

# A Daidzein Metabolite, 6,7,4'-Trihydroxyisoflavone Inhibits Cellular Proliferation through Cell Cycle Arrest and Apoptosis Induction in MCF10CA1a Human Breast Cancer Cells

Jae Hoo Lee · Hong Jin Lee

Received: 12 July 2013 / Accepted: 5 October 2013 / Published Online: 31 December 2013  
© The Korean Society for Applied Biological Chemistry and Springer 2013

**Abstract** Despite recent findings of hepatic daidzein metabolites on prevention of skin and colon cancers, little study has been performed on breast cancer. In this study, we found that 6,7,4'-trihydroxyisoflavone, one of the major hepatic metabolite of the daidzein more significantly inhibited proliferation of MCF10CA1a human estrogen receptor (ER)-negative breast cancer cells, which was derived from arresting cell cycle at S- and G2/M phase. Cyclins and cyclin-dependent kinases (CDKs) involved in S- and G2/M phases, including cyclins A, B, E, CDK1 and CDK2 were regulated by 6,7,4'-trihydroxyisoflavone as well as CDK inhibitor, p21 and p27, in a dose-dependent manner. In addition, 6,7,4'-trihydroxyisoflavone induced apoptosis by enhancing death receptor4 (DR4) expression and suppressing the X-linked inhibitor of apoptosis protein, leading to poly ADP-ribose polymerase cleavage. Taken together, 6,7,4'-trihydroxyisoflavone inhibits cell proliferation via arresting cell cycle at S- and G2/M phases and inducing apoptosis in MCF10CA1a human breast cancer cells. These results suggest that the hepatic metabolite of daidzein, 6,7,4'-trihydroxyisoflavone, may be considered as a more potent agent in inhibiting ER-negative breast carcinogenesis.

**Keywords** Apoptosis · Breast Cancer · Cell Cycle · Metabolite · Trihydroxyisoflavone

## Introduction

Numerous epidemiological studies have demonstrated that soy food intake is associated with reduced risk of breast cancer (Messina et al., 2006; Qin et al., 2006; Shu et al., 2009), and the isoflavones such as genistein has been considered as potent ingredients working through regulating the estrogenic activity (Pugazhendhi et al., 2008). Shu et al. (2009) also reported that soy consumption decreased mortality and recurrence in both estrogen receptor (ER)-positive and ER-negative breast cancers, suggesting that isoflavones may have regulatory activity of ER-independent intracellular signaling pathway (Taylor et al., 2009; Boucher et al., 2013). In line with this finding, genistein is known to regulate the expression of HER2 to sensitize the effect of tamoxifen and regulate the nuclease factor- $\kappa$ B and Akt signaling pathways to inhibit the cell proliferation and induce apoptosis (Messina et al., 2006; Mai et al., 2007), which eventually suppress the development of breast cancer.

The studies showing beneficial effect of genistein on breast cancer chemoprevention have been significantly accumulated although the results still remain controversial (Taylor et al., 2009; Hilakivi-Clarke et al., 2010). However, daidzein, one of the major isoflavones in soy foods, has been drawn less attention on breast cancer chemoprevention than genistein due to lower activity. For example, genistein exerted more significant inhibition of the estradiol binding to ER from MCF-7 cells and suppression of the cellular proliferation of human breast cancer cells including SK-BR-3 and ZR-75-1 than daidzein (Pugazhendhi et al., 2008; Choi and Kim, 2013). Interestingly, daidzein was suggested to be more bioavailable than genistein based on the higher plasma concentration of daidzein after soymilk ingestion (Lu and Anderson, 1998). In addition, Kulling et al. (2000; 2001) reported that daidzein is biotransformed into hydroxylated daidzein, mainly 6,7,4'-trihy-

J. H. Lee · H. J. Lee (✉)  
Department of Food Science and Technology, Chung-Ang University, 4726  
Seodong-daero, Anseong, Gyeonggi-do 456-756, Republic of Korea  
E-mail: hongjin@cau.ac.kr

droxyisoflavone, 7,3',4'-trihydroxyisoflavone, and 7,8,4'-trihydroxyisoflavone in liver microsomes from rat and human. Daidzein metabolites such as trihydroxyisoflavones could be considered as potent chemopreventive agents, although the parent molecule shows less activity on breast cancer.

Recently, several studies have described that the daidzein metabolites, trihydroxyisoflavones showed inhibitory activity on neoplastic cell transformation (Lee et al., 2010), skin cancer (Lee et al., 2011a), and colon cancer (Lee et al., 2011b). For example, 7,3',4'-trihydroxyisoflavone is reported to reduce cellular proliferation and transformation induced by epidermal growth factor through targeting cyclin-dependent kinases (CDKs) and phosphatidylinositol 3-kinase (PI3K), as well as inhibit skin cancer development induced by UV-B via regulation of Cot and MKK4 (Lee et al., 2010, Lee et al., 2011a). In addition, Lo et al. (2012) found 7,3',4'-trihydroxyisoflavone suppressed multidrug-resistant transporters ROS dependently and induced apoptosis in human cervical cancer cells. Another daidzein metabolite, 6,7,4'-trihydroxyisoflavone is also shown to induce cell cycle arrest at S and G2/M phases in HCT-116 human colon cancer cells (Lee et al., 2011b). Therefore, hepatic metabolites of daidzein appeared to possess more significant chemopreventive activity than the parent daidzein. However, the studies demonstrating the roles of trihydroxyisoflavones in regulating cellular proliferation of breast cancer cells have not yet been performed.

In the present study, the effect of a hepatic daidzein metabolites on inhibiting growth of ER-negative breast cancer cells was investigated and found that 6,7,4'-trihydroxyisoflavone significantly suppressed MCF10CA1a breast cancer cell proliferation through arresting cell cycle at S and G2/M phase and inducing apoptosis.

## Materials and Methods

**Reagents.** Daidzein and genistein were purchased from Sigma (USA) and its hepatic metabolites, 7,3',4'-trihydroxyisoflavone, 6,7,4'-trihydroxyisoflavone, 7,8,4'-trihydroxyisoflavones were from Indofine Chemical Company, Inc (USA).

**Cell culture.** Human breast cancer cell line, MCF10CA1a, was obtained from Barbara Ann Karmanos cancer institute (USA) and cultured in DMEM/F12 (Life Technologies, USA) medium supplemented with 5% horse serum, 1% penicillin/streptomycin, and HEPES buffer (Life Technologies) at 37°C, 5% CO<sub>2</sub>.

**Cell proliferation assay.** MCF10CA1a cells were seeded (1,000 cells/well) in a 96-well plate overnight and treated with different concentrations of daidzein, genistein, and trihydroxyisoflavones (1, 10, 30, 50, and 100 µM) for 72 h in growth medium. Before 4 h of harvest, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) solution (200 µL) was added to each well. The dark blue formazan crystals formed in intact cells were dissolved in dimethylsulfoxide, and the absorbance was measured with a microplate reader at 570 nm (Molecular devices, USA). The cell proliferation was described as % cell viability compared

to the control.

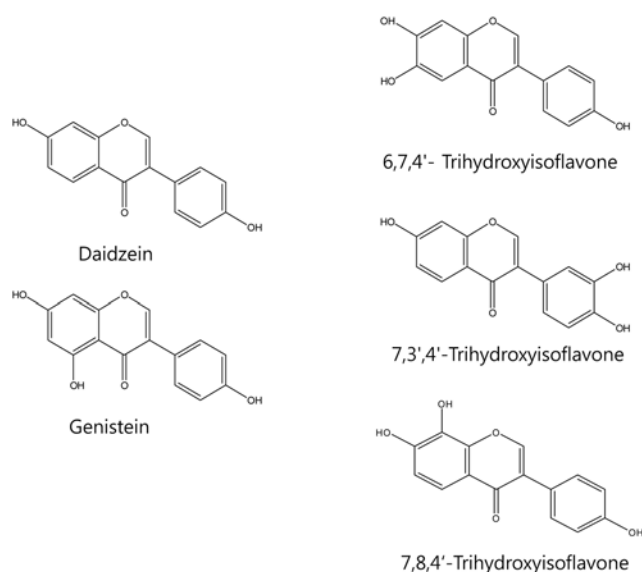
**Cell cycle analysis.** MCF10CA1a cells ( $5 \times 10^5$  cells/well) were seeded in a 6-well plate and cultured overnight. The cells were treated with various concentrations (10, 30, and 50 µM) of 6,7,4'-trihydroxyisoflavone for 72 h. The cells fixed with 70% ethanol were washed twice with phosphate buffered saline (PBS), and incubated with RNase A (20 µg/mL, Sigma) and propidium iodide (200 µg/mL, Sigma) at room temperature for 30 min. The stained cells were analyzed with flow cytometry using the FACSCalibur flow cytometer (BD Biosciences, USA). The percentage of the cells residing in G0/G1, S, G2/M, and sub-G1 phases were determined using ModFit LT (Verity Software House, Inc., USA) and CellQuest pro (BD Biosciences).

**Western blot analysis.** After the cells ( $7 \times 10^5$  cells/10 cm dish) were cultured overnight, they were treated with 6,7,4'-trihydroxyisoflavone at different concentrations (10, 30, and 50 µM) for 72 h. The cells were washed with PBS and then, harvested with radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris-HCl, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, 1% phenylmethylsulfonyl fluoride, and 1% protease inhibitor) to extract the cellular proteins. After the centrifugation at 12,000 g for 15 min, the same amount of protein (30 µg) was loaded in 10% polyacrylamide gel, and electrophoretically separated and transferred to a polyvinylidene difluoride membrane (Millipore, USA). The primary antibodies against cyclin B, cyclin E, CDK2, p21, p27 (Santa Cruz Biotech, Santa Cruz, USA), PARP, XIAP, and DR4 (Cell Signaling Technology, USA), CDK1 (BD Biosciences), β-actin (Sigma), and secondary antibodies against mouse and rabbit (Santa Cruz Biotech) were used. The expression level of proteins were visualized and quantified with EZ capture MG and EZ west Lumi plus (ATTO, Japan)

**Statistical analysis.** Statistical significances were determined using the Student's t-test.

## Results and Discussion

**6,7,4'-Trihydroxyisoflavone exerted a significant inhibition of ER-negative basal-like MCF10CA1a human breast cancer cells.** We first investigated the suppressive activity of isoflavones and its metabolites on MCF10CA1a human breast cancer cells (Fig. 1). As expected, daidzein showed minimal activity on inhibiting cell proliferation compared to genistein (Fig. 2). Interestingly, however, the daidzein metabolites including 7,3',4'-trihydroxyisoflavone, 7,8,4'-trihydroxyisoflavone, and 6,7,4'-trihydroxyisoflavone exerted significant suppression of cell growth in MCF10CA1a cells, with IC<sub>50</sub> of 21.0, 33.6, and 12.4 µM, respectively (Fig. 2). In line with our results, Lee et al. (2011b) also demonstrated daidzein metabolite, 6,7,4'-trihydroxyisoflavone, showed more significant inhibition of HCT116 and DLD1 human colon cancer cell proliferations than the parent compound. Therefore, the hepatic metabolites of daidzein are suggested to act as potent regulators of human cancer



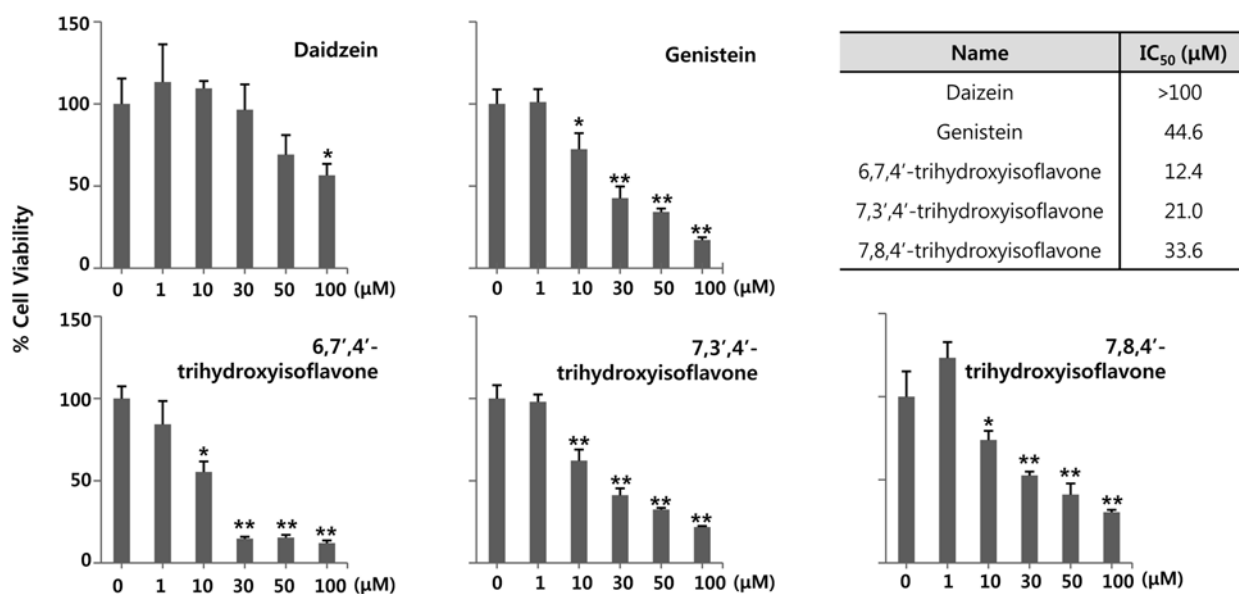
**Fig. 1** Chemical structures of daidzein, genistein, and daidzein metabolites, 6,7,4'-trihydroxyisoflavone, 7,3',4'-trihydroxyisoflavone, and 7,8,4'-trihydroxyisoflavone.

cell proliferation, and the most active metabolite, 6,7,4'-trihydroxyisoflavone, was chosen for further study.

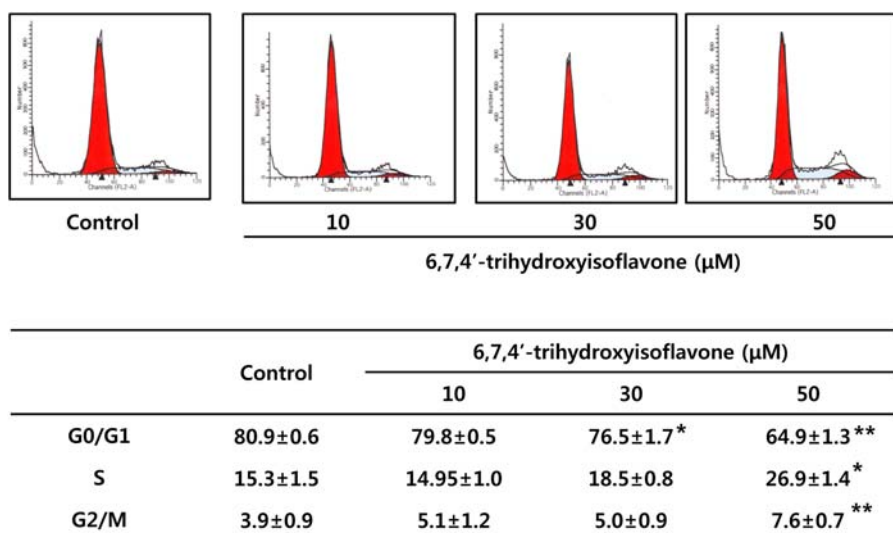
**6,7,4'-Trihydroxyisoflavone effectively arrests S phase and G2/M phase of cell cycle in MCF10CA1a human breast cancer cells.** Carcinogenesis is deeply related to molecular machinery of cell cycle (Kamb, 1995); thus we further performed the cell cycle arrest assay to determine whether the inhibition of cell proliferation by 6,7,4'-trihydroxyisoflavone is caused by

regulating the cell cycle. Treatment of 6,7,4'-trihydroxyisoflavone in MCF10CA1a cells significantly reduced the cell contents at G0/G1 phase from 80.1% of control to 64.9% at 50  $\mu$ M of 6,7,4'-trihydroxy isoflavone (Fig. 3). The cell populations at S and G2/M phases were increased by 6,7,4'-trihydroxyisoflavone from 15.3 to 26.9% and 3.9 to 7.6%, respectively. In addition, different concentration treatments of 6,7,4'-trihydroxyisoflavone (10, 30, and 50  $\mu$ M) exerted dose-dependent cell cycle regulatory activity (Fig. 3). These results indicate that 6,7,4'-trihydroxyisoflavone induced the cell cycle arrest at S and G2/M phases.

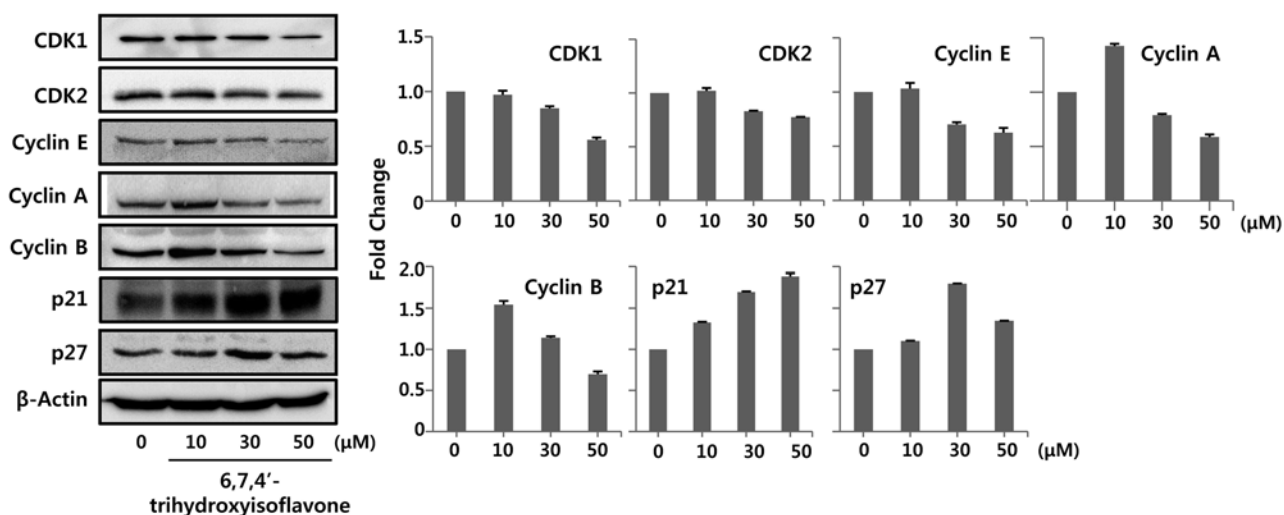
**6,7,4'-Trihydroxyisoflavone regulates the protein expression involved in S and G2/M phases in MCF10CA1a cells.** It is generally known that cyclins and CDKs are critical mediators in regulating the cell cycle (Ivanchuk and Rutka, 2004). During the cell cycle, cyclins D and E are involved in G1 phase, cyclins A and E in S phase, and cyclins A and B in M phase. In the case of CDKs, CDK 2/4/6 are involved in G1 phase, CDK2 in S phase, and CDK 2/1 in M phase (Ivanchuk and Rutka, 2004). Hochegger et al. (2007) also reported that the critical functions of CDK1 in regulating S phase in addition to M phase were demonstrated in vertebrate cells. Furthermore, CDK1 can perform all the events necessary for the cell division, suggesting that CDK1 is the unique essential CDK in mammalian cells (Santamaria et al., 2007). Therefore, we first investigated the expression of CDK1 and found that it was down-regulated by 6,7,4'-trihydroxy-isoflavone treatment in a dose-dependent manner (Fig. 4). In addition, expressions of CDK2, cyclins E, A, and B were significantly suppressed (Fig. 4), CDK inhibitors are the proteins regulating CDK-cyclin complex activity. The well characterized CDK inhibitors, p21 and p27, are known to inhibit CDKs during all



**Fig. 2** 6,7,4'-Trihydroxyisoflavone exerted the strongest activity in inhibiting cell proliferation of MCF10CA1a cells. MCF10CA1a cells (1,000/well in 96-well plate) were treated with the indicated isoflavones for 72 h and cell viability was determined by MTT assay as described in materials and methods. Statistical significance, \* $p$  < 0.05, \*\* $p$  < 0.01



**Fig. 3** 6,7,4'-Trihydroxyisoflavone induced the accumulation of MCF10CA1a cells at S- and G2/M phases and reduced at G0/G1 phase in cell cycle analysis. MCF10CA1a cells ( $5 \times 10^5$ /well in 6-well plate) were treated with 6,7,4'-trihydroxyisoflavone (10, 30, and 50 μM) for 72 h and stained with annexin V and propidium iodide. Statistical significance, \* $p < 0.05$ , \*\* $p < 0.01$



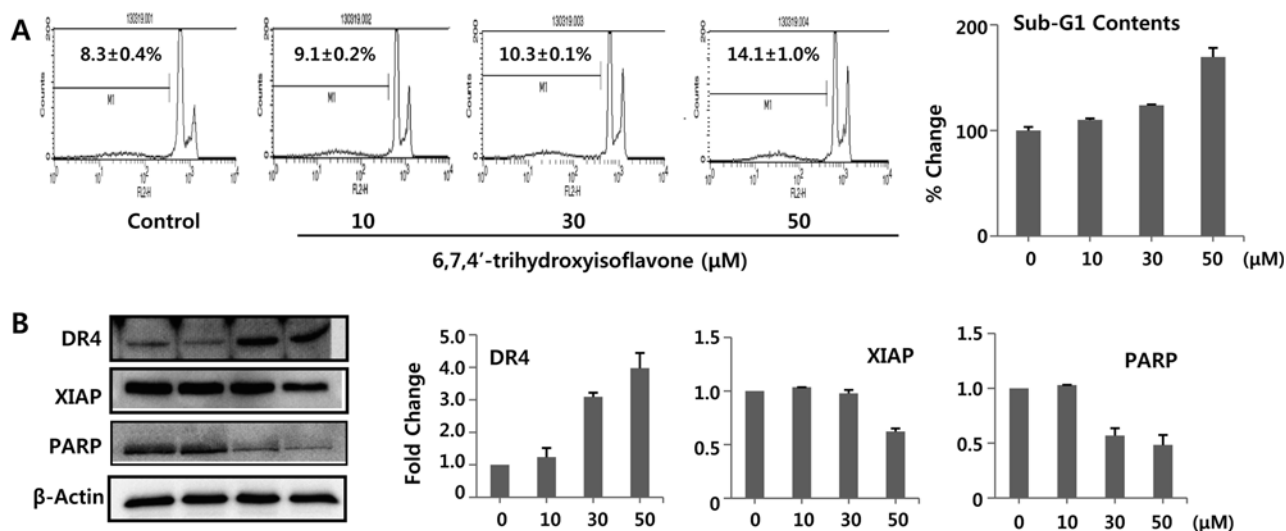
**Fig. 4** 6,7,4'-Trihydroxyisoflavone regulates cyclins A, E, and B, and cyclin-dependent kinases CDK1 and CDK2, and CDK inhibitors, p21 and p27. MCF10CA1a cells ( $7 \times 10^5$  cells in 100 mm dish) were treated 6,7,4'-trihydroxyisoflavone (10, 30, 50 μM) for 72 h and harvested. Protein was extracted using RIPA buffer. The level of protein expression was determined by Western blotting.

phases of the cell cycle (Ivanchuk and Rutka, 2004). Here, we also found that 6,7,4'-trihydroxyisoflavone significantly increased the expressions of p21 and p27 (Fig. 4). These results suggest that 6,7,4'-trihydroxyisoflavone may induce cell cycle arrest through regulation of the cyclins, CDKs, and CDK inhibitors at S and G2/M phases.

Fisher et al. (2012) reported that cyclin B-CDK1 complex played a crucial role in the transition from S phase to mitosis, and 6,7,4'-trihydroxyisoflavone was shown to suppress the CDK1 and CDK2 activities in HCT-116 colon cancer cells (Lee et al., 2011b). Therefore, our results also support the role of 6,7,4'-trihydroxy-

isoflavone in regulating S- and G2/M phase regulatory proteins in MCF10CA1a human breast cancer cells.

**6,7,4'-Trihydroxyisoflavone induces apoptosis through activation of death receptor signaling in MCF10CA1a cells.** To investigate whether 6,7,4'-trihydroxyisoflavone induces apoptosis, we first tested the effects of 6,7,4'-trihydroxyisoflavone on sub-G1 contents using flow cytometry. 6,7,4'-Trihydroxyisoflavone treatment at 10, 30, and 50 μM increased the sub-G1 contents from 8.3 to 9.1, 10.3, and 14.1%, respectively, suggesting the role of 6,7,4'-trihydroxyisoflavone in inducing apoptosis in MCF10CA1a cells (Fig. 5A). Tumor necrosis factor receptor superfamily including



**Fig. 5** The accumulation of sub-G1 was enhanced by 6,7,4'-trihydroxyisoflavone treatment which may have derived from the regulation of apoptotic mediators, DR4, XIAP, and PARP. **A**, MCF10CA1a cells ( $7 \times 10^5$  cells in 100 mm dish) were treated with 6,7,4'-trihydroxyisoflavone (10, 30, 50  $\mu\text{M}$ ) for 72 h, and harvested. Protein was extracted using RIPA buffer. The level of protein expression was determined by Western blotting.

Fas and death receptors (DRs) are known to induce apoptosis through activating of downstream caspase cascade and poly ADP-ribose polymerase (PARP) cleavage (Kaufmann et al., 2012). In addition, X-linked inhibitor of apoptosis (XIAP) is considered as an inhibitor of apoptosis signaling by inhibiting the activity of caspases (Kaufmann et al., 2012; Rajput et al., 2013). In the present study, we confirmed that 6,7,4'-trihydroxyisoflavone enhanced the expression of DR4 and suppressed XIAP at the concentrations of 30 and 50  $\mu\text{M}$ , which led to the activation of cleavage of PARP protein (Fig. 5B). These results suggest that 6,7,4'-trihydroxyisoflavone may induce apoptosis through death receptor signaling activation in MCF10CA1a human breast cancer cells.

In summary, we investigated the effects of a hepatic metabolite of daidzein, 6,7,4'-trihydroxyisoflavone, on growth of MCF10CA1a human breast cancer cells, and found that 6,7,4'-trihydroxyisoflavone inhibited cell proliferation by arresting cell cycle at S and G2/M phases and inducing the cellular apoptosis by regulating death receptor signaling cascade. To the best of our knowledge, this study is the first report demonstrating the role of daidzein metabolite in regulating cell cycle-related proteins and apoptotic mediators in ER-negative MCF10CA1a human breast cancer cells, and suggests the possible use as a more potent agent with higher inhibition of mammary tumorigenesis than its parent molecule, daidzein.

**Acknowledgment** This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2011-0013812) and by the Chung-Ang University Excellent Student Scholarship.

## References

- Boucher BA, Cotterchio M, Anderson LN, Kreiger N, Kirsh VA, and Thompson LU (2013) Use of isoflavone supplements is associated with reduced postmenopausal breast cancer risk. *Int J Cancer* **132**, 1439–50.
- Choi EJ and Kim GH (2013) Antiproliferative activity of daidzein and genistein may be related to ERalpha/c-erbB-2 expression in human breast cancer cells. *Mol Med Rep* **7**, 781–4.
- Fisher D, Krasinska L, Coudreuse D, and Novak B (2012) Phosphorylation network dynamics in the control of cell cycle transitions. *J Cell Sci* **125**, 4703–11.
- Hilakivi-Clarke L, Andrade JE, and Helferich W (2010) Is soy consumption good or bad for the breast? *J Nutr* **140**, 2326S–34S.
- Hochegger H, Dejsuphong D, Sonoda E, Saberi A, Rajendra E, Kirk J et al. (2007) An essential role for Cdk1 in S phase control is revealed via chemical genetics in vertebrate cells. *J Cell Biol* **178**, 257–68.
- Ivanchuk SM and Rutka JT (2004) The cell cycle: accelerators, brakes, and checkpoints. *Neurosurgery* **54**, 692–9.
- Kamb A (1995) Cell-cycle regulators and cancer. *Trends Genet* **11**, 136–40.
- Kaufmann T, Strasser A, and Jost PJ (2012) Fas death receptor signalling: roles of Bid and XIAP. *Cell Death Differ* **19**, 42–50.
- Kulling SE, Honig DM, and Metzler M (2001) Oxidative metabolism of the soy isoflavones daidzein and genistein in humans in vitro and in vivo. *J Agric Food Chem* **49**, 3024–33.
- Kulling SE, Honig DM, Simat TJ, and Metzler M (2000) Oxidative in vitro metabolism of the soy phytoestrogens daidzein and genistein. *J Agric Food Chem* **48**, 4963–72.
- Lee DE, Lee KW, Byun S, Jung SK, Song N, Lim SH et al. (2011a) 7,3',4'-Trihydroxyisoflavone, a metabolite of the soy isoflavone daidzein, suppresses ultraviolet B-induced skin cancer by targeting Cot and MKK4. *J Biol Chem* **286**, 14246–56.
- Lee DE, Lee KW, Jung SK, Lee EJ, Hwang JA, Lim TG et al. (2011b) 6,7,4'-trihydroxyisoflavone inhibits HCT-116 human colon cancer cell proliferation by targeting CDK1 and CDK2. *Carcinogenesis* **32**, 629–35.
- Lee DE, Lee KW, Song NR, Seo SK, Heo YS, Kang NJ et al. (2010) 7,3',4'-Trihydroxyisoflavone inhibits epidermal growth factor-induced proliferation

- and transformation of JB6 P+ mouse epidermal cells by suppressing cyclin-dependent kinases and phosphatidylinositol 3-kinase. *J Biol Chem* **285**, 21458–66.
- Lo YL, Wang W, and Ho CT (2012) 7,3',4'-Trihydroxyisoflavone modulates multidrug resistance transporters and induces apoptosis via production of reactive oxygen species. *Toxicology* **302**, 221–32.
- Lu LJ and Anderson KE (1998) Sex and long-term soy diets affect the metabolism and excretion of soy isoflavones in humans. *Am J Clin Nutr* **68**, 1500S–4S.
- Mai Z, Blackburn GL, and Zhou JR (2007) Genistein sensitizes inhibitory effect of tamoxifen on the growth of estrogen receptor-positive and HER2-overexpressing human breast cancer cells. *Mol Carcinog* **46**, 534–42.
- Messina M, McCaskill-Stevens W, and Lampe JW (2006) Addressing the soy and breast cancer relationship: review, commentary, and workshop proceedings. *J Natl Cancer Inst* **98**, 1275–84.
- Pugazhendhi D, Watson KA, Mills S, Botting N, Pope GS, and Darbre PD (2008) Effect of sulphation on the oestrogen agonist activity of the phytoestrogens genistein and daidzein in MCF-7 human breast cancer cells. *J Endocrinol* **197**, 503–15.
- Qin LQ, Xu JY, Wang PY, and Hoshi K (2006) Soyfood intake in the prevention of breast cancer risk in women: a meta-analysis of observational epidemiological studies. *J Nutr Sci Vitaminol (Tokyo)* **52**, 428–36.
- Rajput S, Kumar BN, Sarkar S, Das S, Azab B, Santhekadur PK et al. (2013) Targeted apoptotic effects of thymoquinone and tamoxifen on XIAP mediated Akt regulation in breast cancer. *PLoS One* **8**, e61342.
- Santamaria D, Barriere C, Cerqueira A, Hunt S, Tardy C, Newton K et al. (2007) Cdk1 is sufficient to drive the mammalian cell cycle. *Nature* **448**, 811–5.
- Shu XO, Zheng Y, Cai H, Gu K, Chen Z, Zheng W et al. (2009) Soy food intake and breast cancer survival. *JAMA* **302**, 2437–43.
- Taylor CK, Levy RM, Elliott JC, and Burnett BP (2009) The effect of genistein aglycone on cancer and cancer risk: a review of in vitro, preclinical, and clinical studies. *Nutr Rev* **67**, 398–415.