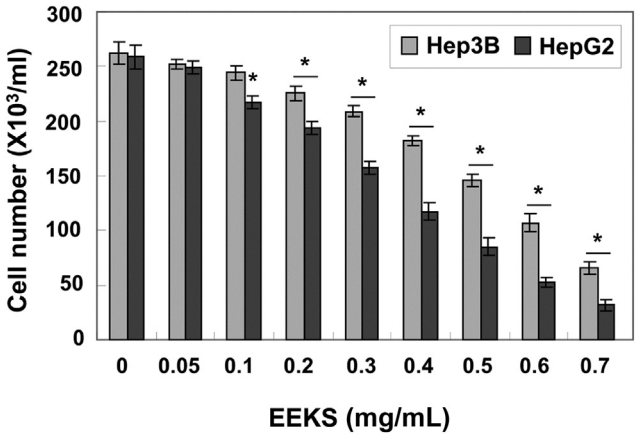

Figure 1. Inhibition of cell proliferation by treatment of human HCC cells with EEKS.
*$P < 0.05$ *vs*. untreated control.

phase HepG2 cells increased after treatment with EEKS in a concentration-dependent manner (Figure 2). This G_1 arrest was accompanied by a reciprocal decrease in the percentage of cells observed in the S phase; however, the percentage of cells in the G_2/M phase was not altered.

3.3. EEKS treatment downregulates pRB phosphorylation and increases binding of pRB with E2Fs in HepG2 cells

The pRB/E2F pathway constitutes the basic machinery controlling the G_1 to S transition and S phase entry [12,14]; therefore, we examined the effects of EEKS on the kinetics of phosphorylation of pRB and the E2F transcription factors. The immunoblotting data indicated that treatment of HepG2 cells with EEKS for 24 h led to a concentration-dependent decrease

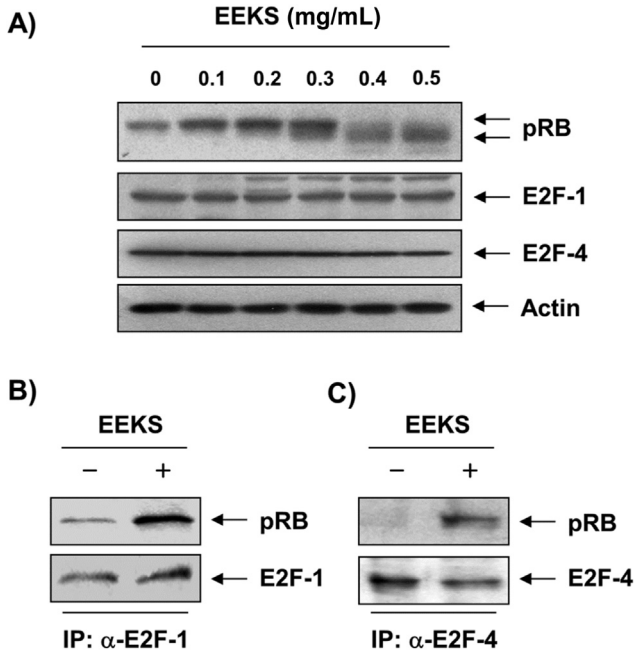

Figure 3. EEKS treatment induces hyperphosphorylation of pRB and enhances association of pRB and E2Fs in HepG2 cells.
(A) Cells were with the indicated concentrations of EEKS for 24 h, then total cell lysates were prepared and separated by electrophoresis on 8% or 10% SDS-polyacrylamide gels. Western blotting was then performed using anti-pRB, anti-E2F-1, and anti-E2F-4 antibodies. Actin was used as an internal control. (B and C) Cells were incubated without or with EEKS (0.5 ìg/mL) for 24 h, and then equal amounts of proteins were immunoprecipitated with the anti-E2F-1 (B) or anti-E2F-4 (C) antibody. Immunocomplexes were separated by 8% SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and probed with the anti-pRB, E2F-1, and E2F-4 antibodies, respectively. Proteins were detected by ECL detection (IP; immunoprecipitation).

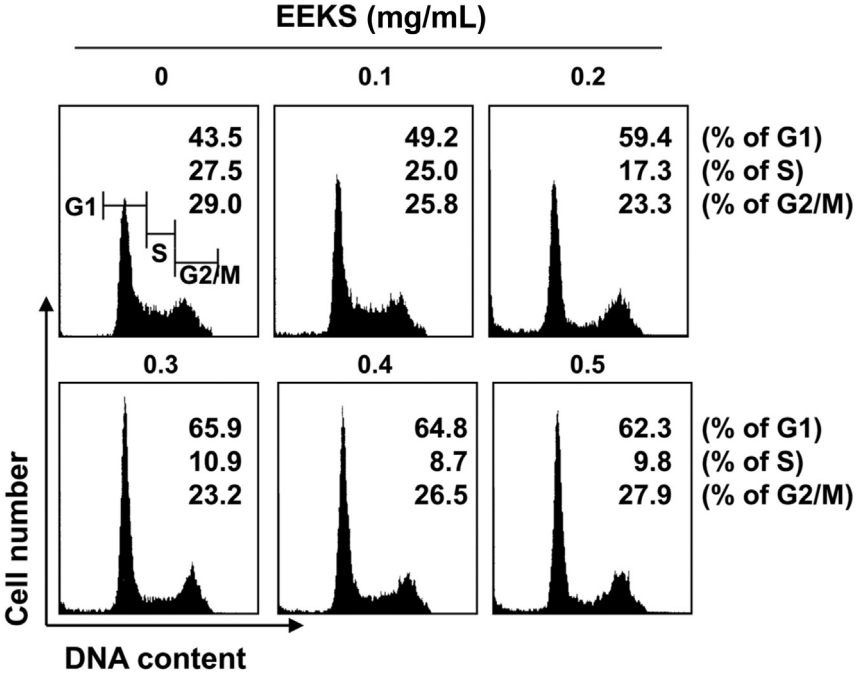

Figure 2. EEKS treatment induces G_1 phase cell cycle arrest in HepG2 cells.
DNA content is represented on the x-axis and the number of cells counted is represented on the y-axis. Each point represents the mean of two independent experiments.

in phospho-pRB expression without affecting expression of E2F transcription factors such as E2F-1 and E2F-4 (figure 3A). We assessed whether EEKS treatment increases the pRB/E2Fs interaction by immunoprecipitation of cell extracts with E2F-1 and E2F-4 antibodies and probing with pRB antibodies, which revealed an increase in the pRB levels in the E2F immunoprecipitates (Figure 3B and C).

3.4. Effects of EEKS treatment on the levels of G_1 phase-associated cyclins and CDKs in HepG2 cells

The G_1 phase CDKs (CDK2, CDK4 and CDK6) activities are positively regulated by the bound cyclins such as D-type cyclins (cyclin D1, D2 and D3) and cyclin E, but negatively by the CDK inhibitors such as p21 and p27 [8,10,11]. We investigated whether these molecular events were causally related to the EEKS-induced G_1 arrest by examining the response of HepG2 cells to EEKS using RT-PCR and Western blot analyses. As shown in Figure 4, the levels of CDK4 mRNA and protein were significantly decreased after treatment with EEKS at both the transcriptional and translational levels, whereas the expression levels of other molecules, including CDK2, CDK6, cyclin D1, and cyclin E, remained unchanged.

3.5. EEKS treatment increases the levels of p21 and p27 expression, inhibits G_1 cyclin-associated kinase activities in HepG2 cells

We examined the effects of EEKS on the expression of CDK inhibitors p21 and p27 at the mRNA and protein levels. As shown in Figure 5, EEKS caused a marked increase in expression of mRNA and protein of both of p21 and p27, whereas the levels of tumor suppressor p53 were not altered following the same treatment. Therefore, we evaluated the potential association between the p21 and p27 proteins induced by EEKS treatment and the CDKs, in order to further define the mechanism of the G_1 arrest response to EEKS. Co-immunoprecipitation analyses revealed no detectable complexes between p21 and CDK4/6 or between p27 and CDK4/6 in lysates from untreated control cells; however, treatment of cells with EEKS resulted in a marked increase in the binding of p21 and p27 with CDK4 and CDK6 (Figure 6A). We further investigated the nature of G_1 arrest following EEKS treatment by evaluating the possible effects of EEKS on the modulation of G_1 cyclin-associated kinase activities using histone H1 as the substrate. As shown in Figure 6B, treatment of HepG2 cells with EEKS reduced the cyclin D1-and E-associated kinase activity in a concentration-dependent manner.

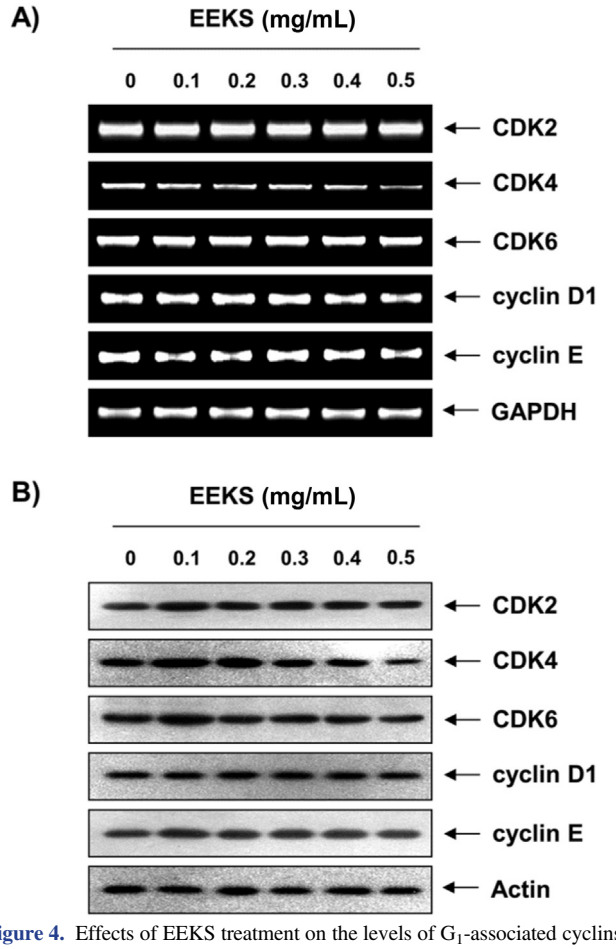

Figure 4. Effects of EEKS treatment on the levels of G_1-associated cyclins and CDKs in HepG2 cells.
(A) Cells were treated with various concentrations of EEKS for 24 h, and then total RNA was isolated and reverse-transcribed. The resulting cDNAs were subjected to PCR with the indicated primers, and the reaction products were separated on 1% agarose gel and visualized by EtBr staining. (B) The cell lysates were prepared, and equal amounts of total cell lysates were subjected to 10% SDS-polyacrylamide gel electrophoresis, transferred, and probed with the indicated antibodies. GAPDH and actin were used as internal controls for the RT-PCR and Western blot assays, respectively.

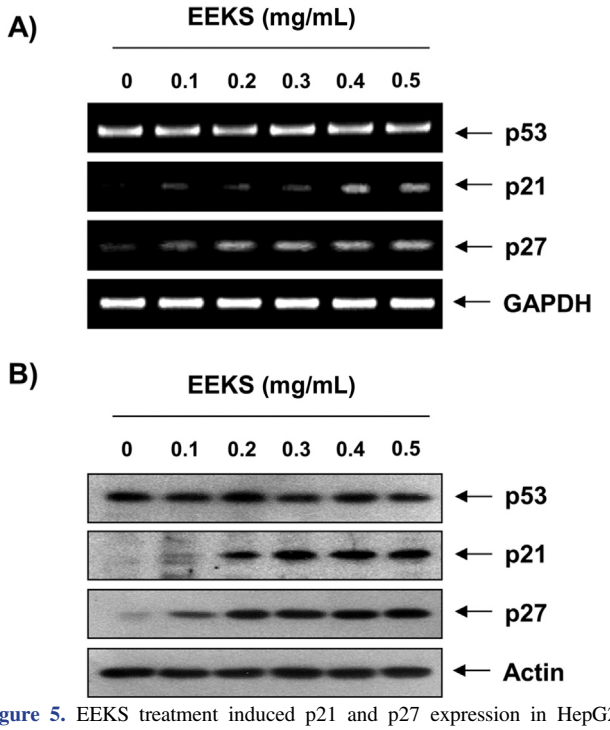

Figure 5. EEKS treatment induced p21 and p27 expression in HepG2 cells.
(A) Cells were treated with various concentrations of EEKS for 24 h, and then total RNA was isolated and reverse-transcribed. The resulting cDNAs were then subjected to PCR with the indicated primers, and the reaction products were separated on 1% agarose gel and visualized by EtBr staining. (B) The cell lysates were prepared, and equal amounts of total cell lysates were subjected to SDS-polyacrylamide gel electrophoresis, transferred, and probed with the indicated antibodies. GAPDH and actin were used as internal controls for the RT-PCR and Western blot assays, respectively.

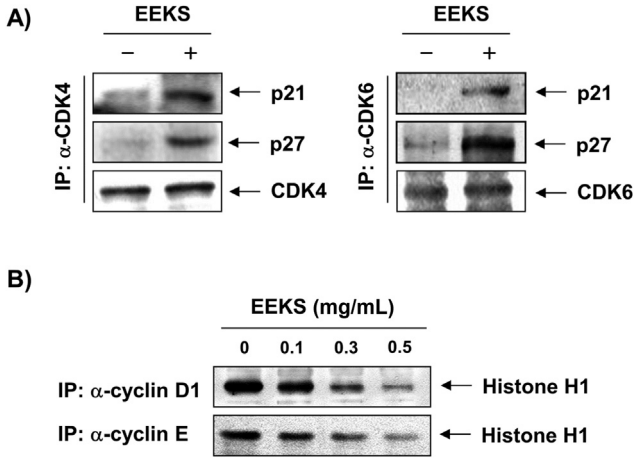

Figure 6. EEKS treatment induced association of p21 and p27 with CDKs, and inhibited cyclin D1-and cyclin E-associated kinase activities in HepG2 cells.

(A) Cells were treated with EEKS (0.5 mg/ml) for 24 h, and then total cell lysates were prepared and immunoprecipitated with the anti-CDK4 or anti-CDK6 antibody. The immuno-complexes were separated on 12% SDS-polyacrylamide gels, transferred onto nitrocellulose membranes, and probed with the indicated antibodies and an ECL detection system. (B) After treatment with various concentrations of EEKS for 24 h, the cell lysates were prepared and immunoprecipitated with the anti-cyclin D1 or anti-cyclin E antibody, and kinase activity was assayed using histone H1 as substrate.

4. Discussion

In the present study, we provide evidence that EEKS treatment induces anti-proliferative activity through cell cycle arrest in the G_1 phase in human HCC cells, and particularly in the HepG2 cell line. The observed increase in the G_1 phase in EEKS-treated HepG2 cells was accompanied by a reciprocal decrease in the number of cells in the S phase, with no change in the G_2/M fraction.

The transition of the G_1 to the S phase of the cell cycle marks an irreversible commitment to DNA synthesis and proliferation, and is strictly regulated by positive and negative growth-regulatory signals [6,7]. The major cyclin-CDK complexes involved in cell cycle progression from the G_1 to the S phase are cyclin D-CDK4/6 and cyclin E-CDK2, respectively [26,27]. The protein products of the pRB family of tumor suppressor proteins, such as pRB, p107 and p130 (referred to as 'pocket proteins,' key regulators of G_1 cell cycle progression), are phosphorylated by a set of these cyclin-CDK complexes [12,13]. Among the pRB family proteins, pRB is a major negative regulator of cell division, and pRB hyperphosphorylation is a hallmark of the G_1 to S transition in the cell cycle. The phosphorylation of pRB causes breakdown of the pRB/E2Fs complex, leading to liberation of the E2F transcription factors that activate a transcription program required for DNA synthesis, S-phase progression, and cell division [14,15]. In contrast, dephosphorylated pRB binds to E2Fs, thereby blocking their inhibitory activity and allowing the cell to progress to the S phase [6,7]. Therefore, decreases in either the levels of protein or in the association between the respective binding partners are expected to cause a concomitant decrease in the degree of pRB phosphorylation. In the present study, EEKS-treated HepG2 cells showed decreased expression of phosphorylated pRB, along with increased binding of pRB to E2Fs, such as E2F-1 and E2F-4. The data indicate that EEKS represses the transcriptional activity of E2Fs for S phase entry by promoting their binding with pRB in HepG2 cells.

The cyclin-CDK complexes are also known to bind to CDK inhibitors, thereby inhibiting their kinase activities and restraining cells in a particular phase of the cell cycle [27,28]. Expression of G_1 cyclins is reported in many human cancers: the D-type cyclins bind to CDK4 and CDK6 in early G_1 and act as growth sensors, providing a link between mitogenic stimuli and the cell cycle [27,28]. On the other hand, cyclin E, which is expressed in late G_1, forms an active kinase complex with CDK2 and contributes to the G_1 to S transition in the cell cycle [6,29]. It was shown that EEKS partially inhibited the expression of CDK4 mRNA and protein; however, little change was detectable in the levels of CDK2, CDK6, cyclin D1, and cyclin E.

The CIP/KIP family is involved in regulation of G_1 and S phase of the cell cycle [8,27]. Generally, p21 blocks cell proliferation in response to DNA damage or replication stress by the inactivation of CDK2 by either cyclin E in the G_1 phase or by cyclin A in the S phase [8,30]. Likewise, p27 also suppresses cyclin E-CDK2 as well as D-type cyclins-CDK4 complexes and helps the cells to withdraw from the cell cycle when they terminally differentiate [10,31]. We observed a significant induction of p21 and p27 at both the transcriptional and translational levels in response to EEKS treatment, whereas the expression of p53 was unchanged. Interestingly, this was accompanied by an enhanced association of p21 and p27 with CDK4 and CDK6, as indicated by co-immunoprecipitation analysis. In addition, the results from the immuno-complex kinase assays demonstrated that EEKS downregulated both cyclin D1-and cyclin E-associated kinase activities. These data suggest that the capability of EEKS to arrest HepG2 cells in the G_1 phase might be linked to the inhibition of CDK kinase activity through binding of the CDK inhibitor proteins p21 and p27 to the cyclin-CDK complexes.

Taken together, our data suggested that EEKS induced G_1 arrest in association with an inhibition of G_1 cyclin-CDK complex activity, rather than by altering the levels of G_1 cyclins and CDK proteins. The result was an accumulation of hypo-phosphorylated, growth-inhibitory pRB that suppressed the E2F pathway in HepG2 cells. Further investigation is needed to identify the biologically active compounds of EEKS responsible for the observed effects; nevertheless, EEKS may have significant potential for development as a cancer treatment.

Conflict of interest statement

We declare that we have no conflict of interest.

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