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Tellimagrandin I enhances gap junctional communication and attenuates the tumor phenotype of human cervical carcinoma HeLa cells in vitro

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Abstract

Tellimagrandin I and chebulinic acid, two hydrolysable tannins, have been shown to exert anti-tumor properties. Dysfunctional gap junctional communication (GJIC) has been recognized as being involved in carcinogenesis. The human cervical carcinoma HeLa cells have been reported to be deficient in functional GJIC. In present study, we investigated whether tellimagrandin I and chebulinic acid might restore functional GJIC in HeLa cells. Both compounds could inhibit the growth of HeLa cells. Either Lucifer yellow transfer assay or calcein transfer assay demonstrated that tellimagrandin I improved GJIC in HeLa cells while chebulinic acid showed no effect on GJIC. The GJIC enhancement by tellimagrandin I occurred along with an increase of *Cx43* gene expression at mRNA and protein levels. Exposure to tellimagrandin I also led to inhibition of proliferation and anchorage-independent growth of HeLa cells. In addition, tellimagrandin I decreased the percentage of cells in the G0/G1 and G2/M phases coinciding with an increase in the percentage of cells in the S phase. The accumulation of cells in S phase was coupled with a decreased expression of cyclin A that was critical to the progression of S phase. These results suggested that restoring GJIC might be one explanation for tellimagrandin I antitumor effects, whereas chebulinic acid exerted antitumor action through other pathways.

Keywords: Anchorage-independent growth; Connexin 43; Gap junctional communication; Tellimagrandin I

1. Introduction

Tellimagrandin I and chebulinic acid both are hydrolysable tannins widely present in plants. Tellimagrandin I (Fig. 1), rich in plants such as *Punica granatum*, *Myrtaceae*, and *Elaeagnaceae,* shows various biological activities including inhibitory effects on carbonic anhydrase and syncytia formation [1,2], antibacterial activity against *Helicobacter pylori* [3], toxicities towards the nematode and the brine shrimp [4], and ability to restore effectiveness of beta-lactams and tetracycline on methicillin-resistant *Staphylococcus aureus* [5]. Chebulinic acid (Fig. 1), present in several medicine plants, such as *Phyllanthus emblica*, *Terminalia arborea*, and *Terminalia chebula,* also demonstrates anti-*neisseria gonorrhoeae* activity [6], inhibitory effects on the contractile responses of cardiovascular muscles [7], and blocking the binding

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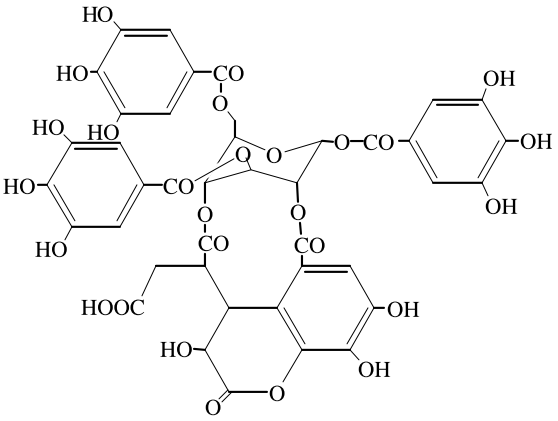
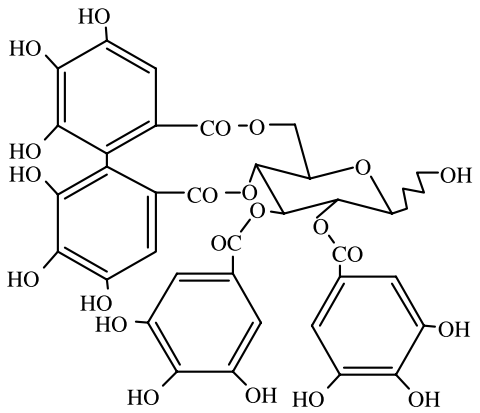


Chebulinic acid



Tellimagrandin I

Fig. 1. The structure of chebulinic acid and tellimagrandin I.

of HIV rgp120 to CD4 [8]. Our recent studies have shown that chebulinic acid and tellimagrandin I demonstrated inhibitory effects on chemically induced differentiation of human leukemia K562 cells [9,10]. Remarkably, chebulinic acid and tellimagrandin I also exhibit antitumor activities. For instance, treatment of chebulinic acid results in growth inhibition and cytoxicity against malignant cell lines such as MCF-7 Human breast adenocarcinoma cells, S115 mouse mammary tumor cells, HOS 1 human osteosarcoma cells, PC-3 human prostate cancer cells, A-549 human lung carcinoma cells and HCT-15 human colon adenocarcinoma cells [11], whereas tellimagrandin I demonstrates anti-tumorigenesis against S-180 mouse sarcoma cells in mice [12]. However, the mechanisms to explain antitumor activities of two tannins remain unclear. To explore the potential mechanisms of action, we focused our study on gap junctional communication (GJIC).

Gap junctions are clusters of tiny aqueous channels that link the cytoplasm of neighboring cells. They consist of two hemi-channels, called connexons, each in turn composed of six molecules of the membrane-spanning connexin protein (Cx). An evolutionarily conserved gene family containing at least 20 members encodes connexin proteins that form homotypic or heterotypic connexons and are expressed in a cell-type specific manner [13]. Intercellular communication through gap junction plays a significant role in maintaining tissue homeostasis by exchanging small molecules, including sugars, nucleotides and amino acids, and second messengers, such as Ca^{2+}, cAMP, cGMP and IP3. GJIC has long been proposed as a mechanism to regulate growth control, development and differentiation [14].

Dysfunctional GJIC has been recognized as being involved in carcinogenesis, and growing evidences have indicated that connexin proteins function as tumor suppressors [14–16]. Firstly, aberrant GJIC is often observed in malignant tumor cells, include reduction of the transcription level of connexin genes, abnormal phosphorylation and/or localization of connexins, and failure of gap junction assembly [15]. Transfection of connexin gene expression vectors into tumor cells restores GJIC, which correlates with reduced tumorigenesis in vivo and inhibition of cell growth in vitro, such as human cervical cancer cells [17,18]. Furthermore, tumorpromoting agents (phorbol esters, DDT, phenobarbital, etc.), oncogenes (e.g. *ras, raf, neu, src, mos*), and growth factors, in most cases, resulted in the down-regulation of GJIC at either the transcriptional, translational or post-translational levels [15,19,20]. Additionally, knockout transgenic mice for *Cx32* gene develop a higher incidence of age-related liver tumors and become more susceptible to the effects of liver-specific chemical carcinogens [21,22], immortalized cell lines derived from the $Cx43^{-/-}$ mice showed several biological properties of transformed cells [23], and deletion of one allele of the *Cx43* gene ($Cx43^{+/-}$) results in a higher susceptibility to lung adenoma formation in mice [24]. Recently, Shao et al. has shown that down-regulation of *Cx43* expression by retroviral delivery of small interfering RNA promotes an aggressive breast cancer cell phenotype [25].

Since the degree of GJIC is often modulated at the transcriptional, translational, and channel gating levels in cancer cells, and mutations have been found to be rare events in chemically induced rat tumors and various forms of human neoplasia [26–31], it has been assumed that pharmacological stimulation to efficiently restore GJIC in tumor cells might represent a strategy

for anti-neoplastic therapies [32]. Indeed, agents that exhibit tumorpreventing properties or cause cell differentiation, and anti-neoplastic agents, such as green tea components [33], resveratrol [34], and retinoids [35], can increase GJIC through up-regulation of connexin expression.

The human cervical carcinoma cell line HeLa has been reported to be deficient in expression of Cx26, Cx32 or Cx43 and without functional GJIC [36], while these proteins express in the normal human cervical tissue. Several reports have demonstrated that GJIC can be induced in HeLa cells. When growing in spheroids and being co-cultured with normal fibroblasts, HeLa cells have abundant gap junctions [37]. In addition, when HeLa cells are co-cultured in close contact with Friend erythroleukemia cells, gap junctions are induced [38]. These results suggested transcriptional regulation might be involved in connexin expression in HeLa cells. Indeed, recent research has indicated that DNA-methylation involved in modulating the expression of Cx43 in HeLa cells [17]. Herein, it was investigated whether chebulinic acid and tellimagrandin I could restore GJIC in HeLa cells. The results showed that tellimagrandin I up-regulated GJIC, but chebulinic acid did not, suggesting that restoring GJIC might be one explanation for tellimagrandin I antitumor effects.

2. Materials and methods

2.1. Chebulinic acid and tellimagrandin I

Chebulinic acid and tellimagrandin I (purity > 95%), kindly provided by Dr Yan-Ze Liu (Henan College of Traditional Chinese Medicine, Zhengzhou, China), were dissolved in ethanol as stock solution.

2.2. Cell culture

HeLa cells were cultured in RPMI 1640 medium (GIBCO) supplemented with 10% fetal bovine serum (HyClone), 100 U/ml penicillin (Sigma) and 100 μg/ml streptomycin (Sigma) in a humidified atmosphere containing 5% CO_2 at 37 °C. For the experiment, the exponentially growing cells were used.

2.3. Cell growth analysis

For measuring the effects of chebulinic acid and tellimagrandin I on cell growth, HeLa cells were seeded and cultured in six-well plates at 1×10^4 cells/well. Twenty-four hours later, the medium was replaced with fresh RPMI 1640 medium containing chebulinic acid or tellimagrandin I at concentrations from 0 to 100 μM. After treatment with chebulinic acid or tellimagrandin I for 72 h, trypsin-digested cells in each well were stained by trypan blue, the live cells and dead cells were counted using a haemocytometer.

2.4. Gap junction intercellular communication assays

Scrape-loading and dye transfer (SL/DT) and calcein transfer assay were used to measure GJIC in confluent HeLa cells treated with chebulinic acid or tellimagrandin I. These techniques were carried out as follows.

2.4.1. SL/DT technique

SL/DT method has been preciously described in detail by El-Fouly et al [39]. Briefly, HeLa cells treated with chebulinic acid or tellimagrandin I were washed with phosphate buffered saline (PBS), scraped, incubated for 3 min with Lucifer yellow CH (0.05%, w/v, in PBS) (Fluka/Sigma), washed with PBS, and fixed in 4% formalin. The degree of Lucifer yellow transfer, obtained from the scraped edge, was visualized with fluorescent microscope (Leica).

2.4.2. Calcein transfer assay

GJIC was also measured using calcein transfer assay as previously described with some modifications [40]. Briefly, for loading with calcein acetoxymethyl ester (calcein AM) (Fluka/Sigma) and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) (Sigma), donor cells were trypsinized with 0.25% trypsin solution, suspended in culture medium, centrifuged, re-suspended in 2 ml of staining solution (10 μM DiI, 5 μM calcein AM, 0.3 M glucose), and fluorescence-labeled for 30 min at 37 °C. The cells were then washed with PBS, centrifuged three times, and added to a monolayer culture of unstained recipient cells. After incubation for 3 h, these co-cultures were trypsinized, suspended in PBS, and analyzed by flow cytometry. As a fluorescent lipophilic tracer, DiI stains plasma membranes of cells with a red fluorescence. The membrane-permeable calcein AM is hydrolyzed by intracellular nonspecific esterase and the resulting green fluorescent hydrophilic calcein is then trapped inside the cells. Therefore, gap junctions are permeable for calcein but not for DiI. In flow cytometry analysis, two peaks of calcein fluorescent density will appear in the obtained diagram (Fig. 3C). The right one represents the donor cells, and the left one means the recipient cells. If cells demonstrate functional GJIC, the left peak will shift to right in co-cultured cells compared with the mixture of donor cells and recipient cells without incubation at 37 °C.

After co-incubation, calcein transfer to neighboring cells was also observed with fluorescent microscope. The number of calcein-stained recipient cell by each donor cell was counted. Fifty donor cells in a continuous field was measured in each sanples. The data represent means ± SD.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted with Trizol Total RNA Minipreps Classic Kit (Sangon, Shanghai, China), according to the manufacturer's instructions. The RNA quantity was identified from the absorbance at 260 nm. First strand cDNA was synthesized using 25 μg total RNA by M-MuLV reverse transcriptase (Sangon, Shanghai, China) in a 100 μl reaction system.

PCR was carried out using 10 μl of cDNA in a 50 μl reaction with UNOII Thermocycler (Biometra). The sense primer for human *Cx43* was 5′-TGAGCAGTCT GCCTTTCGTT G-3′. The antisense primer for human *Cx43* was 5′-CCATCAGTTT GGGCAACCTT G-3′. The expected amplified fragment for *Cx43* was 219 bp. As an internal control, the sense primer for β-actin was 5′-TGGACTTCGA GCAAGAGATG G-3′, the antisense primer for β-actin gene was 5′-ATCTCCTTCT GCATCCTGTC G-3′. The expected amplified fragment for β-actin was 289 bp. The amplification reactions were initiated by a denaturation step for 5 min at 95 °C and then subjected to 28 cycles of 95 °C for 1 min, 60 °C for 45 s, and 72 °C for 45 s. The amplified DNA products were separated on 2% agarose gel, stained with ethidium bromide, visualized and photographed with Image-Master Video Documentation System (Pharmacia Biotech), and semi-quantitated using a Sigma gel analysis software V1.0.

2.6. Western Blotting

Western blot were performed to assay the protein level of *Cx43*, cyclin A, cyclin D1 and cyclin E. Briefly, cells were lysed with 20% SDS containing 1 mM phenylmethylsulfonyl fluoride (Sigma), 1 mM leupeptin (Sigma), 1 mM antipain (Sigma), 0.1 M aprotinin (Sigma), 0.1 mM sodium orthovanadate (Sigma) and 5 mM sodium fluoride. After sonicating the lysates, protein concentrations were determined using the BCA protein assay kit (Beyotime Biotechnology). Proteins were separated on 12.5% SDS polyacrylamide gels and transferred to PVDF membranes (Pall Gelman Laboratory). The separated proteins were reacted sequentially for 1 h at room temperature with primary antibodies for *Cx43* (Zymed), cyclin A (Santa Cruz), cyclin D1 (Santa Cruz), cyclin E (Santa Cruz) and β-actin (Sigma), followed by fluorescein-conjugated secondary antibody, detected with ECF chemifluorescence Western Blotting Kit (Amersham Biosciences), visualized and obtained photos with a Storm 860 PhosphorImager system (Amersham Pharmacia Biotech), and semi-quantitated using a Sigma gel analysis software V1.0.

2.7. Anchorage-independent growth assay

In each plate, one hundred cells in 2.5 ml of 0.33% agarose medium were plated on the top of 2.5 ml pre-hardened 0.5% agarose medium. After incubation at 37 °C for 1 day, the medium containing tellimagrandin I at different concentrations was added on the top of the agar, and then incubation continued. Ten days later, the plates were dried at 37 °C for 1 day and anchorage-independent colonies of cells were stained with 1 mg/ml of 2-(*p*-iodophenyl)-3-(nitrophenyl)-5-phenyltetrazolium chloride (Sigma). The number of colonies in each plate was counted. The cloning efficiency was defined as the number of colonies per one hundred plated cells. The values from three independent experiments were averaged to obtain the final cloning efficiency of anchorage independence.

2.8. Cell cycle analysis

Cells were collected by centrifugation, fixed by 70% ethanol and re-suspended at 1×10^6 cells/ml in propidium iodide (PI) staining buffer (0.1% sodium citrate, 0.1% Triton-X 100, and 50 μg/ml PI) and were treated with 1 mg/ml RNase at room temperature for 30 min. Cell-cycle histograms were generated after analysis of PI-stained cells with a Becton Dickinson FACScan. For each culture, at least 1.5×10^4 events were recorded. Histograms generated by FACS were analyzed by ModFit Cell Cycle Analysis Software V2.0 to determine the percentage of cells in each phase (G1, S, and G2/M).

2.9. Statistical analysis

All data were expressed as means ± SD. Differences between groups were analyzed by Student's *t*-test.

3. Results

3.1. Chebulinic acid and tellimagrandin I inhibit the growth of HeLa cells

We first determined the effects of increasing concentrations of chebulinic acid and tellimagrandin I on cell growth and cell viability. It was observed that after a 72 h exposure to chebulinic acid or tellimagrandin I, the growth of HeLa cells was inhibited in a concentration-dependant manner (Fig. 2). The inhibitory effects begin to appear at the lowest concentration (20 μM) of each compound. Tellimagrandin I up to 60 μM caused over 80% decrease of cell growth with slight cytoxicity, whereas the treatment with 100 μM chebulinic acid led to a growth inhibition to a similar extent but without cytoxicity. An exposure to 50 μM tellimagrandin I also resulted in obvious growth inhibition, but cell death was not found. Therefore, the maximum concentrations of chebulinic acid and tellimagrandin I used in the following experiments were 100 and 50 μM, respectively.

3.2. Tellimagrandin I up-regulates GJIC, but chebulinic acid does not

In several studies, HeLa cells have demonstrated no functional GJIC. So, we determined the changes of GJIC in HeLa cells after treatment with tellimagrandin I or chebulinic acid. Firstly, SL/DT was used to evaluate functional GJIC (Fig. 3A). As expected, control HeLa cells were negative for GJIC which exhibited no ability to transfer Lucifer yellow. After treatment with chebulinic acid at the concentrations from 20 to 100 μM for 72 h, HeLa cells still maintained no GJIC. After a 72 h exposure to tellimagrandin I, low concentrations (from 10 to 30 μM) showed no effects on dye transfer to neighbor cells, but 40 and 50 μM tellimagrandin I enhanced GJIC.

In order to further validate the effects of tellimagrandin I up-regulating GJIC, another fluorescent dye calcein transfer through gap junction was determined using fluorescence microscope and flow cytometry. Under fluorescence microscope, tellimagrandin I-treated cells demonstrated their ability to transfer calcein from double-dyed donor cells to neighboring recipient cells (Fig. 3B), and the number of communicating cells was 12 ± 4 cells/donor cell. The control HeLa cells showed no dye transfer, which suggests there were no functional GJIC. This dye transfer was also analyzed with flow cytometry. After co-culture for 3 h, the recipient cell peak (the left one of two black peaks) of tellimagrandin I-treated cells obviously shifted to right, while the recipient cell peak of control cells only slightly shifted to right, indicating that the GJIC was increased in tellimagrandin I-treated cells (Fig. 3C).

3.3. Tellimagrandin I enhances the expression of Cx43

Connexin 43 protein is one type of general subunit of gap junction semi-channel (e.g. in cervical tissue). To evaluate the mechanism of GJIC restoration by tellimagrandin I, it was detect whether treatment with tellimagrandin I altered the expression of *Cx43* gene. RT-PCR was performed to determine the mRNA level of *Cx43* gene. We observed that the control HeLa cells showed a low basic level of *Cx43* mRNA, whereas HeLa cells treated with tellimagrandin I showed 8.5-fold enhanced level of *Cx43* transcript compared with the solvent control (Fig. 4A). Furthermore, protein expression of *Cx43* and phosphorylation were also analyzed by Western blot. The three typical bands (P0, P1 and P2) of Cx43 could be detected in all samples and separated according to the degree of its phosphorylation state; the band P0 represents nonphosphorylated Cx43 and P2 represents a much higher phosphorylated state than P1 (Fig. 4B). The level of Cx43 protein, which was represented by the total density of the three bands, was up-regulated by tellimagrandin I at time-dependent manner. After treatment with tellimagrandin I for 72 h, HeLa cells showed a 7.3-fold enhanced level of Cx43 protein compared to the control. In addition, the nonphosphorylated form

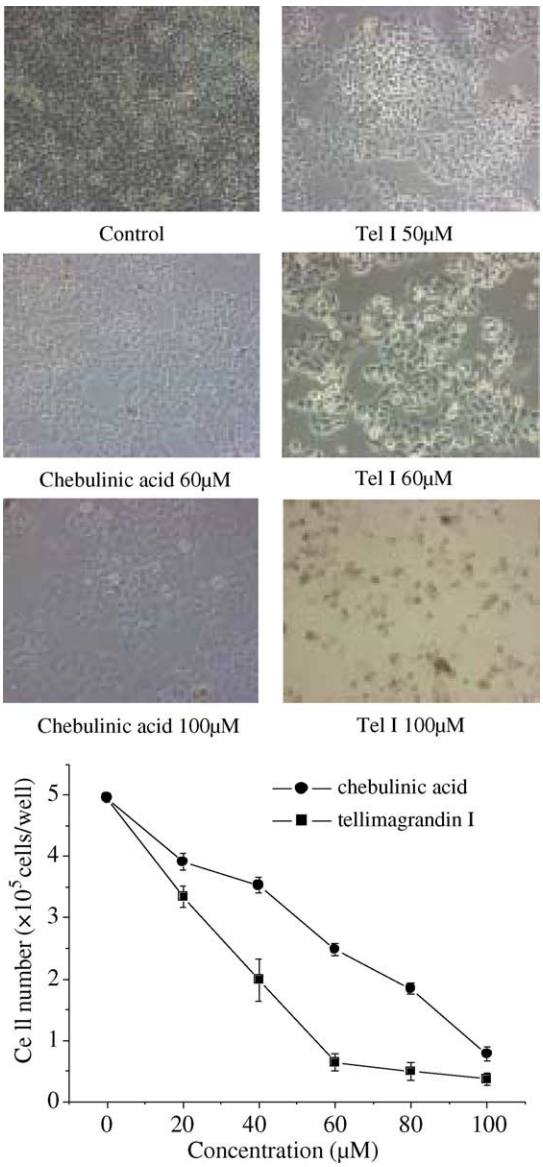


Fig. 2. Effects of chebulinic acid and tellimagrandin I (Tel I) on growth of HeLa cells. The HeLa cells were treated with chebulinic acid and tellimagrandin I at indicated concentrations for 72 h. Then, the number of the cells was counted under light microscopy. Data represent the mean and SD from three independent experiments.

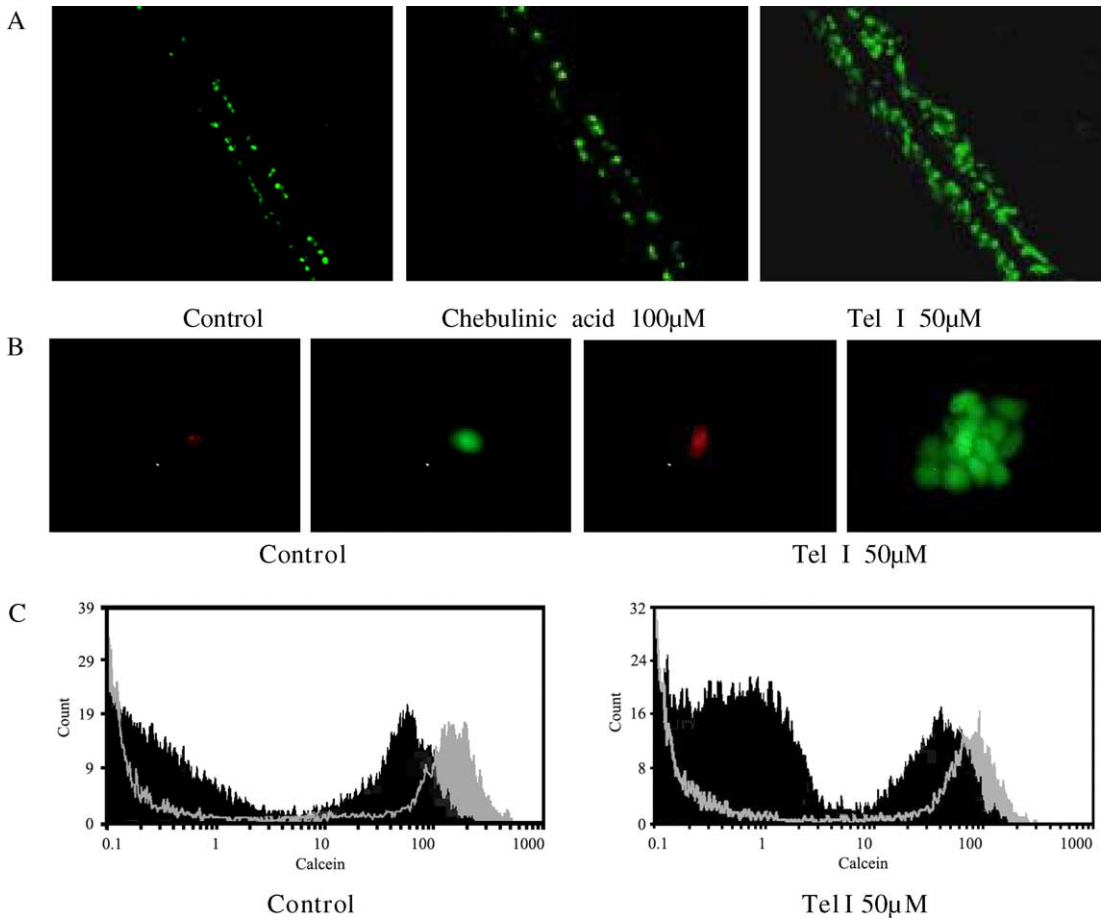


Fig. 3. Effects of chebulinic acid and tellimagrandin I on GJIC of HeLa cells. (A) Lucifer Yellow transfer was assayed by SL/DT in HeLa cells treated with chebulinic acid 100 μM or tellimagrandin I 50 μM for 72 h (×100). (B) calcein transfer was observed under fluorescence microscope in HeLa cells treated with tellimagrandin I 50 μM for 72 h (×100). After treatment with tellimagrandin I, one set of cells were fluorescence-labeled with calcein-AM (green) and DiI (red) as donor cells (white arrow). Then the donor cells were added to a monolayer culture of unstained recipient cells and cultured for 3 h at 37 °C. After that, the transfer of calcein (green) form donor cells to recipient cells was observed under fluorescence microscope. (×100). (C) Calcein transfer was analyzed with flow cytometry in control HeLa cells and tellimagrandin I-treated HeLa cells. After HeLa cells were treated with tellimagrandin I 50 μM for 72 h, one set of cells were fluorescence-labeled with calcein-AM as donor cells. Then the donor cells were added to a monolayer culture of unstained recipient cells and cultured for 3 h at 37 °C. After that, the transfer of calcein form donor cells (right black peak) to recipient cells (left black peak) was analyzed by flow cytometry. The gray peaks indicated the mixture of the donor cells (right peak) and recipient cells (left peak) without co-culture at 37 °C. Here represent one of three independent experiments.

completely accounted for the increased levels of Cx43, but phosphorylated Cx43 (P1 and P2) showed no significant change.

3.4. The effect of tellimagrandin I on anchorage-independent growth of HeLa cells

The effect of tellimagrandin I on anchorage-independent growth of HeLa cells in soft agar was studied. After HeLa cells were plated in soft agar and cultured for 24 h, the medium containing tellimagrandin I was added on the top of the agar. After a 10 days culture, the anchorage-independent growth of the cells was showed in Fig. 5. The control cells and 10 μM tellimagrandin I-treated cells formed colonies in soft agar with high cloning efficiency (over 85%), whereas the treatment with 30 μM tellimagrandin I resulted in lower cloning efficiency (10%), and 50 μM tellimagrandin I-treated HeLa cells grew poorly with almost no colony formation (1.3%) in soft agar.

3.5. Tellimagrandin I induces in an accumulation of HeLa cells in S phase

Flow cytometry analysis was conducted to analyze the cell cycle of HeLa cells treated with tellimagrandin

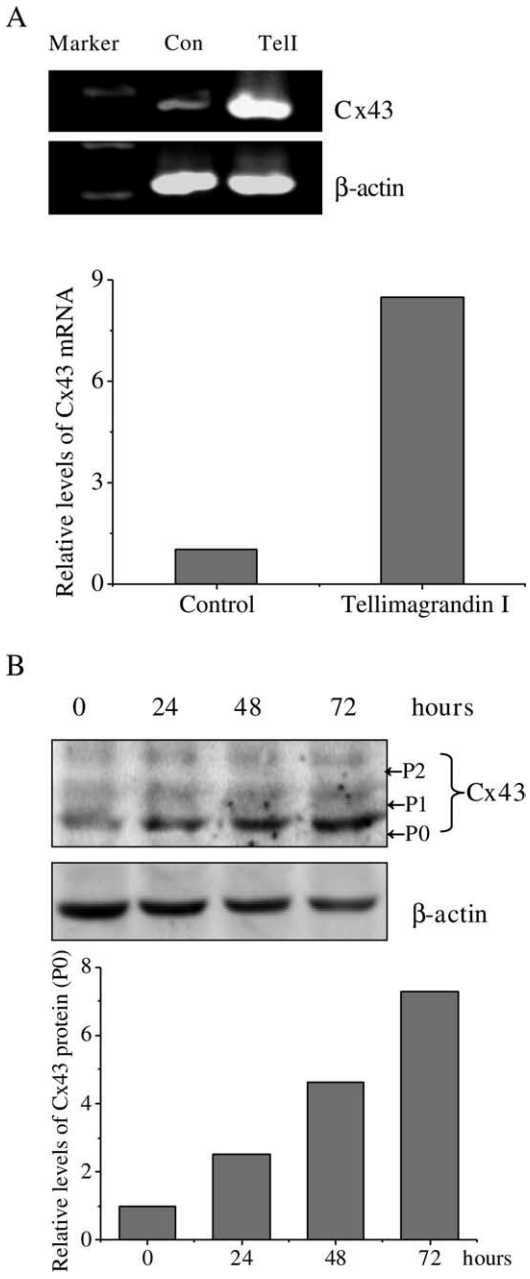


Fig. 4. Effects of tellimagrandin I on expression of Cx43 in HeLa cells. (A) After HeLa cells were treated with tellimagrandin I 50 μM for 72 h, the mRNA level of Cx43 gene was determined by RT-PCR. (B) After HeLa cells were treated with 50 μM tellimagrandin I for the indicated times, the expression of Cx43 protein was determined with Western blotting analysis. The P0 represents non-phosphorylated Cx43. The P1 and P2 represent the different phosphorylation states of Cx43.

I. Tellimagrandin I induced S-phase cell cycle arrest of HeLa cells concomitant with a decrease in G0/G1 and G2/M phases in concentration-dependent manner (Fig. 6A). At 25 μM, the percentage of cells in S phase in tellimagrandin I-treated cells increased to 44.24% from 35.96% compared with control cells, whereas the percentage of cells in G0/G and G2/M phase decreased to 40.16 and 15.6% from 47.3 and 16.74% respectively. At 75 μM, the percentage of cells in S phase was reached to 68.87%, while the progress from S to G2/M was almost completely blocked and the percentage of cells in G0/G1 phase decreased to 31.13%. The tellimagrandin I induced accumulation of cells in S phase was also time-dependent (Fig. 6B).

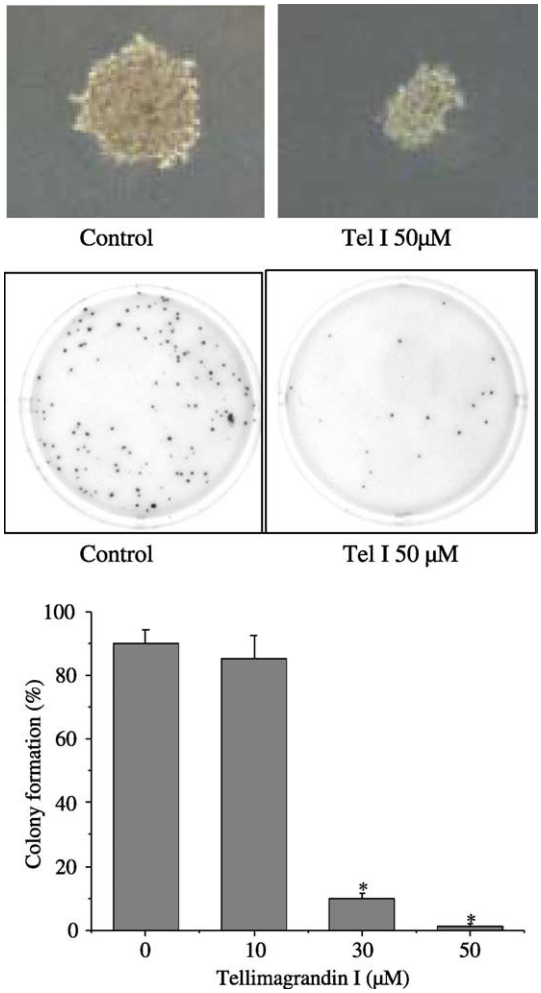


Fig. 5. The effect of tellimagrandin I on anchorage-independent growth of HeLa cells. HeLa cells were plated in soft agar 100 cells pre plate. After 1 day, the medium containing tellimagrandin I at indicated concentrations was added on the top of the agar. Ten days later, the colony formation in soft agar was assayed. Data represent the mean and SD from three independent experiments. $*P<0.01$ *vs.* solvent control.

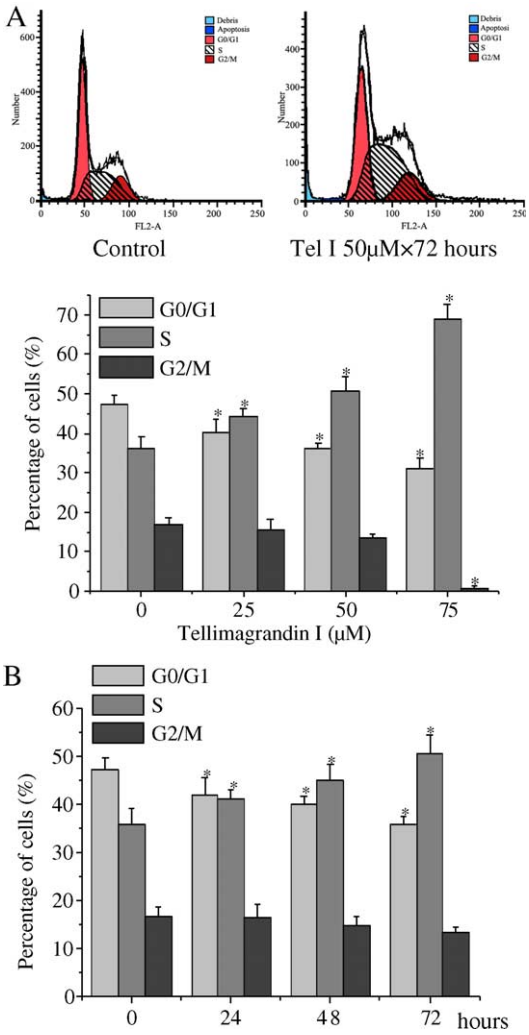


Fig. 6. Cell cycle analysis of HeLa cells after treated with tellimagrandin I. After HeLa cells were treated with tellimagrandin I 50 μM for indicated times (A) or increasing concentrations for 72 h (B), the cells were stained with propidium iodide, and 15,000 cells of each sample were analyzed under FACScan. Data represent the mean and SD from three independent experiments. $*P<0.01$ *vs.* solvent control.

3.6. Tellimagrandin I decreases cyclin A at the protein level

In order to character the mechanism of tellimagrandin I-induced S phase arrest, the specific cell cycle regulatory genes critical to cell cycle progression including cyclin A, D1, and E were analyzed by Western blot in HeLa cells treated with 50 μM tellimagrandin I. Tellimagrandin I induced a decrease of cyclin A protein with time-dependence, while the protein levels of cyclin D1, and E were not affected (Fig. 7).

4. Discussion

Previous studies have shown that chebulinic acid and tellimagrandin I demonstrate anti-tumor activities [11,12]. Present results showed that the two tannins inhibited the proliferation of HeLa cells (Fig. 2). However, two compounds showed different effects on GJIC in HeLa cells, which have been reported to exhibit no functional GJIC [36]. SL/DT assay showed that GJIC in chebulinic acid-treated HeLa cells maintained low level similar to in control cells, whereas the exposure of tellimagrandin I promoted GJIC in HeLa cells. Tellimagrandin I-treated cells also showed the ability to transfer calcein to neighboring cells, further validating the effects of tellimagrandin I up-regulating GJIC. Although chebulinic acid and tellimagrandin I both are ellagitannins, their chemical structure and properties show obvious difference (Fig. 1). Both tannins possess a glucose core. However, three galloyl groups and one chebuloyl group link to glucose core in chebulinic acid, and chebulinic acid

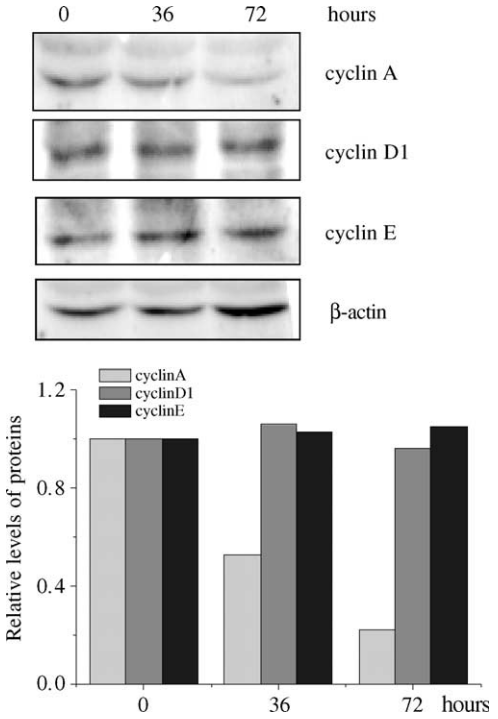


Fig. 7. Effects of tellimagrandin I on protein expression of cyclins in HeLa cells. After HeLa cells were treated with tellimagrandin I 50 μM for indicated times, the protein expression of cyclins was determined with Western blotting.

shows more acidity because of one carboxyl group in chebuloyl group. As to tellimagrandin I, two galloyl groups and one hexahydroxydiphenoyl group link to glucose core with more phenolic hydroxyl groups, so tellimagrandin I demonstrates stronger reduction property. Therefore, the effect difference might be due to the difference on chemical structure and properties.

Several other reports have also demonstrated that GJIC can be induced in HeLa cells, suggesting transcriptional regulation involved in connexin expression in HeLa cells [37,38]. Indeed, HeLa cells and other forms of tumor cells have been reported to be deficient in expression of connexin genes at transcriptional level, whereas connexin genes are rarely mutated in tumors. In our study, RT-PCR and Western Blot analysis demonstrated that treatment with tellimagrandin I resulted in transcription activation of *Cx43* gene and an increase of Cx43 protein in HeLa cells. The results indicated that tellimagrandin I induced a restoration of GJIC through up-regulating the expression of *Cx43* gene.

Recent studies suggested that the methylation of the connexin gene promoter might be a mechanism for the reduced expression of connexin genes frequently observed in neoplastic cells [41–45]. King et al has shown that treatment of Cx43-negative clones from HeLa cells with methylation inhibitor 5-aza-2′-deoxycytidine resulted in expression of *Cx43*, suggesting DNA-methylation involved in modulating the expression of *Cx43* in HeLa cells [17]. Additionally, gene regulation by *trans*-activating factors also appears to be a mechanism regulating the connexin gene promoters. Several functional *cis* elements, including AP-1 and AP-2 response elements, cAMP response element and estrogen response elements, have been described in the human, rat and mouse *Cx43* promoters [46]. Lastly, other mechanisms, including transcript stability, may be important in expression of *Cx43* and other connexin genes. For instance, domains within the 39-untranslated region of the *Cx43* transcript have been identified that control its stability in a hormone-specific manner [47]. Therefore, it will be of interest to understand which mechanisms are responsible for increased expression of *Cx43* gene by tellimagrandin I.

Cx43 and other connexin protein have been indicated to function as a tumor suppressor [14–16]. For HeLa cells, transfection of connexin 43 decreases neoplastic potential as evidenced by attenuated anchorage-independent growth and decreased tumorigenicity in immunodeficient mice [18]. Interestingly, King et al has isolated Cx43-positive clones and Cx43-negative clones from HeLa cells that are previously thought to be completely negative for Cx43 mRNA/protein expression [17]. This endogenous Cx43 expression in HeLa cells was correlated with increased growth control and negatively correlated with neoplastic potential. Consistently, our results show that together with increasing gap junction communication and Cx43 expression, tellimagrandin I significantly decreases the cell proliferation and anchorage-independent growth of HeLa cells.

The inhibition of cell proliferation and anchorage-independent growth of HeLa cells is likely due to a block of cell cycle progression, because tellimagrandin I decreased the percentage of cells in the G0/G1 and G2/M phases coinciding with an obvious increase in the percentage of cells in the S phase. In fact, previous studies have indicated that the re-establishment of gap junctions with the restoration of gap junctional communication affects the phenotype of transformed or tumor cells involved in cell cycle regulation. Transfection and overexpression of *Cx43* gene reverted phenotypically transformed dog kidney epithelial cells to a flat morphology and restored the cells sensitive to density-dependent inhibition of proliferation with their G1 and S phase duration almost doubled [48]. Another study showed that overexpression of Cx43 suppressed proliferation of human osteosarcoma U2OS cells through inhibition of the cell cycle transition from G1 to S phase [49]. Tolbutamide, a sulfonylurea compound, has been shown to enhance gap junction permeability in the poorly coupled C6 glioma cells with an increase of Cx43 expression [50]. The increase in communication is concurrent with the inhibition of the rate of proliferation due to a block of the progression of C6 glioma cells through the S phase of the cell cycle. These Cx43-induced effects on cell cycle progression were coupled with a decreased expression of specific cell cycle regulatory genes including cyclin A, D1, D2, and the cyclin-dependent kinase (CDK) 5 and CDK6 [48]. Consistently, the accumulation of cells in S phase induced by tellimagrandin I was coupled with a decreased expression of cyclin A that was critical to the progression of S phase. Further study will be conducted to elucidate how tellimagrandin I regulates the cyclin A expression and whether other mechanisms involve in the effects of tellimagrandin I on cell cycle progression.

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