Tropical Journal of Pharmaceutical Research July 2017; 16 (7): 1615-1620
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v16i7.21

Original Research Article

Inhibitory effect of puerarin on proliferation of retinoblastoma cells: An in-vitro study

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Sent for review: 19 June 2016 *Revised accepted: 9 June 2017*

Abstract

Purpose: *To investigate the anti-proliferative effect of puerarin on retinoblastoma cells.*
Methods: *The effect of puerarin was examined on human retinoblastoma Y79 cells using cell proliferation assays and reverse transcription-polymerase chain reaction (RT-PCR). The effect of puerarin on the cell cycle was also investigated. Western blot and RT-PCR analyses were also performed to identify the putative mechanism of action.*
Results: *The results showed that cell viability was suppressed by puerarin in a concentration-dependent manner with a half-maximal inhibitory concentration (IC_{50}) of 0.184 ± 0.034 µmol/L. Moreover, puerarin increased the proportion of cells in G1 phase from 42.6 ± 3.1 to 62.83 ± 4.1, 75.76 ± 3.4 and 91.33 ± 5.1 % in a concentration-dependent manner at concentrations of 0.1, 0.2, and 0.4 µmol/L, respectively. The results also indicate that Bmi-1 mRNA and protein levels decreased after puerarin treatment. Additionally, flow cytometry data showed that Bmi-1 knock-down through siRNA resulted in G1-cell cycle arrest. The proportion of cells in G1 were 51.2 ± 2.5 and 71.4 ± 4.5 % for control and Bmi-1 siRNA-treated groups, respectively.*
Conclusions: *The results show that puerarin exert suppressive effects on human retinoblastoma Y79 cells and therefore may find application in the treatment of intraocular tumor.*

Keywords: *Cancer, Puerarin, Retinoblastoma Y79 cells, mTOR inhibition, Intraocular tumor*

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INTRODUCTION

Cancer is one of the deadliest diseases known to mankind, and despite improvements in treatment remains a leading cause of death worldwide [1,2]. Retinoblastoma is a malignant intraocular tumor that affects infants and children [3]. If undiagnosed or untreated, patients with retinoblastoma die within two years due to intracranial extensions of the tumor [4]. The primary treatment consists of chemo-reduction with local consolidation with or without systemic chemotherapy [5]. However, all available therapies have major limitations and the use of chemotherapy can be challenging in pediatric patients [6]. Consequently, there is a pressing need to develop new therapeutic options to improve the outcomes of patients diagnosed with retinoblastoma. Natural products or derivatives are often good sources of agents that associated are with numerous biological activities [7]. These derivatives have high therapeutic activity due to their high chemical and molecular diversity. They are also associated with biochemical specificity and optimal molecular characteristics that make them promising targets for future drug discovery [8]. Out of the millions of naturally occurring compounds, only a few have been developed for use as cancer therapies [9]. These include vinca alkaloids [10], paclitaxel, docetaxel [11],

etoposide, teniposide [12], and camptothecin and camptothecin derivatives, topothecan, irinothecan [13], and anthracycline [14].

Therefore, we aimed to elucidate the anti-proliferative effects of Puerarin on retinoblastoma cells [15]. Puerarin is a major bioactive ingredient obtained from the *Puerarialobata* (Willd.) *Ohwi* root [16]. It is widely known as *Gegen* in traditional Chinese medicine.

EXPERIMENTAL

Chemicals

Puerarin (98 %), rapamycin, penicillin, streptomycin, propidium iodide (PI), and other chemicals were purchased from Sigma Chemical Co (Missouri, US).

Reagents

Roswell Park Memorial Institute (RPMI) 1640 medium was purchased from Gibco BRL (Waltham, US), fetal bovine serum (FBS) was obtained from Life Technologies Corporation (Shanghai, China), and cell counting kit-8 (CCK-8) was obtained from Beyotime Corporation (Jiangsu, China). Mammalian target of rapamycin (mTOR), phosphorylated-mTOR (p-mTOR), cyclin E1, Bmi-1, and β-actin antibodies were procured from Cell Signaling Technology Inc (Shanghai, China). Primer sequences, including specific siRNA sequences for Bmi-1 and nonspecific control siRNA, were designed and developed by GenePharma Co. Ltd (Shanghai, China). An Avian Myeloblastosis Virus (AMV) reverse transcriptase system was purchased from Promega (Wisconsin, United States).

Cell lines and cell culture

The human retinoblastoma cell line Y79 (ATCC, USA) was cultured in RPMI 1640 medium supplemented with 10 % FBS, 1 % penicillin, and streptomycin at 37 °C in a humidified incubator containing 5 % CO_2.

Cell proliferation assays

To determine cell proliferation, the CCK-8 assay was used to assess cell viability. Briefly, the cells were seeded in 96-well plates with an average of 3, 000 cells per well for 24 h. Ten microliters of rapamycin was then diluted with culture medium and different concentrations of puerarin were added to the wells for an additional 48 h. After that, 10 µL of CCK-8 solution was added to each well and incubated at 37 °C for an additional 2 h. The optical density (OD) value of the solution was then evaluated at 450 nm, and the IC_{50} values were calculated from the survival curve. The entire experiment was replicated three times.

Puerarin effect on the cell cycle

Briefly, Y79 cells were treated with different concentrations of puerarin for 48 h. The treated cells were then trypsinized, washed in PBS, and fixed in ice-cold 75 % ethanol/PBS. The resulting cells were stained with Propidium Iodide (PI) for 30 min and were analyzed by flow cytometry (Beckman Coulter, Inc). The corresponding cell cycle profiles were determined using MultiCycle AV software. All experiments were replicated three times.

Western blot analysis

Briefly, Y79 cells were treated with various concentrations of puerarin for 48 h, and then lysed using cold lysis buffer. The resulting cell lysates were centrifuged at 10, 000 rpm for 20 min, and the supernatant was collected. Fifty micrograms of protein lysate was then separated using an 8-12 % SDS-PAGE gel and later transferred onto a PVDF membrane (Millipore, USA). The loaded membrane was blocked with 5 % non-fat milk powder (w/v) in tris-buffered saline with tween (TBST) (10 mm Tris, 100 mm NaCl, 0.1 % Tween 20) for 2 h, and then incubated with primary antibodies at 4 °C overnight. Subsequently, suitable HRP-linked secondary antibodies were added and incubated for an additional 1 h. Lastly, Western Blotting Luminal Reagent (Millipore) was used for the detection of specific protein bands using β-actin as an endogenous control. All experiments were replicated three times.

Reverse transcription-PCR

Y79 cells were treated with the desired concentrations of puerarin for 48 h before extracting total RNA. The single-stranded cDNA was reverse transcribed using the AMV Reverse Transcriptase System, and the PCR reactions were performed using an ABI Prism 9700 PCR System. For cDNA development, 35 cycles (94 °C; 30 s, 60 °C; 30 s, and 72 °C; 1 min) were used and the PCR primers selected were 5'-GTA TTC CCT CCA CCT CTT CTT G-3' (forward) and 5'-TGC TGA TGA CCC ATT TAC TGA T-3' (reverse) for Bmi-1, and 5'-GGG ACC TGA CTG ACT ACC TCA-3' (forward) and 5'-GAC TCG TCA TAC TCC TGC TTG-3' (reverse) for β-actin (control), respectively. The reactions were replicated three times.

Bmi-1 siRNA studies

siRNA was transfected using Lipofectamine 2000 in accordance with the manufacturer's instructions. Briefly, 5×10^5 Y79 cells were plated in six-well plates and transfected with 50 nmol/L Bmi-1 or negative control siRNA duplex components using 5 µL Lipofectamine 2000 per well. The cells were incubated for 48 h, and the total extracted protein was subjected to Western blot analysis for Bmi-1.

Statistical analysis

Data were statistically analyzed using Graphpad Prism software. Independent t-test was used between two groups, and comparisons among multiple groups were performed using a one-way analysis of variance (ANOVA) followed by Dunnett's test. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of puerarin on the proliferation of human retinoblastoma Y79 cells

In this assay, we investigated the effect of puerarin on the proliferation of human retinoblastoma Y79 cells. We treated the cells with various concentrations of puerarin, (0.1, 0.2, and 0.4 µmol/L) for 48 h using a CCK-8 assay. The results show that cell viability was suppressed by puerarin in a concentration-dependent manner. The IC_{50} of puerarin (0.184 ± 0.034 µmol/L) further indicated the potent inhibitory potential of puerarin on human retinoblastoma Y79 cells.

Effect of puerarin on the cell cycle in human retinoblastoma Y79 cells

In this assay, Y79 cells were treated with or without puerarin for 48 h. The puerarin concentrations used were: 0.1, 0.2, and 0.4 µmol/L. After treatment, the cells were fixed, nuclear DNA was stained with PI, and flow cytometry was used to determine the population of cells in each cell cycle stage. As shown in Figure 1A, the percentage of cells in G1 and S increased and decreased, respectively, after treatment with puerarin. As shown in Figure 1, the percentage of cells in G1 increased from 42.6 ± 3.1 to 62.83 ± 4.1, 75.76 ± 3.4 and 91.33 ± 5.1 % at concentrations of 0.1, 0.2, and 0.4 µmol/L, respectively (Figure 1B). The results suggest that puerarin results in G1 arrest in a time-dependent manner.

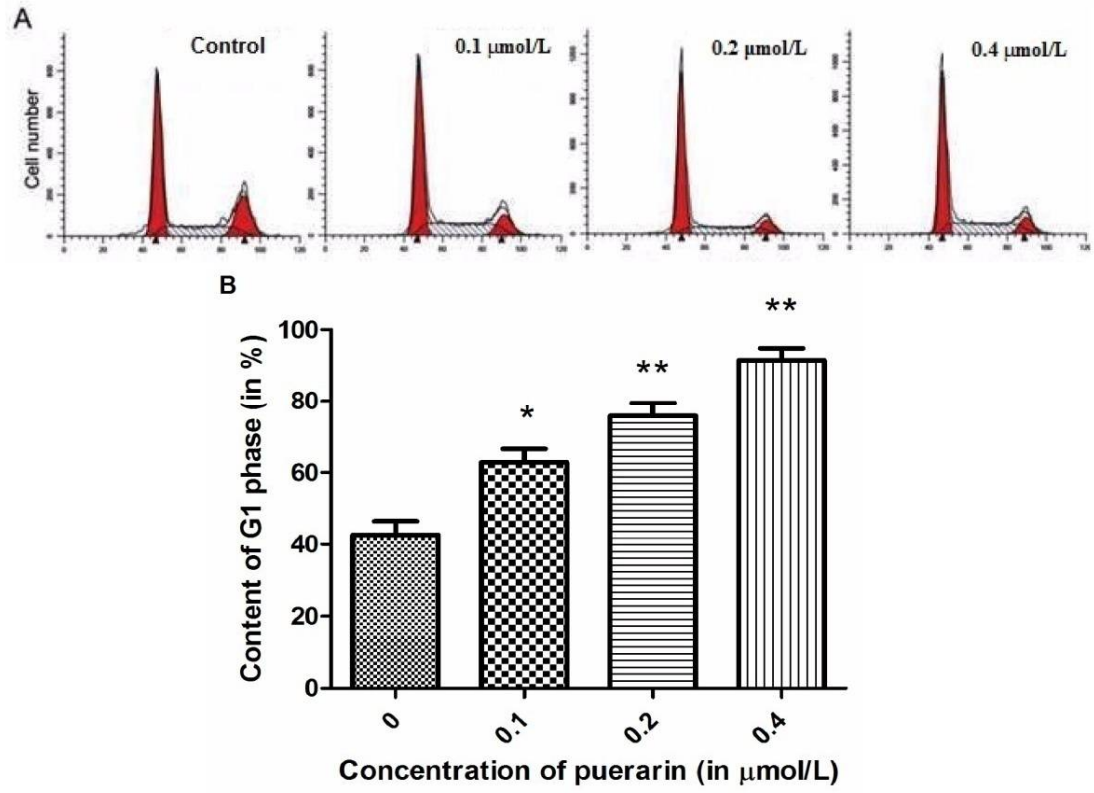

Figure 1: Effect of puerarin on cell cycle phase in human retinoblastoma Y79 cells;*$p < 0.05$; **$p < 0.01$ compared with respective controls (n = 3)

Effect of puerarin on mTOR phosphorylation in human retinoblastoma Y79 cells

The role of mTOR in the progression of human cancers has been shown and reviewed in multiple previous studies. Thus, in this study, we analyzed the effect of puerarin on mTOR protein levels in human retinoblastoma Y79 cells by Western blot analysis. As depicted in Fig. 2A, administration of puerarin resulted in decreased phosphorylation of mTOR (p-mTOR) and had no effect on overall mTOR levels. The results of the Western blot analysis confirmed that puerarin effectively attenuated the mTOR pathway signaling in Y79 cells.

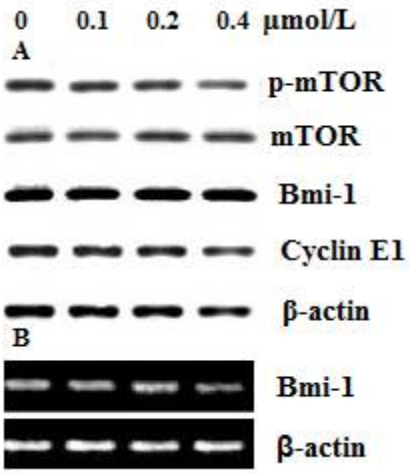

Figure 2: Effect of puerarin on protein expression determined by Western blot analysis. A) Inhibition of the mTOR pathway via puerarin and reduction of BMI-1 and cyclin E1 proteins. B) Puerarin decreased mRNA expression of Bmi-1.

Effect of puerarin on Bmi-1 and cyclin E1 protein levels

Various studies have described the role of cyclin E1 in the progression of G1/S phase and the downregulation of this action through the mTOR pathway [17]. Therefore, we examined the effect of puerarin on cyclin E1 expression by Western blot analysis (Fig. 2A). The results show that puerarin results in cyclin E1 downregulation. The results also indicate that Bmi-1 protein and mRNA levels were reduced after treatment with puerarin. This suggests that the inactivation of Bmi-1 and downregulation of cyclin E1 could be responsible for mTOR inhibition.

Bmi-1 siRNA reduced cyclin E1 levels and subsequently amplified the percentage of cells in G1 phase

Lastly, we investigated the effect of Bmi-1 inhibition on human retinoblastoma Y79 cell viability. The cells were transfected with specific Bmi-1 siRNA and negative control siRNA. As shown in Fig. 3A, cyclin E1 levels decreased after treatment with Bmi-1 siRNA. This suggests that cyclin-E1 suppression is mediated via Bmi-1. Furthermore, Figure 3B shows that attenuation of Bmi-1 resulted in G1-cell cycle arrest. Flow cytometry data revealed that 51.2 ± 2.5 and 71.4 ± 4.5 % of cells were in G1 phase before and after Bmi-1 siRNA treatment, respectively.

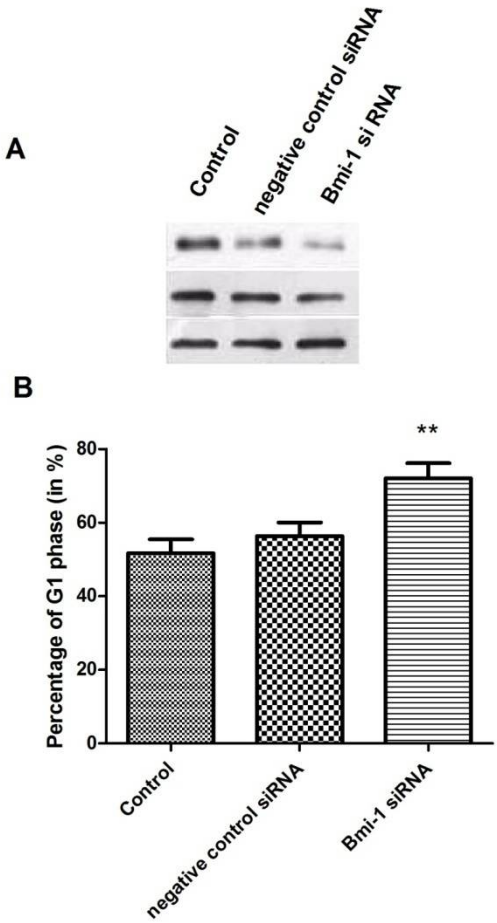

Figure 3: Reduction of Bmi-1 reduced cyclin E1 expression and increased the number of cells in G1. A. Knock-down of Bmi-1 reduced cyclin E1 using negative control siRNA. B. Depletion of Bmi-1 increased the numbers of cells in G1 phase. ** $p < 0.01$ compared with control (n = 3)

DISCUSSION

Since puerarin was first isolated from *Gegen* in 1950, its pharmacological activity has been widely investigated. Puerarin treatment has been shown to have beneficial effects on cardiovascular disease, CNS disorders, Parkinson's disease (PD), Alzheimer's disease (AD), cancer, and diabetes, and to suppress serum ALT and AST levels and Bcl-2 mRNA

expression [16, 18]. Moreover, it has also been found to stimulate apoptosis in activated hepatic stellate cells in rats with liver fibrosis induced with CCl_4. The viability of acute myeloid leukemia cell lines, including U937, Kasumi-1, HL-60, and NB4 cells, was considerably decreased after treatment with puerarin via inhibition of the cell cycle. Further studies confirmed the role of puerarin in the reduction of motility migration, adhesion, and invasion of human HO-8910 omophoria cells [19]. More recently, puerarin has been shown to decrease the apoptosis of retinal pigment epithelial cells by preventing damage in peroxynitrites [20]. Thus, the effect of puerarin on retinoblastoma cells and the possible mechanisms of action was explored [15].

Retinoblastoma is a rare form of cancer that affects the retina in children. Unfortunately, we are still facing challenges and hurdles in treating this disease effectively. Therefore, it is necessary to find novel agents for retinoblastoma management. The initial part of this study was aimed at defining the effect of puerarin on the cellular viability of human retinoblastoma Y79 cells. The results showed that cell viability was significantly diminished after puerarin treatment in a concentration-dependent manner. The results confirmed that puerarin has anti-proliferative effects. Next, we investigated the effect of puerarin on the cell cycle. The flow cytometry results suggest that puerarin significantly increases the number of Y79 cells in G1 phase in a concentration-dependent manner.

Cyclins (cyclins D and E), which are activated through cyclin-dependent kinase (Cdk) enzymes, play a vital role in the progression of the cell cycle. The E type cyclins (E1 and E2) initiate entry into S-phase and are required for G1/S cell cycle development. Therefore, inhibition of E cyclins are a potentially lucrative target for inhibition of tumorigenesis in human retinoblastoma Y79 cells. We found that puerarin inhibits the expression of cyclin E1. Various studies have confirmed the role of the signaling pathway in the down regulation of cyclin E1 [17]. Therefore, we investigated the effect of puerarin on mTOR expression.

mTOR plays a critical role in the metabolism, growth, and proliferation of cells, and is a novel target for anticancer agents. The results of this study show that puerarin inhibited the growth of human retinoblastoma Y79 cells by downregulating Bmi-1 protein and mRNA through mTOR inhibition. These results are similar to those reported in previous studies, and suggest that puerarin can inhibit the progress of retinoblastoma through inhibition of mTOR.

CONCLUSION

This study showed that puerarin has beneficial effects on human retinoblastoma Y79 cells. Downregulation of Bmi-1 protein and mRNA expression through mTOR inhibition may be a possible mechanism for the anti-proliferative effect of puerarin in human retinoblastoma Y79 cells. The findings also indicate that puerarin may find therapeutic.

DECLARATIONS

Acknowledgement

None declared.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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