# Hexane/ethanol extract of *Glycyrrhiza uralensis* and its active compound isoangustone A induce G1 cycle arrest in DU145 human prostate and 4T1 murine mammary cancer cells<sup>☆</sup>

Mi Ra Seon[a,1], So Young Park[a,1], Soo Jin Kwon[a], Soon Sung Lim[a], Hyun Ju Choi[a], Heesook Park[a], Do Young Lim[a], Jong-Sang Kim[c], Choong Hwan Lee[d], Jongdai Kim[e,f], Jung Han Yoon Park[a,b,e,*]

[a]*Department of Food Science and Nutrition, Hallym University, Chuncheon, 200-702, Republic of Korea*
[b]*Center for Efficacy Assessment and Development of Functional Foods and Drugs, Hallym University, Chuncheon, 200-702, Republic of Korea*
[c]*Department of Animal Science and Biotechnology, Kyungpook National University, Daegu, 702-701, Republic of Korea*
[d]*Department of Bioscience and Biotechnology, Konkuk University, Seoul, Republic of Korea, 143-701*
[e]*Medical and Bio-Materials Research Center, Kangwon National University, Chuncheon, 200-701, Republic of Korea*
[f]*Department of Food Science & Biotechnology, Kangwon National University, Chuncheon, 200-701, Republic of Korea*

## Abstract

Although licorice is known to exert anticarcinogenic effects, it contains large quantities of glycyrrhizin (GL), which causes severe hypertension. We have previously demonstrated that the hexane/ethanol extract of *Glycyrrhiza uralensis* (HEGU) contains no detectable GL and suppresses doxorubicin-induced apoptosis in H9c2 rat cardiac myoblasts. The principal objective of this study was to determine whether and by what mechanism HEGU and its active component, isoangustone A, inhibit cell-cycle progression in DU145 human prostate and 4T1 mouse breast cancer cells. HEGU and isoangustone A dose-dependently decreased DNA synthesis and induced G1 phase arrest in both DU145 and 4T1 cells. HEGU and isoangustone A reduced the levels of CDK2 and CDK4 as well as cyclin A and cyclin D1 proteins, and also induced a decrease in CDK2 activity. The addition of HEGU to drinking water significantly suppressed the orthotopic growth of 4T1 allografts and the expression of the proliferating nuclear cell antigen, CDK2 and CDK4 proteins in the tumor tissues. These results demonstrate the potential of HEGU containing isoangustone A as an antitumor agent.

## 1. Introduction

The mammalian cell cycle is generally divided into four distinct phases: the G1, S, G2 and M phases. Cell-cycle checkpoints ensure accurate chromosome replication and separation, thereby maintaining genetic stability. One checkpoint — the restriction point — occurs at mid-G1 phase, and the disruption of restriction-point regulation is a hallmark of cancer (reviewed in Ref. [1]). Thus, the identification of bioactive compounds with the potential to restore restriction-point regulation may prove to be an effective strategy for the suppression of cancer development. In this regard, the anticancer activity of phytochemicals has become the focus of a great deal of attention, owing to the demonstrated efficacy and low or nontoxicity of this class of compounds.

Progression through the restriction point is controlled by cyclins and cyclin-dependent kinases (CDKs) [2]. The activities of cyclin D-dependent CDK4 and CDK6 are detected first in mid-G1 phase and then increase as cells approach the G1/S boundary [3]. Cyclin E is periodically expressed at maximum levels near the G1/S transition, binding to a different catalytic subunit, CDK2 [4]. The CDK inhibitors are tumor suppressor proteins that interact with distinct cyclin–CDK complexes and thereby inhibit the activity of these enzymes [5]. The INK4 (inhibitors of CDK4) family has four members — p16<sup>INK4a</sup>, p15 <sup>INK4b</sup>, p18 <sup>INK4c</sup> and p19 <sup>INK4d</sup> — and are known to control the G1 to S phase transition of the cell cycle (reviewed in Ref. [6]). The CIP/KIP families (p21<sup>WAF1/CIP1</sup> and p27<sup>KIP1</sup>) also regulate the G1 to S phase transition and contribute to the maintenance of cell-cycle arrest in the G2 phase [7,8].

*Glycyrrhiza uralensis* (licorice) is used widely as a flavoring and sweetening agent in tobacco products, chewing gum, candy,

* Corresponding author at: Hallym University, Department of Food Science and Nutrition, Chuncheon, 200-702, Republic of Korea. Tel.: +82 33 248 2134; fax: +82 33 256 0199.

E-mail address: jyoon@hallym.ac.kr (J.H.Y. Park).

[1] The first two authors contributed equally to this work.

toothpaste and beverages. In addition, licorice is frequently prescribed as a treatment in Oriental herbal medicine. Licorice root has also been recognized by the National Cancer Institute as exerting a cancer chemopreventive effect (reviewed in Ref. [9]). Among the many compounds present in licorice, glycyrrhizin (GL) is quantitatively the most significant component, with detected levels varying between 3.63 and 13.06 g/100 g in dried licorice roots. Thus, GL is frequently utilized as a tool for the identification of *Glycyrrhiza* species (reviewed in Ref. [10]). GL has been shown to be hydrolyzed into its aglycone, glycyrrhetinic acid (GA), by human intestinal bacteria [11], and GA has been reported to inhibit the proliferation and secretion of prostate specific antigen in LNCaP prostate cancer cells [12] and to induce apoptosis in human hepatoma, promyelotic leukemia and stomach cancer cells [13]. However, the chronic consumption of large quantities of licorice has been reported to result in severe hypertension, hypokalemia and other signs of mineralocorticoid excess (reviewed in Ref. [9]), as GA is a well-known inhibitor of 11β-hydroxysteroid dehydrogenase type 2 [14]. Because licorice has been consumed by humans for several thousand years and is known to be safe for human consumption (except for its hypertension-inducing effects), it is worthwhile to determine whether licorice extracts lacking GL still exert a cancer chemopreventive effect.

We have demonstrated previously that the hexane/ethanol extract of *G. uralensis* (HEGU) contains no detectable amounts of GL and suppresses doxorubicin-induced apoptosis in H9c2 rat cardiac myoblasts. In addition, we have determined that HEGU reduces the numbers of viable HT-29 human colon, MDA-MB-231 human breast and DU145 human prostate cancer cells [15]. Furthermore, we identified isoangustone A (Fig. 1) as an active compound in HEGU and also noted that isoangustone A induced apoptosis in DU145 and MDA-MB-231 cells [16]. In the present study, we then attempted to determine whether and via what mechanisms HEGU and isoangustone A regulate cell-cycle progression in DU145 human cancer cells and 4T1 murine mammary cancer cells. We also attempted to determine whether HEGU suppresses the growth of solid tumors when 4T1 cells were implanted in the mammary fat pads of syngeneic BALB/c mice. The 4T1 cell line was derived from a spontaneously arising BALB/c mammary tumor, and thus, the 4T1 breast cancer model is a highly physiological mouse model, which closely resembles breast cancer in humans [17].

## 2. Materials and methods

### 2.1. Materials

The reagents used in this study were as follows. RNase, anti-β-actin and anti-p16[INK4a] (Sigma, St. Louis, MO, USA); protein A sepharose, [3H]thymidine (5 μCi/mmol),



Fig. 1. Structure of isoangustone A.

horseradish peroxidase (HRP)-conjugated antirabbit, antimouse IgG (Amersham, Arlington Heights, IL,USA); [γ-32P]ATP (NEN Life Sciences, Boston, MA, USA); proliferating cell nuclear antigen (PCNA), CDK4, cyclin A, cyclin E, p21[CIP/WAF1] and p27[KIP1] antibody (Santa Cruz, Delaware, CA, USA); cyclin D1 antibody (NeoMarkers, Fremont, CA, USA) and histone H1 (Roche Applied Science, Mannheim, Germany). If not otherwise specified, all other materials were purchased from Sigma. The HEGU was prepared from *G. uralensis* (Daekwang Co. Ltd., Chuncheon, Korea) as described previously [15], and isoangustone A was purified from HEGU as previously described [16].

### 2.2. Cell culture

DU145 human prostate cancer and 4T1 mouse breast cancer cells (American Type Culture Collection, Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 (DU145) and DMEM (4T1) supplemented with 100 ml/L fetal bovine serum (FBS), 100,000 U/L penicillin and 100 mg/L of streptomycin. In order to evaluate the effects of HEGU and isoangustone A, we plated the cells in multiwell plates with DMEM/F12 containing 100 ml/L FBS. Prior to treatment with HEGU or isoangustone A, the cell monolayers were rinsed and serum deprived for 24 h with DMEM/F12 containing 10 ml/L of charcoal-stripped FBS (serum-deprivation medium). After serum deprivation, the medium was replaced with fresh serum-deprivation medium, with or without various concentrations of HEGU (2.5, 5 or 7.5 μg/ml) or isoangustone A (2.5 or 5 μg/ml). The extracts were dissolved in dimethylsulfoxide (DMSO), and all cells were treated with DMSO at a final concentration of 1 ml/L.

### 2.3. [3H]Thymidine incorporation

To determine [3H]thymidine incorporation, cells were plated in 96-well plates at 6000 cells/well and treated with various concentrations of HEGU or isoangustone A. [3H]Thymidine (0.5 μCi/well) was simultaneously added, and the cells were incubated for 3 h to measure incorporation into the DNA.

### 2.4. Cell-cycle analysis by flow cytometry

Cells were plated in 24-well plates at a density of 50,000 cells/well and treated for 6 h with HEGU or isoangustone A. Cell-cycle analysis was conducted using a Guava PCA-96 system (Guava Technologies, Inc., Hayward, CA, USA) in accordance with the manufacturer's protocols. The data were analyzed using Guava CytoSoft version 2.5 software.

### 2.5. Western blot analysis

Total cell lysates [18] and tumor lysates [19] were prepared as described previously, and Western blot analyses were conducted as previously described [18]. The relative abundance of each band was quantified using the Bio-profile Bio-ID application (Vilber-Lourmat, Marne-la-Vallée, France), and the expression levels were normalized to β-actin.

### 2.6. Immunoprecipitation and in vitro kinase assay (CDK2) activity

The cell lysates (750 μg protein) were precleaned with normal rabbit IgG and a protein-A-sepharose bead slurry (Amersham), and immunoprecipitation was conducted using 1.5 μg of anti-CDK2 antibody and protein A sepharose as previously described [18]. The in vitro kinase assay for CDK2 activity was conducted as previously described [18].

### 2.7. In vivo tumor growth study

Four-week-old female BALB/c mice (12 mice) were purchased from Orient Bio Inc. (Gapyeong, Korea) and fed on a rodent chow diet (Dae Han Biolink Co. Ltd., Eumseong, Korea) and tap water ad libitum. After a 7-day acclimatization to the laboratory conditions, 4T1 cells ($5\times10^4$) were suspended in 50 μl of PBS and 50 μl matrigel and injected through the skin using 28-G needles into the inguinal mammary fat pad. Seven days after tumor cell implantation, they were divided randomly into two groups. One group was provided with HEGU (5 mg/kg body weight per day) in their drinking water, and the control group was provided with water containing a vehicle (corn oil). The water intake of the mice was estimated daily, and the concentrations of HEGU in drinking water were adjusted such that the intake of HEGU per mouse was 5 mg kg$^{-1}$ d$^{-1}$. Tumor masses were measured with calipers along two perpendicular diameters. Tumor volume was calculated as follows: volume=length×width$^2$÷2. All animal experiments were approved by the Committee on Animal Experimentation of Hallym University and conducted in compliance with the university's Guidelines for the Care and Use of Laboratory Animals.

### 2.8. Immunohistochemical detection of PCNA

Forty days after 4T1 cell inoculation, the mice were killed by CO$_2$ asphyxiation, and the tumors were excised and stored in 4% paraformaldehyde/phosphate-buffered saline (PBS). Five-micrometer tissue sections of formalin-fixed, paraffin-embedded tumor specimens were deparaffinized in xylene, rehydrated in graded alcohol and
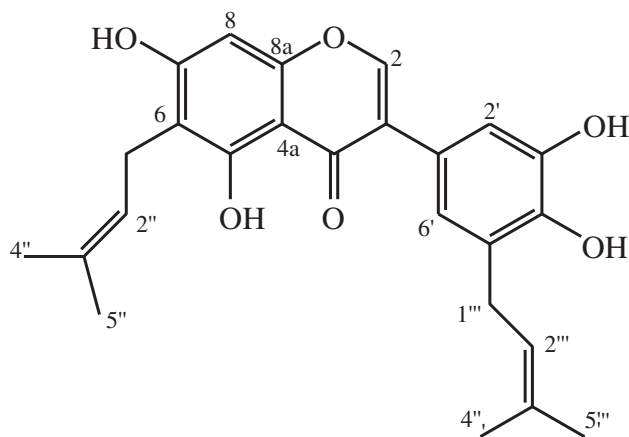
transferred to PBS. Endogenous peroxidases were blocked via incubation in 3% hydrogen peroxide. The sections were blocked with PBS containing 5% BSA and incubated with mouse monoclonal anti-PCNA antibody (1:250 dilution). The sections were then stained using a DAKO LSAB+ System-HRP Kit (DAKO Corporation, Carpinteria, CA, USA) and counterstained with Harris hematoxylin (Sigma-Aldrich Co.). The slides were mounted and the PCNA-positive nuclei (brown) were quantified by counting at five randomly selected fields at ×400 magnification.

### 2.9. Statistical analysis

The results were expressed as the means±S.E.M. and analyzed by analysis of variance. Differences between the treatment groups were analyzed via Duncan's multiple range tests or Student's *t* test. Differences were considered significant at *P*<.05. All statistical analyses were conducted with SAS statistical software, version 8.12.

## 3. Results

### 3.1. HEGU and isoangustone A induce G1 cell-cycle arrest in DU145 human prostate and 4T1 mouse breast cancer cells

In the previous study, we noted that HEGU and isoangustone A reduced viable cell numbers [20] and induced apoptosis in DU145 human prostate, HT-29 human colon cancer and MDA-MB-231 human breast cancer cells [16]. In order to determine whether HEGU and isoangustone A regulate cell-cycle progression, DU145 and 4T1 cells were treated with various concentrations of either HEGU or isoangustone A, and DNA was stained with propidium iodide. Treatment of the DU145 cells with HEGU or isoangustone A resulted in a dose-dependent increase in the percentage of cells in G1 phase, and this G1 phase accumulation was accompanied by a corresponding reduction in the percentages of cells in S and G2/M phase (Table 1). Basically, the same results were observed with 4T1 cells. In addition, both HEGU and isoangustone A increased the number of cells in sub-G1 phase. This result is consistent with our previous observation that HEGU and isoangustone A induced apoptosis in DU145 cells [16]. Consistent with the induction of G1 arrest, the incorporation of [3H] thymidine into the DNA was reduced dramatically in both DU145 and 4T1 cells treated with HEGU or isoangustone A (Table 2). When the effects of HEGU were compared with those of isoangustone A, the

**Table 1**
HEGU and isoangustone A induce cell-cycle arrest at G1 phase in cancer cells

|  | Sub-G1 | G1 | S | G2/M |
|---|---|---|---|---|
| *DU145 cells* |  |  |  |  |
| HEGU (μg/ml) |  |  |  |  |
| 0 | 5.1±0.36[b] | 55.6±1.38[c] | 12.4±0.39[a] | 26.9±1.57[a] |
| 2.5 | 8.1±0.54[b] | 58.6±0.96[bc] | 11.8±0.38[ab] | 21.5±1.07[b] |
| 5 | 12.6±1.03[a] | 60.5±0.93[ab] | 10.8±0.39[b] | 16.1±0.76[c] |
| 7.5 | 15.3±1.74[a] | 62.9±0.87[a] | 10.6±0.69[b] | 11.2±0.79[d] |
| Isoangustone A (μg/ml) |  |  |  |  |
| 0 | 2.9±0.19[c] | 48.1±0.63[c] | 17.6±0.77[a] | 31.4±0.12[a] |
| 2.5 | 5.5±0.15[b] | 52.1±0.99[b] | 15.1±0.23[ab] | 27.3±1.06[b] |
| 5 | 9.9±0.74[a] | 56.0±0.59[a] | 14.6±0.35[b] | 19.5±0.81[c] |
| *4T1 cells* |  |  |  |  |
| HEGU (μg/ml) |  |  |  |  |
| 0 | 1.8±0.24[b] | 56.4±0.33[c] | 18.1±0.66[a] | 23.7±1.45[a] |
| 2.5 | 2.2±0.18[b] | 56.5±0.56[c] | 17.8±0.41[ab] | 23.5±0.57[a] |
| 5 | 3.2±0.22[a] | 59.5±0.34[b] | 16.3±0.23[bc] | 21.0±0.92[ab] |
| 7.5 | 3.8±0.32[a] | 61.0±0.40[a] | 15.8±0.62[c] | 19.4±0.50[b] |
| Isoangustone A (μg/ml) |  |  |  |  |
| 0 | 1.8±0.24[b] | 56.4±0.33[c] | 18.1±0.66[a] | 23.7±1.45[a] |
| 2.5 | 3.4±0.51[a] | 61.8±0.84[b] | 17.1±1.09[ab] | 17.7±2.15[b] |
| 5 | 4.1±0.55[a] | 67.2±0.66[a] | 14.4±0.56[b] | 14.3±0.24[b] |

Cells were plated on 24-well plates at a density of 50,000 cells/well with DMEM/F12 supplemented with 100 ml/L of FBS. Twenty-four hours after plating, the monolayers were serum deprived in DMEM/F12 supplemented with 1% charcoal-stripped FBS and treated for 6 h with HEGU or isoangustone A. The nuclei were stained with propidium iodide, and the cell cycle was analyzed via flow cytometry. Results are expressed as means±S.E.M. (*n*=6). Means without a common letter[a,b,c,d] significantly differ, *P*<.05.

**Table 2**
HEGU and isoangustone A inhibit [3H]thymidine incorporation in cancer cells

|  | DU145 cells | 4T1 cells |
|---|---|---|
| HEGU (μg/ml) |  |  |
| 0 | 1138.7±48.2[a] | 4668.8±310.7[a] |
| 2.5 | 826.3±25.8[b] | 2400.3±219.9[b] |
| 5 | 277.4±9.6[c] | 1742.0±541.0[c] |
| 7.5 | 84.6±18.7[d] | 1253.5±227.0[d] |
| Isoangustone A (μg/ml) |  |  |
| 0 | 549.4±39.0[a] | 4374.0±138.9[a] |
| 2.5 | 225.8±8.4[b] | 3173.3±60.2[b] |
| 5 | 94.7±4.9[c] | 324.8±34.5[c] |

Values are expressed as mean±S.E.M. (*n*=6). Means without a common letter[a,b,c,d] significantly differ, *P*<.05.

same concentrations (5 μg/ml) of isoangustone A were shown to be more effective than HEGU in inhibiting cell-cycle progression and [3H]thymidine incorporation in DU145 cells (Fig. 2).

### 3.2. HEGU reduces the levels of cyclins and CDKs and inhibits CDK2 activity in DU145 and 4T1 cells

We subsequently evaluated the effects of HEGU on the expression of the CDKs and cyclins that regulate the G1 phase transition of the cell cycle. The cells were treated for 6 h with various concentrations of HEGU. Western blot analysis of the DU145 cell lysates revealed that HEGU reduced the levels of CDK2, CDK4, cyclin A and cyclin D1 in a concentration-dependent manner (Fig. 3A, B). In addition, HEGU
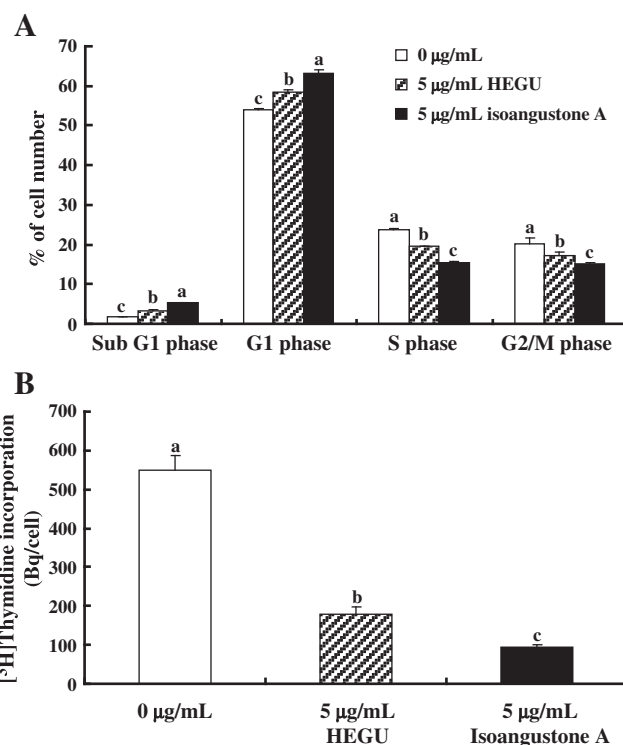
Fig. 2. Isoangustone A is more effective than HEGU in inhibiting cell-cycle progression and [3H]thymidine incorporation in DU145 cells. DU145 cells were plated in 24-well plates at a density of 50,000 cells/well (A) or in 96-well plates at a density of 6000 cells/well (B) and serum deprived. (A) Cells were treated for 6 h with 5 μg/ml of HEGU or isoangustone A and stained with propidium iodide, and the cell cycle was analyzed via flow cytometry. (B) [3H]Thymidine was added at the same time as HEGU or isoangustone A, and the cells were incubated for 3 h to measure the incorporation into DNA. Each bar represents the mean±S.E.M. (*n*=6). Means without a common letter differ, *P*<.05.
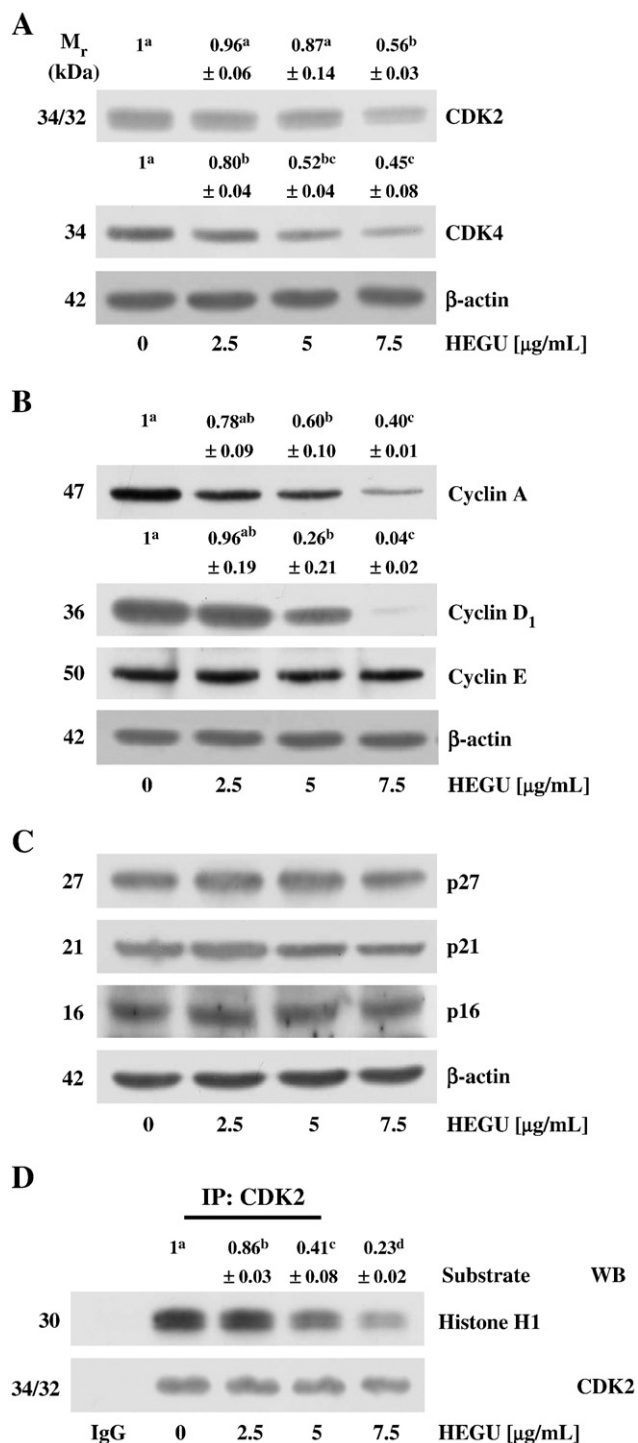
Fig. 3. HEGU reduces the protein levels of CDKs and cyclins and inhibits CDK2 activity in DU145 cells. Cells were plated in 100-mm dishes at a density of 1,000,000 cells/dish and treated with HEGU for 6 h. (A–C) Total cell lysates were analyzed via Western blotting with the indicated antibodies. Photographs of chemiluminescent detection of the blots, which were representative of three independent experiments, are shown. The relative abundance of each band to its own β-actin (A–C) was quantified, and the control levels were set to 1. (D) CDK2 was immunoprecipitated and analyzed via an *in vitro* kinase assay using histone H1 as a substrate. An autoradiograph of the dried gel, which is representative of three independent experiments, is shown. The relative abundance of each band was quantified, and the control levels were set to 1. (A–D) The adjusted mean±S.E.M. ($n=3$) of each band is shown above each blot. Means without a common letter differ, $P<.05$.
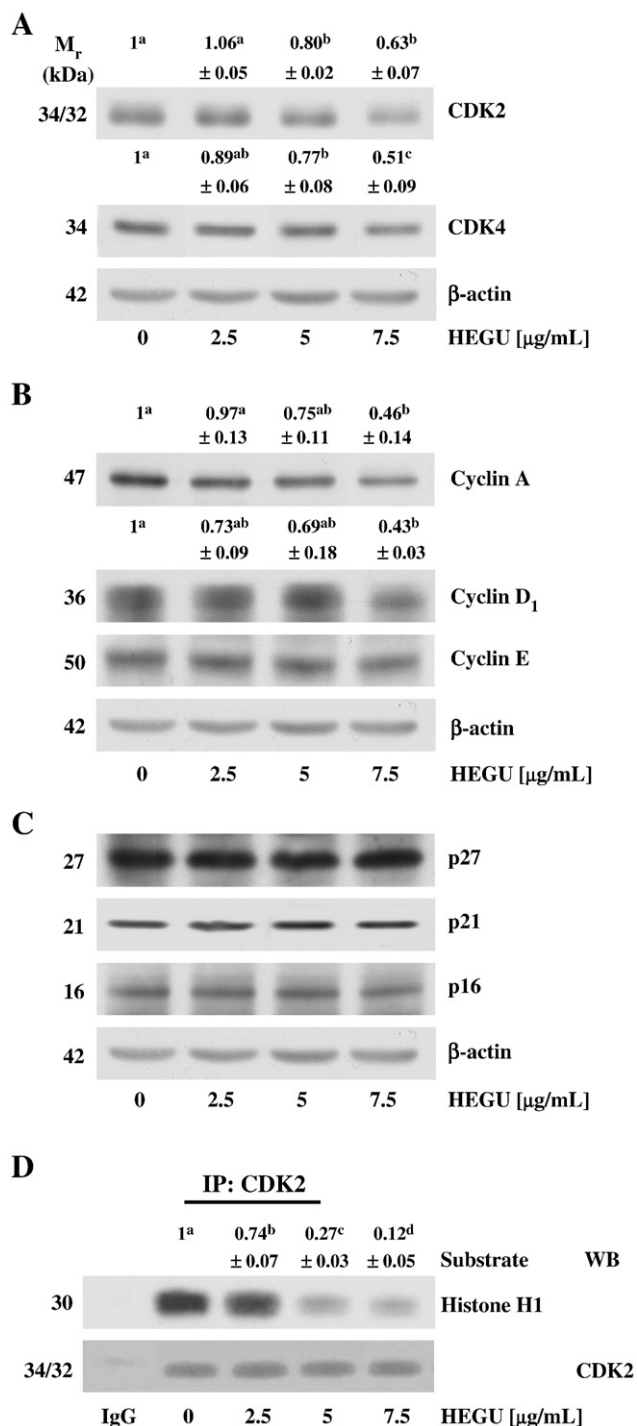
Fig. 4. HEGU reduces the protein levels of CDKs and cyclins and inhibits CDK2 activity in 4T1 cells. Cells were plated in 100-mm dishes at a density of 1,000,000 cells/dish and treated for 6 h with HEGU. (A–C) Total cell lysates were analyzed via Western blotting with the indicated antibodies. Photographs of chemiluminescent detection of the blots, which were representative of three independent experiments, are shown. The relative abundance of each band to its own β-actin (A, B, C) was quantified, and the control levels were set to 1. (D) CDK2 was immunoprecipitated and analyzed via an *in vitro* kinase assay using histone H1 as a substrate. An autoradiograph of the dried gel, which is representative of three independent experiments, is shown. The relative abundance of each band was quantified, and the control levels were set to 1. (A, B, D) The adjusted mean±S.E.M. ($n=3$) of each band is shown above each blot. Means without a common letter differ, $P<.05$.

reduced the levels of CDK2, CDK4, cyclin A and cyclin D1 in 4T1 cells (Fig. 4A, B). However, HEGU treatment did not alter the levels of p16[INK4a], p21[WAF1/CIP1], p27[KIP1] (Figs. 3C and 4C) or cyclin E (Figs. 3B and 4B) in either DU145 cells or 4T1 cells.

To measure CDK2 activity, the enzyme complex was immunoprecipitated with CDK2 antibody, followed by *in vitro* kinase assays using histone H1 as a substrate. The treatment of DU145 and 4T1 cells with increasing concentrations of HEGU resulted in dose-dependent reductions in CDK2 activity (Figs. 3D and 4D).

### 3.3. Isoangustone A reduces the levels of cyclins and CDKs and inhibits CDK2 activity in DU145 and 4T1 cells

In both DU145 and 4T1 cells, isoangustone A treatment reduced the levels of the CDK2, CDK4, cyclin A, cyclin D1 and cyclin E proteins (Figs. 5A, B, and 6A, B). In addition, treatment with isoangustone A resulted in a dose-dependent reduction in CDK2 activity (Figs. 5D and 6D).

### 3.4. HEGU reduces the growth of 4T1 breast cancer cell allografts in BALB/c mouse

To assess the effects of HEGU on tumor growth *in vivo,* we injected 4T1 mouse breast cancer cells into the inguinal mammary fat pads of syngeneic, immunocompetent BALB/c mice. The addition of HEGU to drinking water did not affect the body weights of the mice (Fig. 7A), but the tumor volumes were significantly lower in the HEGU-treated mice (Fig. 7B). In order to determine whether the administration of HEGU exerts antiproliferative effects on tumor cells *in vivo,* tumor tissue samples were immunohistochemically stained with an antibody against PCNA, a ubiquitous proliferation marker. HEGU administration significantly reduced PCNA expression in tumor tissues as compared with the control group (Fig. 8A). Western blot analyses of tumor tissue lysates revealed that CDK2 and CDK4 protein expression was reduced significantly in the HEGU-treated mice (Fig. 8B).

## 4. Discussion

Licorice root has been recognized by the National Cancer Institute as exhibiting cancer-preventive properties [21] and is used for the treatment of prostate cancer patients as an ingredient of PC-SPES, a commercially available combination of eight herbs [22]. As the principal component of licorice, GL has been reported to exert anticarcinogenic effects and is known to induce hypertension (reviewed in Ref. [9]); in a previous study, we attempted to prepare a licorice extract that contained no GL. We noted that HEGU lacking GL and its active compound, isoangustone A, inhibited cell growth and induced apoptosis in a variety of human cancer cells [16]. The principal objective of this study was to determine whether HEGU and isoangustone A delay cell-cycle progression in DU145 human prostate and 4T1 murine breast cancer cells.

Hanahan and Weinberg [23] previously noted that, in cancer cells, the regulatory pathways that govern normal cell proliferation and homeostasis are disrupted. As a result of these defects in the regulation of cell-cycle machinery in cancer cells, cell-cycle regulatory proteins are considered promising molecular targets for cancer therapy and/or prevention. In this study, we have demonstrated that HEGU and isoangustone A (a) inhibited DNA synthesis; (b) induced G1 cell-cycle arrest; (c) reduced the protein levels of CDK2, CDK4, cyclin A and cyclin D1 and (d inhibited CDK2 activity in cancer cells. Alterations in cell proliferation and cell-death pathways are key features of transformed cells; therefore, the induction of apoptosis and the reduction of cell-cycle progression in cancer cells as the result of HEGU and isoangustone A treatment may be an effective means for
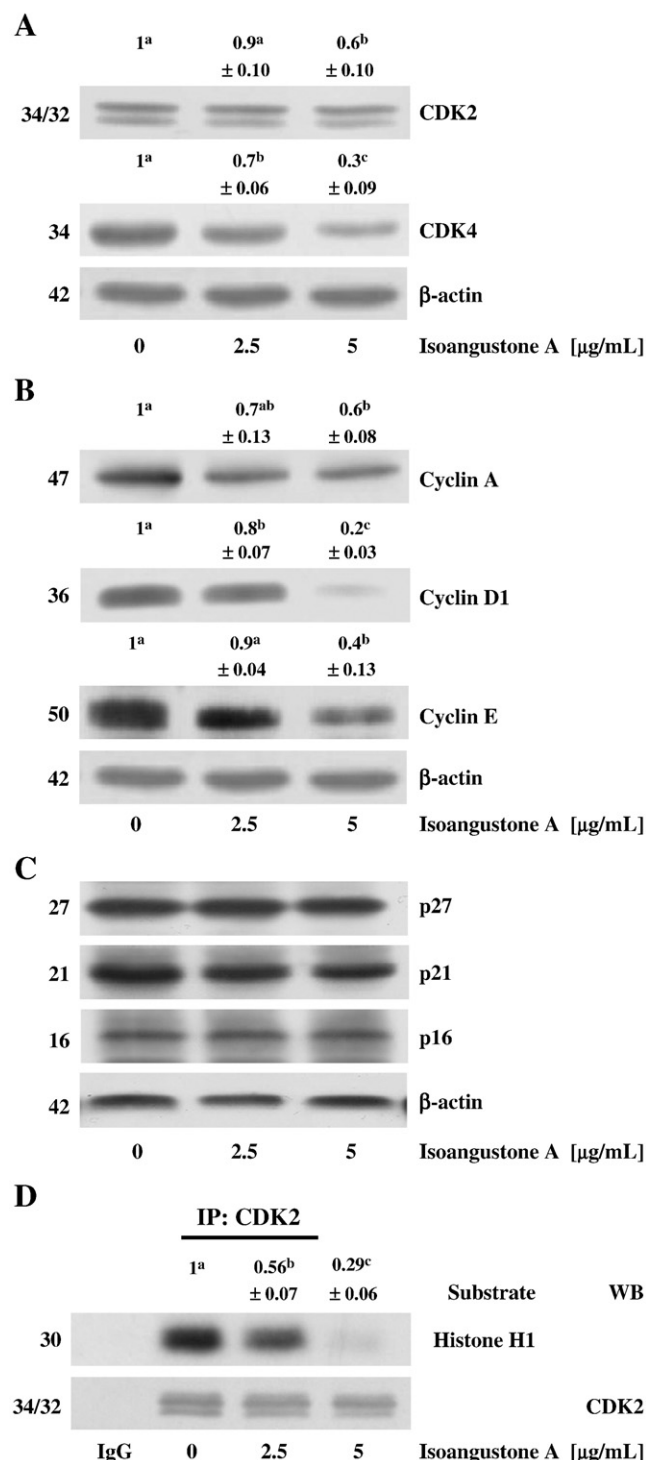


Fig. 5. Isoangustone A inhibits CDK2 activity in DU145 cells. DU145 cells were plated in 100-mm dishes at a density of 1,000,000 cells/dish and treated for 6 h with isoangustone A. (A–C) Total cell lysates were analyzed via Western blotting with the indicated antibodies. Photographs of chemiluminescent detection of the blots, which were representative of three independent experiments, are shown. The relative abundance of each band to its own β-actin was quantified, and the control levels were set to 1. (D) Total cell lysates were immunoprecipitated with CDK2 antibody and analyzed via an *in vitro* kinase assay using histone H1 as a substrate. An autoradiograph of the dried gel, which is representative of three independent experiments, is shown. The relative abundance of each band was quantified, and the control levels were set to 1. The adjusted mean±S.E.M. ($n=3$) of each band is shown above each blot. Means without a common letter differ, $P<.05$.
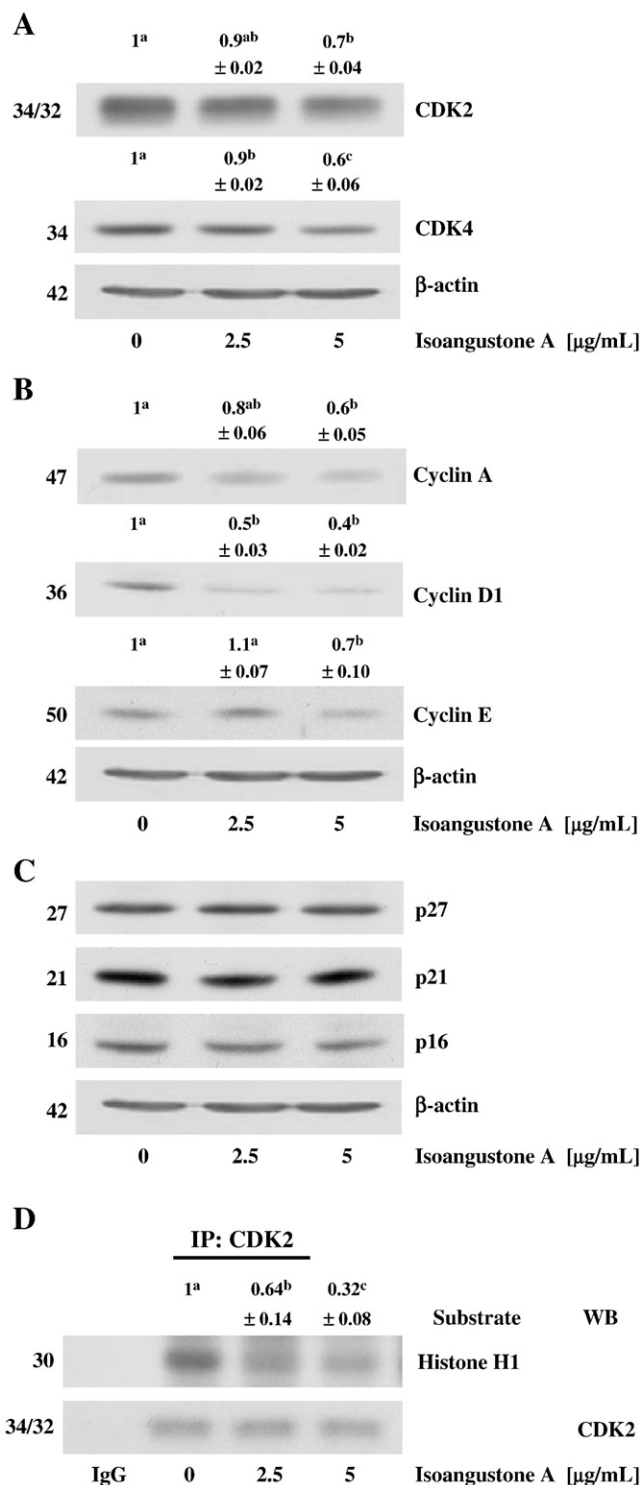
Fig. 6. Isoangustone A inhibits CDK2 activity in 4T1 cells. 4T1 cells were plated in 100-mm dishes at a density of 1,000,000 cells/dish and treated for 6 h with isoangustone A. (A–C) Total cell lysates were analyzed via Western blotting with the indicated antibodies. Photographs of chemiluminescent detection of the blots, which were representative of three independent experiments, are shown. The relative abundance of each band to its own β-actin was quantified, and the control levels were set to 1. (D) Total cell lysates were immunoprecipitated with CDK2 antibody and analyzed via an *in vitro* kinase assay using histone H1 as a substrate. An autoradiograph of the dried gel, which is representative of three independent experiments, is shown. The relative abundance of each band was quantified, and the control levels were set to 1. (A, B, D) The adjusted mean±S.E.M. (*n*=3) of each band is shown above each blot. Means without a common letter differ, *P*<.05.
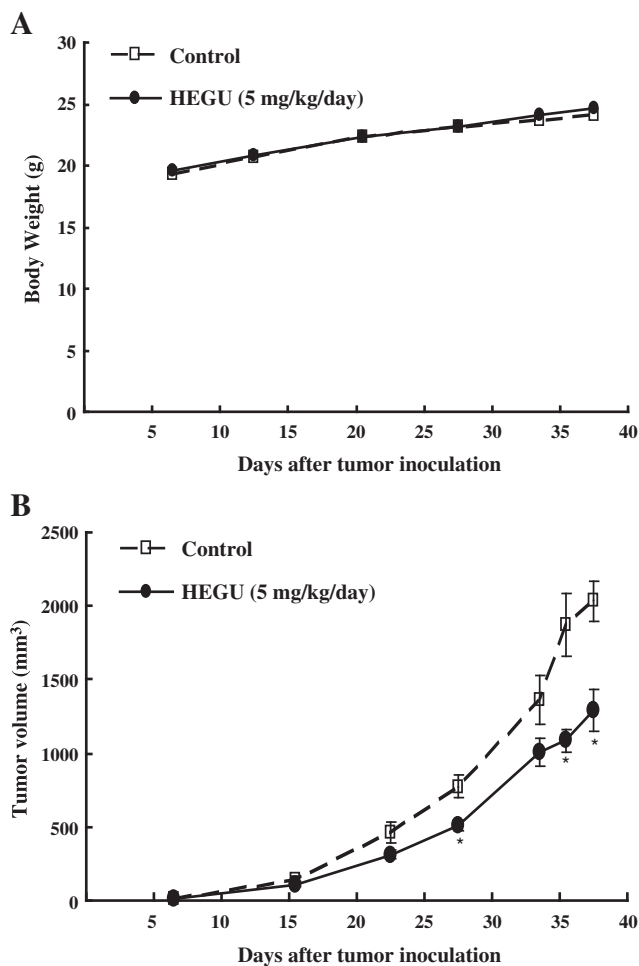


Fig. 7. HEGU feeding inhibits the growth of 4T1 cell allografts in BALB/c mice. (A) Changes in body weight. (B) Tumor volumes were estimated as described in the "Materials and methods" section (means±S.E.M.; *n*=6). *Significantly different from vehicle-administered control group, *P*<.05.

the prevention of cancer development. In fact, in this study, we have demonstrated that HEGU effectively reduced tumor size when 4T1 cells were implanted in the mammary fat pads of BALB/c mice.

HEGU and isoangustone A effectively inhibited DNA synthesis and induced G1 phase arrest in both DU145 and 4T1 cells in a concentration-dependent manner (Tables 1 and 2). At a concentration of 5 μg/ml, the ability of isoangustone A to induce G1 phase arrest and inhibit DNA synthesis was greater than that of HEGU (Fig. 2), thereby indicating that isoangustone A is one of the compounds responsible for the observed anticarcinogenic effects of HEGU. However, the efficacy of isoangustone A was not sufficiently high to draw the conclusion that isoangustone A is the only active compound in HEGU. Several active compounds other than isoangustone A in HEGU may have the ability to inhibit cell-cycle progression in cancer cells. It also seems possible that other components of HEGU protect isoangustone A against oxidation and/or degradation, such that isoangustone A is more stable in a mixture with other compounds in HEGU than as an isolated single compound.

The G1 to S phase transition is regulated by CDK4 and CDK2. In this study, the treatment of DU145 cells with HEGU or isoangustone A was shown to reduce the protein levels of cyclin A and CDK2 (Figs. 3 and 4). In addition, CDK2 activity was reduced in a dose-dependent manner by treatment with HEGU or isoangustone A (Figs. 3D and 4B, D). These results demonstrate that the reduced protein levels of CDK2 and cyclin A contributed to the observed
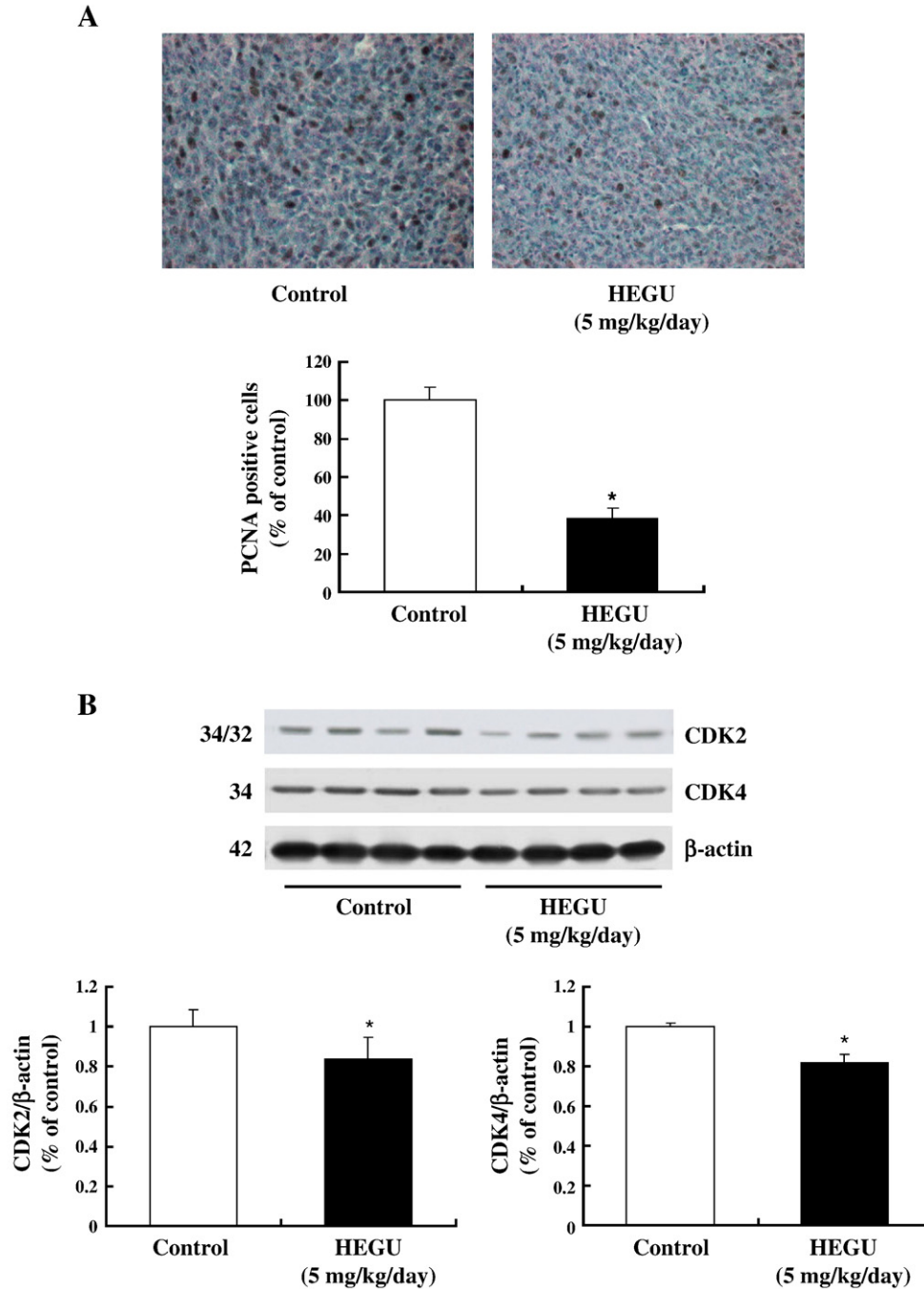
A



B



Fig. 8. HEGU feeding suppresses the expression of the PCNA, CDK2 and CDK4 proteins in the tumor tissues of BALB/c mice. (A) Immunohistochemical detection of cells expressing PCNA in the tumor tissue (×400 magnification). The number of PCNA positive nuclei was expressed as the percentage of the control (mean±S.E.M.; $n=5$). (B) Tumor tissue lysates were analyzed via Western blotting with the CDK4 and CDK2 antibody. Photographs of chemiluminescent detection of the blot are shown. The relative abundance of each band to its own β-actin was quantified, and the adjusted mean±S.E.M. ($n=4$) of each band is shown. *Significantly different from vehicle-administered control group, $P<.05$.

reductions in CDK2 activity. The treatment of cells with HEGU reduced the levels of cyclin D1 and CDK4, but did not affect the levels of cyclin E (Fig. 3A, B). HEGU exerted no effects on the levels of p16[INK4a], p21[WAF1/CIP1] or p27[KIP1] (Fig. 3C). In this study, we did not specifically measure CDK4 activity. However, these results indicate that the reduced levels of cyclin D1 and CDK4 proteins may have induced a reduction in CDK4 activity. Isoangustone A treatment reduced the levels of cyclin E (Fig. 5A), whereas HEGU treatment did not affect cyclin E levels (Fig. 3B). These differences in efficacy between HEGU and isoangustone A may be one

explanation for the finding that isoangustone A is more effective in inducing G1 cell-cycle arrest than HEGU at a concentration of 5 μg/ml (Fig. 2).

In an attempt to ascertain the *in vivo* effects of HEGU on cancer development, we used the murine 4T1 cell line, which was derived originally from a spontaneous mammary carcinoma in BALB/c mice [24]. As the 4T1 allograft in the mammary fat pads of BALB/c mice is considered to mimic closely the growth and metastatic patterns associated with human breast cancer, this allograft model constitutes an appropriate *in vivo* system for the modeling of human breast

cancer with regard to tumor growth [17]. Our results clearly demonstrate that HEGU effectively inhibits mammary tumor growth (Fig. 7) and the expression of PCNA, CDK2 and CDK4 (Fig. 8). In future studies, it will be necessary to determine whether HEGU can delay breast cancer growth in humans.

In conclusion, the results of this study demonstrate that HEGU and its active component, isoangustone A, induce G1 cell-cycle arrest in DU145 human prostate and 4T1 mouse breast cancer cells and that this effect may be mediated via the inhibition of CDK activity. In addition, HEGU treatment reduced the sizes of solid tumors in 4T1 cell-inoculated BALB/c mice. Our study provides some of the molecular basis for further evaluations of HEGU and isoangustone A as potential anticarcinogenic agents.

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