Department of Biochemistry[1], Chung-Ang University College of Medicine, Seoul, Republic of Korea; Pharmacy Practice[2], Thomas J. Long School of Pharmacy, University of the Pacific, Stockton, CA, USA

Arbutin inhibits TCCSUP human bladder cancer cell proliferation via up-regulation of p21

HAILAN LI[1], YUN-MI JEONG[1], SU YEON KIM[1], MYO-KYOUNG KIM[2], DONG-SEOK KIM[1]

Received September 20, 2010, accepted October 22, 2010

Dong-Seok Kim, Ph.D., Department of Biochemistry, Chung-Ang University College of Medicine, 221 Heukseok-dong Dongjak-gu, Seoul 156-756, Korea
ds_kim@cau.ac.kr

Pharmazie 66: 306–309 (2011) *doi: 10.1691/ph.2011.0785*

Arbutin is a glycosylated hydroquinone extracted from the bearberry plant (*Arctostaphylos* species). In the present study, we determined the effects of arbutin on TCCSUP human bladder carcinoma cell proliferation. Arbutin did not exhibit any cytotoxic effects in TCCSUP cells at concentrations of < 500 μg/ml. To determine the effects of arbutin on cell proliferation, TCCSUP cells were treated with arbutin at various concentrations, and the cell proliferation was measured using the MTT assay. Arbutin significantly decreased TCCSUP cell proliferation in a concentration- and time-dependent manner. Furthermore, cell cycle analysis revealed that arbutin strongly disrupted the cell cycle in a time-dependent manner. Western blot analysis demonstrated that arbutin led to the inactivation of extracellular signal-regulated kinase (ERK), which is known to critically regulate cell proliferation. In addition, arbutin markedly increased the expression of $p21^{WAF1/CIP1}$ (p21), which is known to be highly involved in cell cycle regulation. Therefore, this study suggests that arbutin inhibits TCCSUP cell proliferation via ERK inactivation and p21 up-regulation.

1. Introduction

Arbutin (hydroquinone-*O*-β-D-glucopyranoside) is a substance of the bark and leaves of the bearberry plant (genus *Arctostaphylos*) (Blaut et al. 2006). Arbutin is known to inhibit tyrosinase, the rate-limiting enzyme of mammalian melanogenesis (Maeda and Fukuda 1996; Sugimoto et al. 2004). Thus, arbutin is widely used as a skin-whitening cosmetic agent for the treatment of cutaneous hyperpigmentary disorders, such as melasma and freckles (Ertam et al. 2008; Maeda and Fukuda 1996). Moreover, arbutin has been shown to be safe and mild in humans through clinical use.

Bladder cancer is one of the five most common malignant cancers in industrialized countries (Zieger 2008). At all stages, bladder cancer is capable of rapid tumor progression and metastasis within a few months. Due to the high recurrence rates for bladder cancer, surgery or external beam radiation therapy has been the mainstay of treatment options. However, these radical treatments cause severe complications, treatment-related morbidity, and a reduced quality of life. Furthermore, many elderly patients with bladder cancer are not generally suitable for these procedures (Zieger 2008). Therefore, the development of novel, molecular-targeted therapeutics is urgently needed.

It has recently been suggested that arbutin has a potential role as an anti-tumor agent from the microarray results of A375 human malignant melanoma cells treated with arbutin (Cheng et al. 2007). Moreover, it is well known that *Arctostaphylos uva-ursi* extract has been used in traditional medicine to treat urinary tract infections (Schindler et al. 2002). To examine the possible use of arbutin for bladder cancer, we investigated the effects of arbutin on TCCSUP human bladder carcinoma cell proliferation.

It is generally accepted that activation of the extracellular signal-regulated protein kinase (ERK) signaling pathway plays a critical role in mitogenic signaling of a number of growth factors (Blenis 1993; Blumer and Johnson 1994). Therefore, we determined whether or not arbutin has an influence on ERK activation.

D-type cyclins, such as cyclin D_1 are known to be involved in cell cycle progression (Blagosklonny and Pardee 2002; Prietzsch et al. 2002). Thus, the cyclin-dependent kinase inhibitor, $p21^{WAF1/CIP1}$ (p21), leads to cell growth inhibition via cell cycle arrest (Gartel and Tyner 2002; Tang et al. 2002; Weiss and Randour 2000). To examine the mechanisms of arbutin, we also studied changes in the expression of cell cycle-related proteins.

2. Investigations and results

2.1. Arbutin did not exhibit cytotoxicity against TCCSUP human bladder cancer cells

The chemical structure of arbutin is shown in Fig. 1. To examine the cytotoxicity of arbutin on cell viability, TCCSUP cells were treated with arbutin at 0–500 μg/ml for 24 h. Cell viability was assessed by crystal violet staining. Arbutin did not exhibit cytotoxic effects against TCCSUP cells (Fig. 2).

2.2. Effects of arbutin on TCCSUP cell proliferation

Cell proliferation was examined in the presence of 100 μg/ml of arbutin for 4 days by the MTT assay. Arbutin was shown to inhibit the proliferation of TCCSUP cells in a time-dependent manner. Proliferation was inhibited approximately 40% after

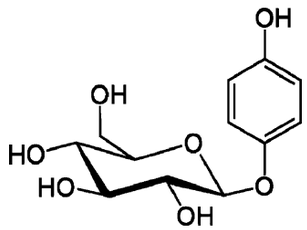


Fig. 1: The chemical structure of arbutin.

4 days of treatment with arbutin (Fig. 3A). In addition, arbutin inhibited TCCSUP cell proliferation in a dose-dependent fashion (Fig. 3B).

2.3. Effects of arbutin on the TCCSUP cell cycle

Cell cycle changes were analyzed by flow cytometry. Cell cycle disruption was observed when TCCSUP cells were treated with arbutin (Fig. 4). This finding suggests that arbutin has an influence on the cell cycle and subsequently inhibits cell growth. To examine the signal transduction pathway by which arbutin inhibits TCCSUP cell proliferation, we studied the activation of the ERK pathway in a time course experiment. Western blot assay showed that 100 μg/ml of arbutin inactivated ERK phosphorylation in TCCSUP cells (Fig. 5A). To further investigate the mechanism of the anti-proliferative effect of arbutin, levels of cell cycle-related proteins, such as cyclin D_1 and p21, were examined by Western blot analysis. As shown in Figure 5B, arbutin treatment increased the levels of p21, whereas arbutin had little effect on the level of cyclin D_1.

3. Discussion

Arbutin is widely used as a safe and mild skin-whitening agent because it inhibits tyrosinase, the rate-limiting enzyme of melanin synthesis. However, other effects of arbutin have not been thoroughly studied. It has been reported that quinones, including arbutin, showed growth inhibitory effects on HCT-15 human colon carcinoma cells (Kamei et al. 1998). Similarly, we also showed that arbutin inhibited the proliferation of TCCSUP human bladder carcinoma cells. These studies showed that arbutin exerts an anti-proliferative activity in some cancer cells; however, the mechanism of arbutin on cell growth inhibition was not investigated.

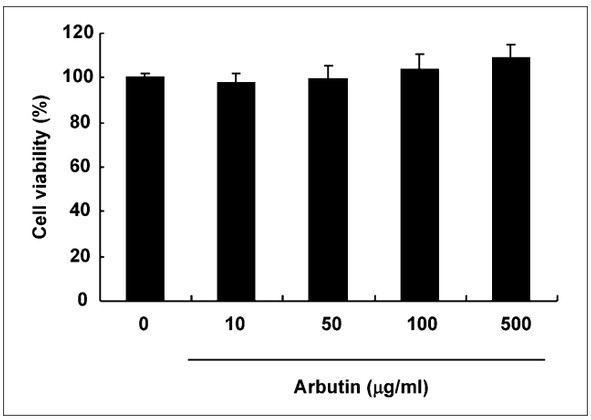
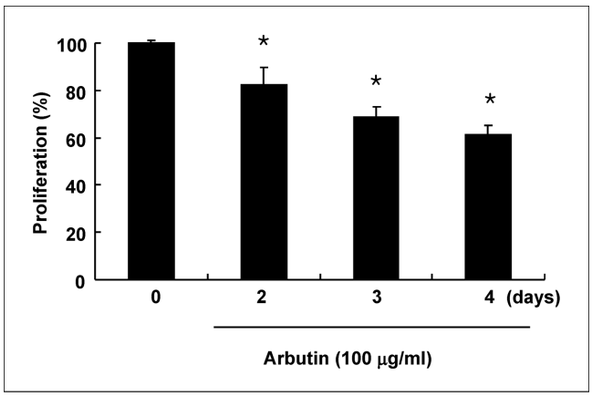
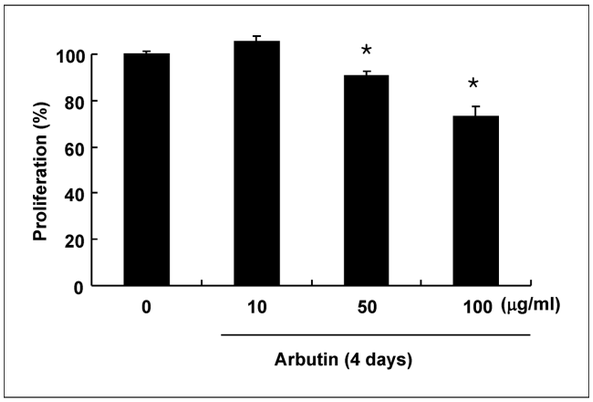


Fig. 2: Effects of arbutin on TCCSUP cell viability. Cells were treated with arbutin at 0–500 μg/ml. After 24 h, cell viability was measured by the crystal violet staining assay. The data represent the means ± S.D. of triplicate assays expressed as percentages of the control.



(A)



(B)

Fig. 3: Effects of arbutin on TCCSUP cell proliferation. (A) Cells were treated with 100 μg/ml of arbutin for 0–4 days. (B) Cells were treated with 0–100 μg/ml of arbutin for 4 days. Cell proliferation was measured using the crystal violet assay after 24 h of treatment, as described in the Experimental section. Data represent the means ± S.D. of triplicate assays expressed as percentages of the control. Each experiment was repeated independently at least twice, and the representative results are shown. * $P<0.01$.

The ERK signaling pathway is known to play a critical role in cellular proliferation (Kiely et al. 2002; Pebay et al. 2001). Thus, we determined whether or not arbutin inhibits cell growth via the regulation of the ERK pathway in TCCSUP cells. Our results showed that arbutin significantly inhibited ERK phosphorylation. As ERK is a major mitogenic signal (Davis 1993; Kim et al. 2006), inactivation of the ERK pathway by arbutin appears to be accountable for its anti-proliferative effects.

Cyclin D_1 is a rate-limiting and critical factor for cell cycle progression through the G_1 phase (Blagosklonny and Pardee 2002; Prietzsch et al. 2002). Additionally, the cyclin-dependent kinase inhibitor, p21 is also highly involved in cell cycle regulation. Thus, the increased expression of p21 may induce cell cycle arrest and subsequent cell growth inhibition (Gartel and Tyner 2002; Tang et al. 2002; Weiss and Randour 2000). The present study showed that arbutin treatment increased p21 expression in a time-dependent manner, whereas arbutin had little effect on the level of cyclin D_1 protein. Moreover, arbutin induced time-dependent cell cycle disruption. These results suggest that p21 up-regulation is related to arbutin-induced growth inhibition.

In summary, we investigated the anti-proliferative effects of arbutin and the mechanism of action. This study demonstrated that arbutin inhibits TCCSUP human bladder carcinoma cell proliferation as a result of ERK inactivation and p21 up-regulation. Therefore, further investigation of arbutin is warranted to develop arbutin for a potential new bladder cancer therapy with safety.

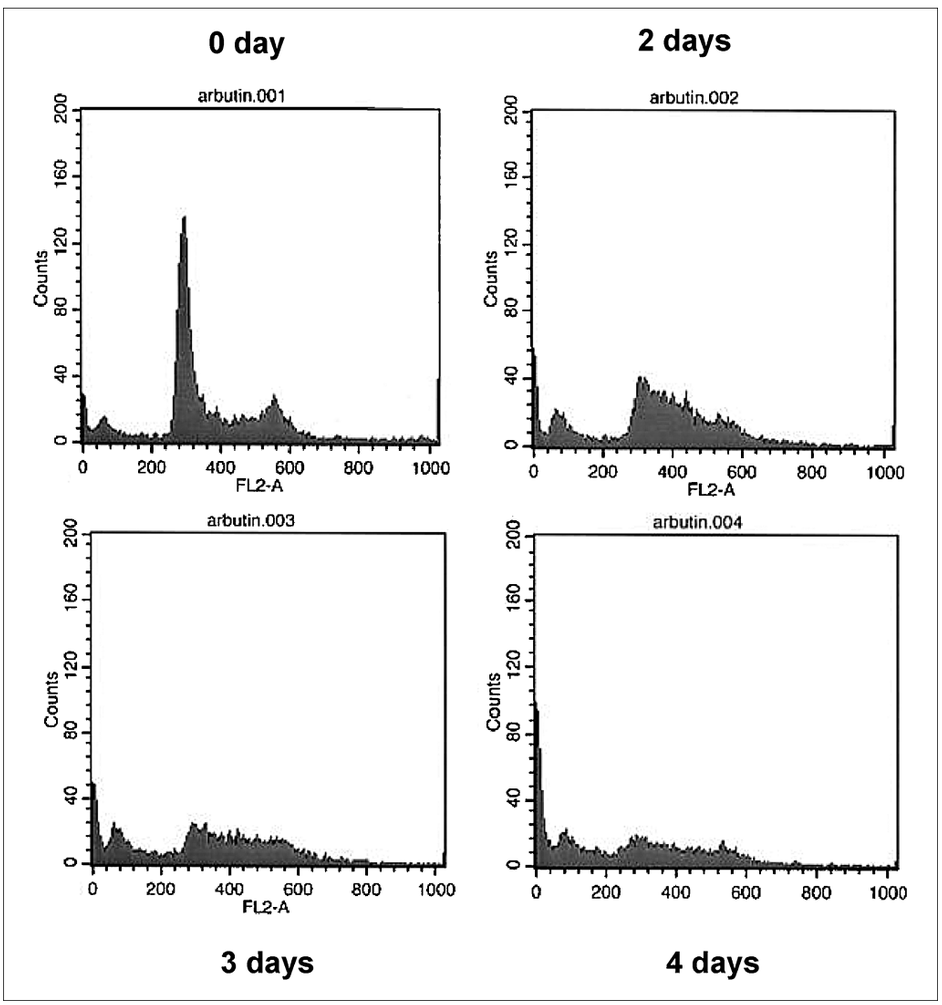


Fig. 4: Effects of arbutin on the TCCSUP cell cycle. Cells were treated with 100 μg/ml of arbutin for 0–4 days. Cells were then analyzed by flow cytometry, as described in the Experimental section.

4. Experimental

4.1. Materials

Arbutin (Fig. 1) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were obtained from Sigma (St. Louis, MO, USA). Antibodies against phospho-ERK (CST-9101) and total ERK (CST-9102) were obtained from Cell Signaling (Danvers, MA, USA). Antibodies against cyclin D_1 (sc-718), p21 (sc-397), and actin (sc-1616) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

4.2. Cell cultures

TCCSUP human urinary bladder carcinoma cells were obtained from ATCC (Rockville, MD, USA). The cells were grown in minimum essential medium (MEM) supplemented with 10% FBS, 50 μg/ml of streptomycin, and 50 μg/ml of penicillin at 37 °C in 5% CO_2.

4.3. Crystal violet assay for cell viability

Cells were observed under a phase contrast microscope (Olympus Optical Co., Tokyo, Japan) and photographed using a DCM300 digital camera for a microscope (Scopetek, Inc., Hangzhou, China), which was supported by ScopePhoto software (Scopetek, Inc.). Cell viability was assessed using the crystal violet staining assay (Jeong et al. 2009). After treating cells with arbutin for 24 h, the culture medium was removed. Cells were stained with 0.1% crystal violet in 10% ethanol for 5 min at room temperature, then rinsed 4 times with distilled water. The crystal violet retained by the adherent cells was extracted with 95% ethanol, and the absorbance was determined in lysates at 590 nm using an ELISA reader (VERSAMax; Molecular Devices, Sunnyvale, CA, USA).

4.4. MTT assay for cell proliferation

Cells (5×10^4 cells/well), seeded into 12-well plates for 24 h, were incubated with various concentrations of arbutin (0–100 μg/ml) in MEM containing 10% FBS at 37 °C in 5% CO_2 for 4 days, or treated with 100 μg/ml arbutin for 0–4 days. After adding 100 μl/well of MTT solution (5 mg/ml), the plates were incubated for another 4 h. Supernatants were then removed and the formazan crystals were solubilized in 1 ml of dimethylsulfoxide. Optical density was determined at 540 nm using a VERSAMax.

4.5. Cell cycle analysis

Cells were trypsinized, adjusted to 5×10^5–1×10^6 cells/tube, washed with ice-cold phosphate-buffered saline (PBS), and re-suspended in 2 ml of ethanol. After incubation at 4 °C for 1 h, the ethanol was removed, and 100 μl of ribonuclease solution (10 mg/ml) was added to each test tube. The tubes were then re-incubated at room temperature for 30 min, and 500 μl of analysis solution (37 mM EDTA and 0.1% Triton X-100 in PBS) and 100 μl of propidium iodide solution (400 μg/ml) were then added. Samples were stored in the dark at 4 °C and analyzed using a flow cytometer (FACSCalibur^{TM}, Becton Dickinson, San Jose, CA, USA).

4.6. Western blotting

Cells were grown in 60-mm culture dishes, serum-starved for 24 h, and treated with arbutin at the indicated time points. Cell lysates were prepared in M-PER mammalian protein reagent (Pierce, Rockford, IL, USA) containing a complete protease inhibitor mixture (Roche, Mannheim, Germany). Samples were separated on 12% SDS-polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes, which were blocked with 5% dried milk in PBS containing 0.5% Tween 20. The blots were incubated with

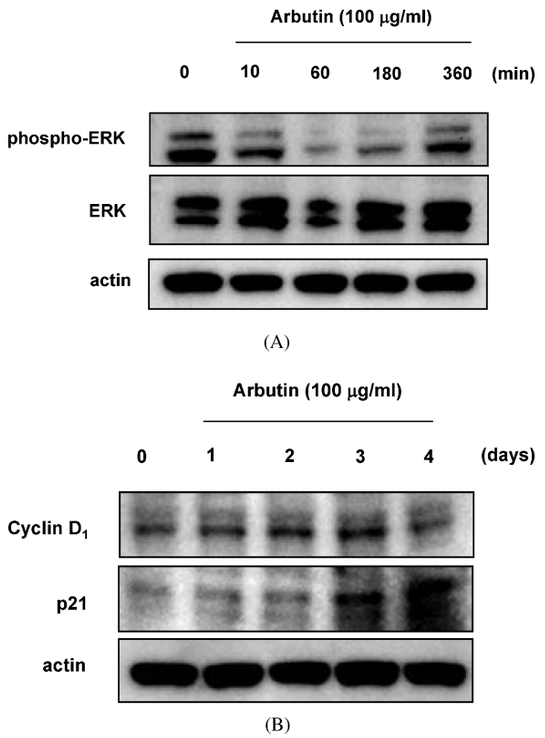


Fig. 5: Effects of arbutin on the ERK pathway and cell cycle-related proteins. TCCSUP cells were treated with 100 μg/ml of arbutin for the indicated times. Whole cell lysates were then subjected to Western blot analysis with antibodies against phospho-specific ERK (A), cyclin D_1, and p21 (B). Equal protein loading was confirmed using anti-actin antibody.

the appropriate primary antibodies at a dilution of 1:1000. Membrane-bound primary antibodies were detected using secondary antibodies conjugated with HRP and chemiluminescent substrate (Pierce). The images of the blotted membranes were obtained using a LAS-1000 lumino-image analyzer (Fuji Film, Tokyo, Japan).

4.7. Statistics

The statistical significance of the differences among groups was tested using the ANOVA and Tukey's HSD test, after testing for normality. P values < 0.05 were considered significant.

References

Blagosklonny MV, Pardee AB (2002) The restriction point of the cell cycle. Cell Cycle 1: 103–110.

Blaut M, Braune A, Wunderlich S, Sauer P, Schneider H, Glatt H (2006) Mutagenicity of arbutin in mammalian cells after activation by human intestinal bacteria. Food Chem Toxicol 44: 1940–1947.

Blenis J (1993) Signal transduction via the MAP kinases: proceed at your own RSK. Proc Natl Acad Sci U S A 90: 5889–5892.

Blumer KJ, Johnson GL (1994) Diversity in function and regulation of MAP kinase pathways. Trends Biochem Sci 19: 236–240.

Cheng SL, Liu RH, Sheu JN, Chen ST, Sinchaikul S, Tsay GJ (2007) Toxicogenomics of A375 human malignant melanoma cells treated with arbutin. J Biomed Sci 14: 87–105.

Davis RJ (1993) The mitogen-activated protein kinase signal transduction pathway. J Biol Chem 268: 14553–14556.

Ertam I, Mutlu B, Unal I, Alper S, Kivcak B, Ozer O (2008) Efficiency of ellagic acid and arbutin in melasma: a randomized, prospective, open-label study. J Dermatol 35: 570–574.

Gartel AL, Tyner AL (2002) The role of the cyclin-dependent kinase inhibitor p21 in apoptosis. Mol Cancer Ther 1: 639–649.

Jeong YM, Lee JE, Kim SY, Yun HY, Baek KJ, Kwon NS, Kim DS (2009) Enhanced effects of citrate on UVB-induced apoptosis of B16 melanoma cells. Pharmazie 64: 829–833.

Kamei H, Koide T, Kojima T, Hashimoto Y, Hasegawa M (1998) Inhibition of cell growth in culture by quinones. Cancer Biother Radiopharm 13: 185–188.

Kiely PA, Sant A, O'Connor R (2002) RACK1 is an insulin-like growth factor 1 (IGF-1) receptor-interacting protein that can regulate IGF-1-mediated Akt activation and protection from cell death. J Biol Chem 277: 22581–22589.

Kim AL, Zhu Y, Zhu H, Han L, Kopelovich L, Bickers DR, Athear M (2006) Resveratrol inhibits proliferation of human epidermoid carcinoma A431 cells by modulating MEK1 and AP-1 signalling pathways. Exp Dermatol 15: 538–546.

Maeda K, Fukuda M (1996) Arbutin: mechanism of its depigmenting action in human melanocyte culture. J Pharmacol Exp Ther 276: 765–769.

Pébay A, Toutant M, Premont J, Calvo CF, Venance L, Cordier J, Glowinshi J, Tencé H (2001) Sphingosine-1-phosphate induces proliferation of astrocytes: regulation by intracellular signalling cascades. Eur J Neurosci 13: 2067–2076.

Prietzsch H, Brock J, Kleine HD, Liebe S, Jaster R (2002) Interferon-alpha inhibits cell cycle progression by Ba/F3 cells through the antagonisation of interleukin-3 effects on key regulators of G(1)/S transition. Cell Signal 14: 751–759.

Schindler G, Patzak U, Brinkhaus B, von Niecieck A, Wittig J, Kráhmer N, Glőckl J, Veit M (2002) Urinary excretion and metabolism of arbutin after oral administration of Arctostaphylos uvae ursi extract as film-coated tablets and aqueous solution in healthy humans. J Clin Pharmacol 42: 920–927.

Sugimoto K, Nishimura T, Nomura K, Kuriki T (2004) Inhibitory effects of alpha-arbutin on melanin synthesis in cultured human melanoma cells and a three-dimensional human skin model. Biol Pharm Bull 27: 510–514.

Tang PP, Hsieh SC, Wang FF (2002) Modulation of caspase activation and p27(Kip1) degradation in the p53-induced apoptosis in IW32 erythroleukemia cells. Cell Signal 14: 961–968.

Weiss RH, Randour CJ (2000) The permissive effect of p21(Waf1/Cip1) on DNA synthesis is dependent on cell type: effect is absent in p53-inactive cells. Cell Signal 12: 413–418.

Zieger K (2008) High throughput molecular diagnostics in bladder cancer - on the brink of clinical utility. Mol Oncol 1: 384–394.