RESEARCH ARTICLE

Molecular mechanism of suppression of MDR1 by puerarin from *Pueraria lobata via* NF-κB pathway and cAMP-responsive element transcriptional activity-dependent up-regulation of AMP-activated protein kinase in breast cancer MCF-7/adr cells

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Multidrug resistance (MDR) is a major obstacle in cancer chemotherapy and its inhibition is an effective way to reverse cancer drug resistance. In the present study, we investigated that puerarin, a natural isoflavonoid from *Pueraria lobata*, down-regulated MDR1 expression in MCF-7/adriamycin (MCF-7/adr), a human breast MDR cancer cell line. Puerarin treatment significantly inhibited MDR1 expression, MDR1 mRNA and MDR1 promoter activity in MCF-7/adr cells. The suppression of MDR1 was accompanied by partial recovery of intracellular drug accumulation, leading to increased toxicity of adriamycin and fluorescence of rhodamine 123, indicating that puerarin reversed the MDR phenotype by inhibiting the drug efflux function of MDR1. Moreover, nuclear factor κ-B activity and IκB degradation were inhibited by puerarin. Puerarin stimulated AMP-activated protein kinase (AMPK), acetyl-CoA carboxylase and glycogen synthase kinase-3β phosphorylation, but puerarin decreased cAMP-responsive element-binding protein phosphorylation. The puerarin-induced suppression of MDR1 expression was reduced by AMPK inhibitor (compound C). Furthermore, both MDR1 protein expression and the transcriptional activity of cAMP-responsive element (CRE) were inhibited by puerarin and protein kinase A/CRE inhibitor (H89). Taken together, our results suggested that puerarin down-regulated MDR1 expression *via* nuclear factor κ-B and CRE transcriptional activity-dependent up-regulation of AMPK in MCF-7/adr cells.

Keywords:
AMP-activated protein kinase / cAMP responsive element / Multidrug resistance 1 / Nuclear factor κ-B / Puerarin

1 Introduction

Cancer cells can develop resistance to structurally diverse and mechanistically unrelated anticancer drugs, a phenomenon termed multidrug resistance (MDR) [1]. P-glycoprotein, encoded by the multidrug resistance 1 (MDR1) gene, is a multidrug transporter that functions as an adenosine triphosphate-dependent drug efflux pump possessing broad substrate specificity [2]. MDR1 was puri-

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Abbreviations: ACC, acetyl-CoA carboxylase; **aicar,** 5-aminoimidazole-4-carboxamide-1-β-d-ribofuranoside; **AMPK,** adenosine 5′-monophosphate-activated protein kinase; **CRE,** cAMP-responsive element; **CREB,** cAMP-responsive element-binding protein; **GSK-3β,** glycogen synthase kinase-3β; **MCF-7/adr,** MCF-7/adriamycin; **MDR1,** multidrug resistance 1; **MTT,** 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **NF-κB,** nuclear factor κ-B; **PKA,** protein kinase A; **Rh-123,** rhodamine 123; **TNF-α,** tumor necrosis factor α

fied in 1979 [3] and strong evidence in support of its role in pleiotropic drug resistance came in 1982, when it was shown that transfer of DNA from resistant cell lines to nonresistant cells conferred resistance to the latter in a manner that correlated with the expression of the protein [4]. MDR1 is understood to act as a pump that detects and removes its substrate from inside the cells to outside; however, another model has been proposed in which MDR1 acts as a flippase, carrying its substrate from the inner leaflet of the lipid bilayer to the outer leaflet [5]. In tumor cells, over-expressed MDR1 is responsible for the efflux of various structurally and functionally related or unrelated hydrophobic chemotherapeutic agents from cells, which results in a low efficacy of chemotherapy [6]. MDR1 demonstrates a broad spectrum of substrate specificities toward vinca alkaloids, anthracyclines, taxens, and epipodophylotoxins and is responsible for the intrinsic and acquired drug resistance in numerous human cancers. MDR1-mediated drug resistance can be effectively overcome by either blocking its drug pump function or inhibiting its expression [7].

Nuclear factor κ-B (NF-κB) is a protein complex that acts as a transcription factor. In tumor cells, NF-κB is active either due to mutations in genes encoding the NF-κB transcription factor or in genes that control NF-κB activity, such as IκB genes. In addition, some tumor cells secrete factors that activate NF-κB. Blocking NF-κB can cause tumor cells to stop proliferating, to die, or to become more sensitive to the action of anti-tumor agents [8]. Previous studies demonstrated MDR1 activation through NF-κB activation [9–11].

AMP-activated protein kinase (AMPK) is considered a key sensor of cellular energy, because AMPK phosphylates and hence regulates the activity of enzymes such as acetyl-CoA carboxylase (ACC) and 3-hydroxy-3-methylglutaryl-CoA reductase [12]. AMPK is down-regulated by cAMP-dependent protein kinase A (PKA) [13, 14]. In human tumor cells, *e.g.* in breast cancer cells [14] and prostate tumor spheroids [15], involvement of cAMP and PKA in regulation of MDR1 expression has been demonstrated previously without establishment of a connection to a special receptor or receptor agonist. In a previous study, cAMP-dependent activation of PKA and cAMP-responsive element-binding protein (CREB) in MDR1 gene activation was reported in primary rat hepatocytes [16, 17]. Furthermore, over-expression of the dominant-negative CREB mutant KCREB in primary rat hepatocytes repressed intrinsic and misoprostol-induced MDR1 promoter activity. KCREB interfered with intrinsic and misoprostol-induced MDR1 promoter activation, which may be interpreted either in terms of a direct or an indirect effect [16, 17].

Plant-derived compounds are gaining increasing interest as potential cancer therapeutics, particularly for treatment-refractory cancers. Some natural compounds in fruits, vegetables, oilseeds, and herbs [18–20] modulate MDR1 activity were reported [19, 21, 22]. Especially, those were found in traditional medicine and dietary supplements have the potential to be developed as MDR1 reversing agents, which could lead to more successful chemotherapy. *Pueraria lobata* (Willd.) Ohwi has been used as a food as well as in traditional oriental medicine for diaphoretic, febrifuge, colds, dysentery, and fever in China [23]. *P. lobata* is disturbuted in eastern Asia. Today it occurs throughout the southeastern US at least as far north as Ohio and Connecticut, and in many others parts of the world, including South America and South Africa [24]. Puerarin, an isoflavonoid found in *P. lobata*, has been reported to possess antioxidant activities [25], anti-hypercholesterolemia [26], and antihyperglycemic effects [27]. Previous studies found that puerarin also possessed anti-cancer properties [28]. In this study, we demonstrate that puerarin is a down-regulator of MDR1 *via* inhibition NF-κB activation in MCF-7/adriamycin (MCF-7/adr) cells. Furthermore, we have investigated cAMP-responsive element (CRE) transcriptional activity through up-regulation of AMPK as a novel, regulated signaling molecule and provide evidence that this protein plays an important role in the regulation of MDR1 expression by puerarin in MCF-7/adr cells.

2 Materials and methods

2.1 Materials

Puerarin, verapamil, rhodamine, adriamycin, H89, forskolin, 5-aminoimidazole-4-carboxamide-1-β-d-ribonucleoside (aicar), and compound C were purchased from Sigma Chemical Company (St Louis, MO, USA), DMEM, fetal bovine serum, and trypsin were purchased from GIBCO-BRL (Grand Island, NY, USA). RNAiso was purchased from Takara (Japan). Antibodies against phospho-IκB-α, phospho-AMPK, AMPK, phospho-CREB, phospho-glycogen synthase kinase-3β (GSK-3β), and GSK-3β, and horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA), antibodies against β-actin and MDR1 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from USB Corporation (Cleveland, OH, USA). The cytotoxicity detection kit used to measure lactate dehydrogenase release was from Roche Applied Science (Indianapolis, IN, USA). All other chemicals were of the highest purity available.

2.2 Cell culture

MCF-7 (human breast adenocarcinoma) cells and the multidrug-resistant subline MCF-7/adr were provided by Dr. Marilyn E. Morris (Buffalo University, NY, USA, USA). Cells were grown in DMEM containing 10% fetal bovine serum. Both cell lines were cultured at 37°C in a humidified CO_2 incubator.

2.3 Measurement of cell viability

The cells were plated in 48-well plates and cell viability was determined by the conventional MTT reduction assay and LDH assay. After incubation for 24 h, various concentrations of puerarin were added the each well and the plates were incubated at 37°C for 24 h. After the supernatant was used for LDH determination at 490 nm using microplate reader (Varioskan, Thermo Electron, Waltham, MA). The cells were treated with MTT solution for 1 h. The dark blue formazan crystals that formed in intact cells were solubilized with DMSO, and the absorbance at 570 nm was measured with a microplate reader (Varioskan, Thermo Electron). Percent cell viability was calculated based on the absorbance measured relative to the absorbance of cells exposed to the control vehicle.

2.4 RT-PCR for MDR1

RT-PCR was performed to evaluate the mRNA expression level of MDR1 after puerarin treatment. Total RNA was isolated with an RNA isolation kit (Takara) according to the manufacturer's protocol, and the quality of RNA was checked by optical density measurement with A260/A280 > 1.8. The cDNA was then amplified by PCR using Taq DNA polymerase under the following conditions: 25 cycles of 94°C for 30 s; 56°C for 30 s; and 72°C for 45 s. The PCR primers were: 5′-GCCTGGCAGCTGGAAGACAAATACACAAAATT-3′ and 5′-CAGACAGCAGCT GACAGTCCAAGAACAGGACT-3′ for MDR1; 5′-GATGATATCGCCGCGCTCGT CGTCGAC-3′ and 5′-AGCCAGGTCCAGACGCAGGATGGCATG-3′ for β-actin. PCR products were electrophoresed on 1.5% agarose gel, visualized by ethidium bromide staining and photographed under ultraviolet light.

2.5 Western blot analysis

After treatment, the cells were collected and washed with PBS. The harvested cells were then lysed on ice for 30 min in 100 μL lysis buffer (120 mM NaCl, 40 mM Tris (pH 8), 0.1% NP40 (Nonidet P-40)) and centrifuged at 12 000 rpm for 30 min. Supernatants were collected from the lysates and protein concentrations were determined using the BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Aliquots of the lysates (60 μg of protein) were boiled for 5 min and electrophoresed on 10% SDS-polyacrylamide gels. Proteins in the gels were transferred onto nitrocellulose membranes, which were then incubated with MDR1, phospho-IκB-α, phospho-AMPK, AMPK, phospho-CREB, phospho-GSK-3β, GSK-3β, antibodies, or mouse monoclonal β-actin antibodies. The membranes were further incubated with secondary anti-mouse or anti-rabbit antibodies. Finally, protein bands were detected using an enhanced chemiluminescence Western blotting detection kit (Pierce Biotechnology).

2.6 Transient transfection and luciferase assay

To determine promoter activity, we used a dual-luciferase reporter assay system (Promega, Madison, WI, USA). Briefly, cells were plated in 24-well plates overnight and transiently co-transfected with hMDR1-Luc, CRE-Luc, or NF-κB-Luc construct and pRL-SV plasmid (Renilla luciferase expression for normalization) (Promega) using LipofectAMINE^{TM} 2000 reagent (Invitrogen, Carlsbad, CA, USA). The cells were then exposed to puerarin, tumor necrosis factor α (TNF-αforskolin, and compound C for 24 h. Luciferase activities in cell lysates were measured using a luminometer (TD-20; Turner Designs, Sunnyvale, CA, USA). Relative luciferase activities were calculated by normalizing MDR1 or NF-κB promoter-driven firefly luciferase activity to Renilla luciferase activity (Luminoskan Ascent, Thermo Electron).

2.7 Rhodamine-123 accumulation assay

MCF-7/adr cells were seeded into 24-well plates at a density of 10^5 cells/well. Cells were pretreated with 20, 50, 100 μM puerarin and verapamil 20 μM for 48 h. Verapamil was used as a positive control as an MDR inhibitory agent [29]. After the pretreatment, cells were incubated with 5 μM rhodamine 123 (Rh-123) in culture medium in the dark at 37°C in 5% CO_2 for 90 min. After Rh-123 accumulation, cells were trypsinized from the subconfluent monolayer of cells, and the cell pellet was washed twice with ice-cold PBS. The cells were then suspended with 1 mL PBS. The fluorescence as an indicator for intracellular Rh-123 accumulation was measured at 488−530 nm using the Varioskan reader.

2.8 Statistical analysis

All experiments were repeated at least three times. For quantitation analysis, the sum of the density of bands corresponding to protein blotting with the antibody under study was calculated, and the amount of β-actin normalized. One-way analysis of variance was used to determine the significance of differences between treatment groups. The Newman–Keuls test was used for multi-group comparisons. Statistical significance was accepted for p values of <0.01.

3 Results

3.1 Characterization of MCF-7 and MCF-7/adr cells

It has been reported that MDR1 is over-expressed in the MCF-7/adr cell line [30]. To confirm this, MDR1 expression was examined in MCF-7/adr cells compared with that in MCF-7 by Western blot analysis. As shown in Fig. 1A, MDR1 was over-expressed in MCF-7/adr. MCF-7 and MCF-7/adr cells were exposed to various concentrations of

adriamycin (0.01−40 μg/mL) for 48 h. The cytotoxicity of adriamycin in MCF-7 is higher than that in MCF-7/adr cells (Fig. 1B). These results confirmed that MDR1 is over-expressed in the resistant (MCF-7/adr) cells.

3.2 Increased adriamycin toxicity in MCF-7/adr cells with puerarin

As shown in Fig. 2A, puerarin was not toxic in MCF-7/adr cells at concentrations from 20 to 200 μM based on MTT assay. Some studies reported that adriamycin caused MDR1 in breast cancer cells [31, 32]. To examine whether puerarin affected the cytotoxic effect of adriamycin in MCF-7/adr and MCF-7 cells, cells were pretreated with puerarin followed by addition of various concentrations of adriamycin, and the incubation was continued for an additional 48 h. Cell viabilities were analyzed by the MTT assay and LDH assay. Figures 2B and C present that the co-incubation of puerarin and adriamycin remarkably increased cytotoxicity against MCF-7/adr cells compared with adriamycin alone. However, they did not affect the cytotoxicity of adriamycin in MCF-7 cells (Figs. 2D and E). These results indicate that puerarin increased the cytotoxic effect of adriamycin in MCF-7/adr cells.

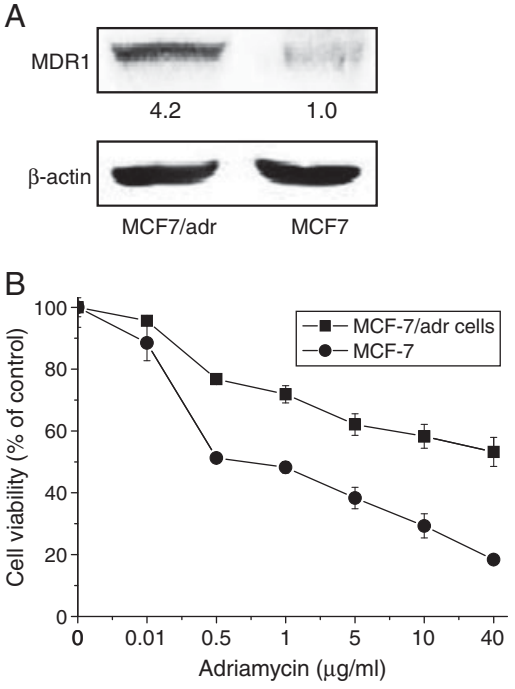

Figure 1. Characterization of MCF-7 and MCF-7/adr cells. (A) MDR1 was detected in MCF-7/adr and MCF-7 cells by Western blot analysis as described in Section 2 . MDR1 is over-expressed in the resistant (MCF-7/adr) cells. (B) After treatment with adriamycin in MCF-7 (sensitive) and MCF-7/adr (resistant) cells for 48 h, cell viability was determined by MTT assay. Significantly different from untreated cells ($p<0.01$).

3.3 Decreased expression of MDR1 mRNA and protein in MCF-7/adr cells by puerarin

Over-expression of MDR1 gene and protein has been related to MDR phenotype [7]. RT-PCR was performed to detect the change in mRNA levels of MDR1 when the cells were treated with puerarin. As shown in Fig. 3A, the level of MDR1 mRNA decreased in a dose- and time-dependent manner. Quantities of RNA in each lane were normalized to β-actin expression. The observed changes in MDR1 protein expression in MCF-7/adr cells were confirmed by Western blot analysis following incubation of the cells for 24 h with various concentrations of puerarin or puerarin 100 μM for the indicated time (3−48 h). The MDR1 protein level of MDR1 was decreased in MCF-7/adr cells in a dose- and time-dependent manner (Fig. 3B). Taken together, these data suggested that puerarin suppressed MDR1 in MCF-7/adr cells.

3.4 Increased intracellular Rh-123 accumulations in MDR1 sublines by puerarin

The efflux of fluorescent Rh-123 is known to be MDR1-dependent and consequently has been used extensively to determine efflux from drug-resistant cell lines expressing MDR1 [33, 34]. After passive diffusion into the cells, Rh-123 is actively transported out of the cell by MDR1. Measurement of cellular Rh-123 accumulation revealed that the observed changes of MDR1 expression were reflective of changes in MDR1 function. Following pretreatment with various concentrations of puerarin and 20 μM verapamil, cells were incubated with 5 μM Rh-123 for 90 min. Figure 4 shows that decreased MDR1 expression led to significant accumulation of intracellular Rh-123 in MCF-7/adr cells after treatment with puerarin and verapamil.

3.5 MDR1 was suppressed by puerarin through inhibition of NF-κB activation and IκB-α phosphorylation

To elucidate the effect of puerarin on NF-κB and MDR1, MCF-7/adr cells were transfected with reporter plasmids containing NF-κB and MDR1 promoter. Cells were then treated with various concentrations of puerarin and TNF-α. Figures 5A and B depict that puerarin dramatically inhibited the activity of NF-κB and MDR1 promoter. Furthermore, the co-treatment of puerarin and TNF-α remarkably decreased the activity of NF-κB and MDR1 promoter compared with TNF-αalone. However, co-treatment with puerarin and TNF-α remarkably increased the activity of NF-κB and MDR1 promoter compared with puerarin alone.

We next determined whether TNF-α induction of NF-κB and MDR1 activation occurs through phosphoryla-

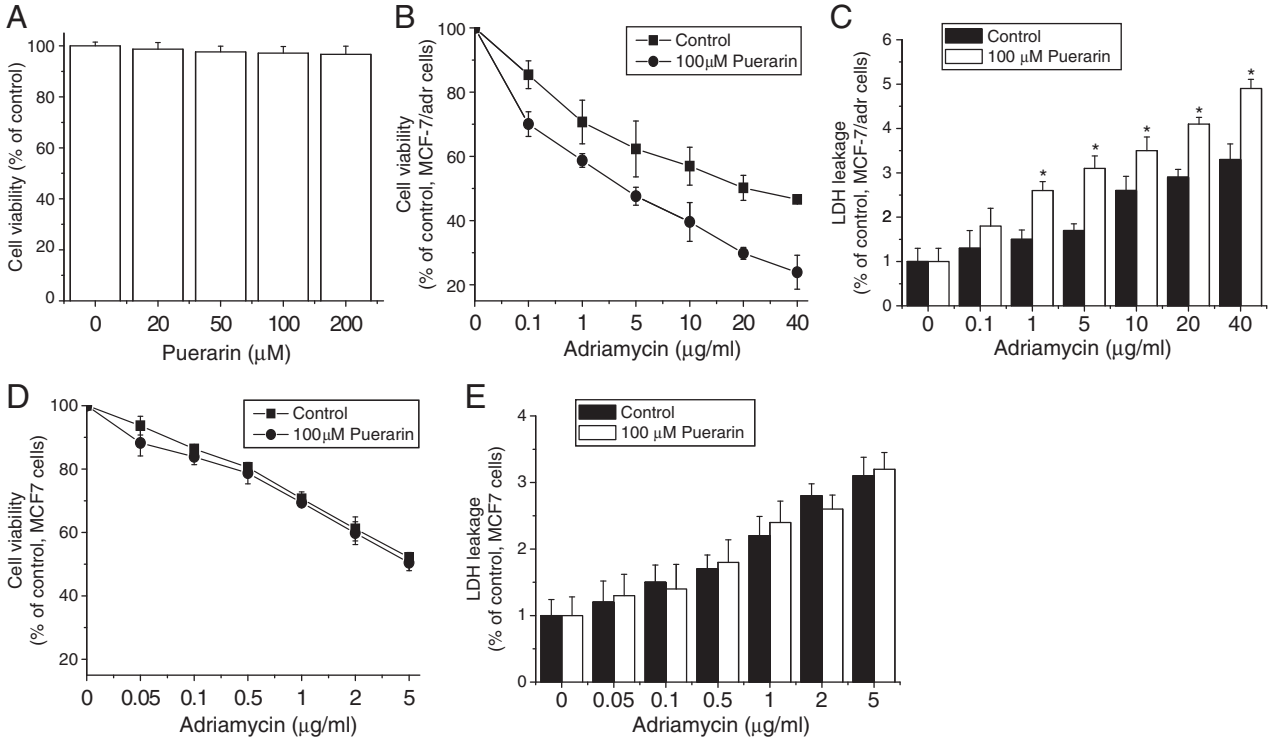

Figure 2. Puerarin enhanced the cytotoxic of adriamycin in MCF-7/adr cells. (A) Cytotoxicity of puerarin (20–200 μM) treatment in MCF-7/adr for 24 h was measured by MTT assay. Effects of co-incubation of puerarin and adriamycin on MCF-7/adr (B and C) and MCF-7 (D and E) cells. Cells were pretreated with or without 100 μM puerarin, followed by addition of various concentrations of adriamycin, and the incubation was continued for an additional 48 h. Cell viability was determined by MTT assay and LDH assay. *Significantly different from untreated cells ($p < 0.01$).

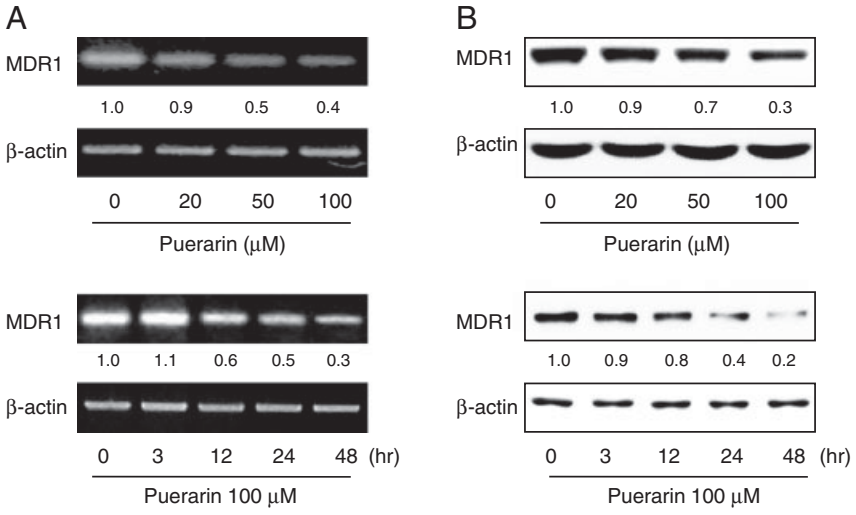

Figure 3. Effect of puerarin on MDR1 gene and protein expression in MCF-7/adr cells. (A) The cells were treated with puerarin (20–100 μM) for 24 h and 100 μM puerarin for 3–48 h. Total RNA was extracted. MDR1 expression was analyzed by semi-quantitative RT-PCR. The β-actin band is shown to confirm integrity and equal loading of RNA. (B) Lysates of cells treated with various concentrations of puerarin for 24 h or 100 μM puerarin for 3–48 h were electrophoresed and the expression of MDR1 was detected with an MDR1-specific antibody. The β-actin band is shown to confirm integrity and equal loading of protein.

tion and subsequent degradation of IκB-α. To examine whether puerarin inhibited IκB-α phosphorylation in MCF-7/adr cells, Western blot analyses were performed using anti-MDR1, anti-phospho-Ser32 of IκB-α and β-actin antibodies. As shown in Fig. 5, MDR1 expression was inhibited by puerarin and increased by TNF-α (Fig. 5C). Furthermore, puerarin decreased the levels of phosphorylation of IκB-α observed at 60 min (Fig. 5D). Thus, puerarin suppressed MDR1 through inhibition of NF-κB activation.

3.6 Phosphorylation of AMPK and ACC were increased by puerarin in MCF-7/adr cells

Activation of AMPK in experimental model systems is often achieved by administration of the AMPK activator aicar [35].

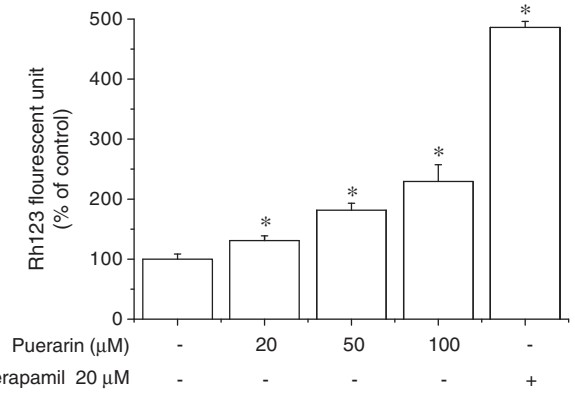

Figure 4. Effect of puerarin on intracellular Rh-123 accumulation in MCF-7/adr cells. Cells were treated with vehicle, 20–100 μM puerarin and 20 μM verapamil (positive control) for 48 h and then exposed to 5 μM of Rh-123 for 90 min. The intracellular Rh-123 accumulation was then measured. *Significantly different from untreated cells ($p < 0.01$).

Cells were treated with 20, 50, 100 μM puerarin and 1 mM aicar for 1 h or 100 μM puerarin for 15–90 min, followed by Western blotting with anti-phospho-AMPK and anti-phospho-ACC antibodies. The result presents that phosphorylation of AMPK and ACC were increased by puerarin in MCF-7/adr cells (Figs. 6A and B). To address the role of AMPK expression by puerarin, we examined the effect of AMPK inhibitor (compound C) in MCF-7/adr cells. As shown in Fig. 6C, compound C blocked puerarin- and aicar (AMPK activator)-induced phosphorylation of AMPK.

3.7 Puerarin increased GSK-3β phosphorylation but decreased CREB phosphorylation in MCF-7/adr cells

A previous study reported that aicar increased phosphorylations of GSK-3β, AMPK, ACC and decreased phosphorylation of CREB [36]. To examine the role of GSK-3β and the CREB pathway in AMPK and MDR1 expression, MCF-7/adr cells were treated with puerarin 100 μM for 0.25–6 h. As shown in Fig. 6D, the phosphorylation of GSK-3β AMPK, and ACC was significantly increased, whereas CREB phosphorylation was decreased.

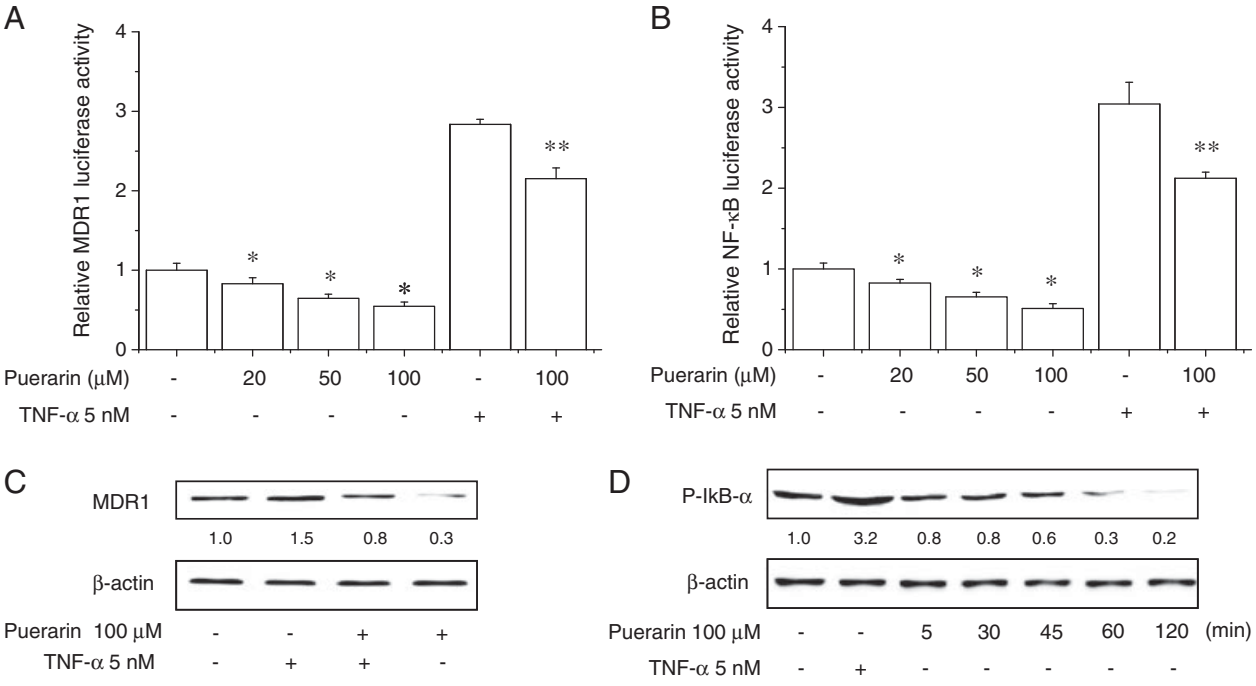

Figure 5. Effect of puerarin on activity of MDR1 and NF-κB in MCF-7/adr cells. Cells were transiently transfected with MDR1 (A) and NF-κB reporter genes (B). Following transfection, cells were treated with 20–100 μM puerarin and TNF-α for 24 h prior to lysis and measurement of luciferase activity. *Significantly different from untreated cells ($p < 0.01$), **Significantly different from TNF-α treated cells ($p < 0.01$). (C) Cells were incubated for 24 h with 100 μM puerarin and 5 nM TNF-α. Cells were then lysed and subjected to Western blot analysis using MDR1 and β-actin antibodies. (D) MCF-7/adr cells were incubated with 100 μM puerarin for 5–120 min followed by TNF-α for 30 min. The cells were lysed and subjected to Western blot analysis using phospho-IκB-α and β-actin antibodies. Puerarin time-dependently inhibited phospho-IκB-α protein levels.

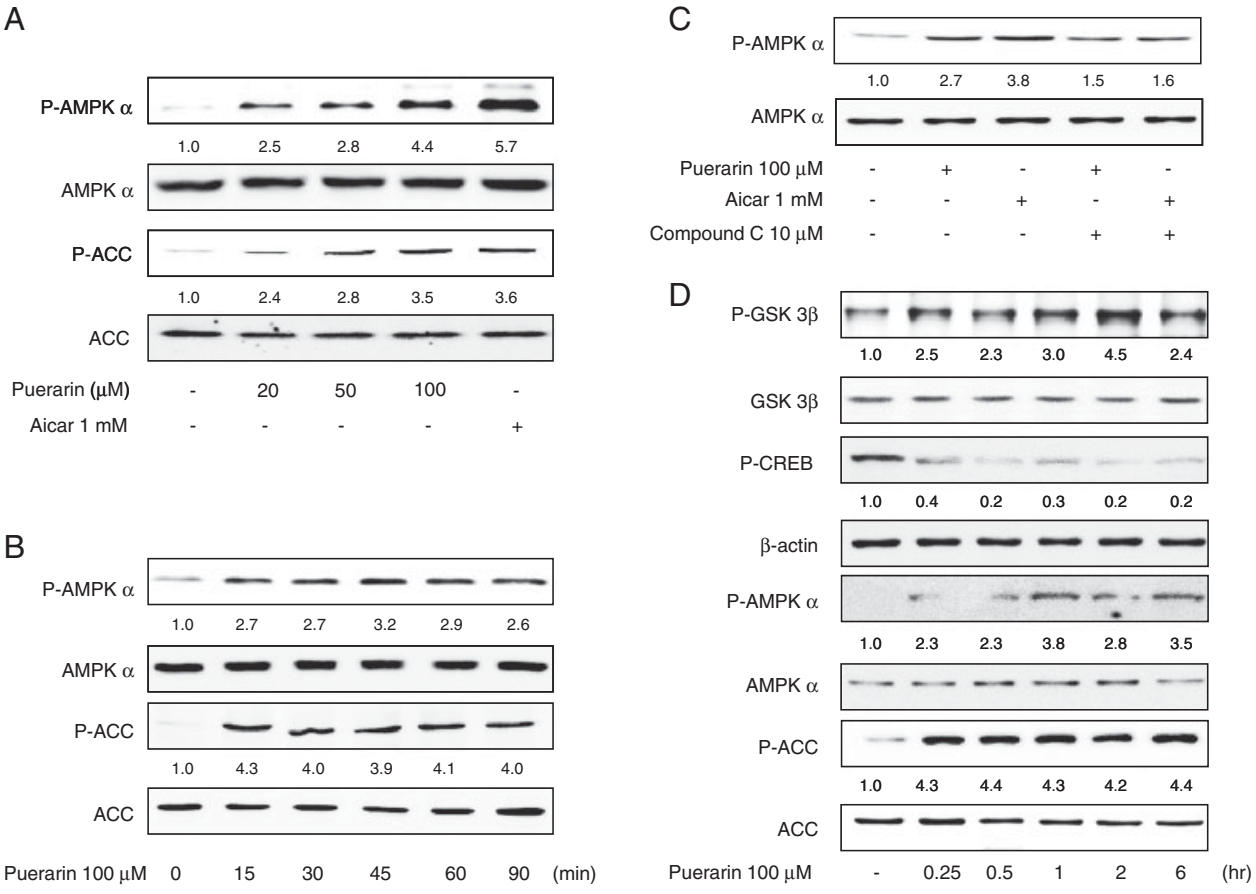

Figure 6. Puerarin induced AMPK, ACC, GSK-3β phosphorylation, and inhibited CREB phosphorylation. (A) MCF-7/adr cells were treated at various doses of puerarin and puerarin 100 μM for the indicated time (B), phosphorylation of AMPK and ACC were detected by Western blot analysis. (C) MCF-7/adr cells were treated for 30 min with 10 μM compound C (AMPK inhibitor) and then treated with puerarin 100 μM and 1 mM aicar for 30 min. Phosphorylation of AMPK and AMPK were detected by Western blot analysis. (D) Western blot analyses of time-dependent effects were performed with MCF-7/adr cells extracts prepared using phospho-AMPK, AMPK, phospho-ACC, ACC, phospho-GSK-3β (Ser 9), GSK-3β, phospho-CREB (Ser 133), and β-actin antibodies after treatment of puerarin 100 μM for 0.25–6 h.

3.8 MDR1 suppression by puerarin involves AMPK pathway

To further elucidate the effect of AMPK pathway on MDR1 suppression by puerarin, MCF-7/adr cells were treated with 100 μM puerarin and 10 μM compound C. As shown in Fig. 7A, puerarin-inhibited MDR1 expression was abrogated by co-treatment of puerarin and compound C, whereas compound C alone did not affect MDR1 expression. Similarly, MDR1 promoter activity was abrogated by co-treatment with puerarin and compound C (Fig. 7B). These results indicate that puerarin suppression of MDR1 involves the AMPK pathway.

3.9 Inhibition of MDR1 by puerarin *via* CRE transcriptional activity through up-regulation of AMPK

To further elucidate the signaling pathway involved in puerarin inhibition of MDR1, CREB and induction of AMPK, ACC, GSK-3β phosphorylation, MCF-7/adr cells were treated with 100 μM puerarin and 10 μM H89 for 30 min or 24 h for detection of AMPK and MDR1 expression, respectively. As shown in Fig. 8A, phosphorylation of AMPK was increased by puerarin and H89. In addition, MDR1 expression was inhibited by puerarin alone, H89 alone or co-treatment of puerarin and H89 (Fig. 8B). Similarly, Fig. 8C shows that CRE transcriptional activity was also decreased by puerarin and H89, but co-treatment of puerarin and forskolin (CRE activator) remarkably decreased the activity of CRE transcriptional compared with forskolin alone. These results suggest that puerarin inhibits MDR1 *via* CRE transcriptional activity through up-regulation of AMPK.

4 Discussion

MDR, the principal mechanism by which many cancers develop resistance to chemotherapy drugs, is a major factor in the failure of many forms of chemotherapy [1, 6]. It affects

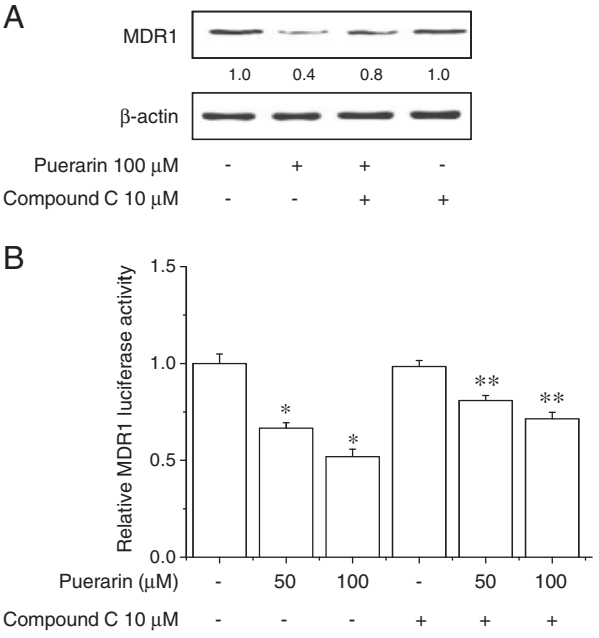

Figure 7. Involvement of AMPK pathway in MDR1 inhibition by puerarin. (A) The effect of puerarin and compound C on MDR1 expression in MCF-7/adr cells. Cells were incubated for 24 h with 100 μM puerarin and 10 μM compound C. Cells were then lysed and subjected to Western blot analysis using MDR1 and β-actin antibodies. (B) Effect of puerarin and compound C on activity of MDR1 in MCF-7/adr cells. Cells were transiently transfected with an MDR1 promoter reporter gene. Following transfection, cells were treated with 50 or 100 μM puerarin and 10 μM compound C for 24 h prior to lysis and measurement of luciferase activity. *Significantly different from untreated cells ($p<0.01$), **Significantly different from compound C-treated cells ($p<0.01$).

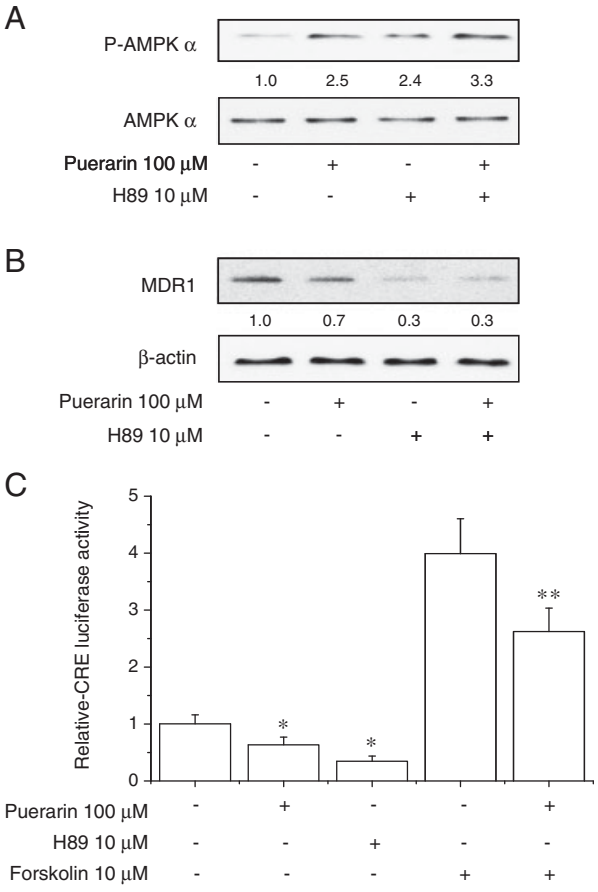

Figure 8. Puerarin inhibited MDR1 *via* CRE transcriptional activity. (A) Effect of puerarin and H89 on phosphorylation of AMPK. MCF-7/adr cells were incubated with 100 μM puerarin and 10 μM H89 for 30 min. The cells were lysed and subjected to Western blot analysis using phospho-AMPK and AMPK. (B) Effect of puerarin and H89 on expression of MDR1. MCF-7/adr cells were incubated with 100 μM puerarin and 10 μM H89 for 24 h. The cells were lysed and subjected to Western blot analysis with MDR1 and β-actin antibodies. (C) Cells were transiently transfected with CRE reporter gene. Following transfection, cells were treated with 100 μM puerarin, 10 μM H89 and 10 μM forskolin for 24 h prior to lysis and measurement of luciferase activity. *Significantly different from untreated cells ($p<0.01$), **Significantly different from forskolin-treated cells ($p<0.01$).

patients with a variety of blood cancers and solid tumors including breast, ovarian, lung, and lower gastrointestinal tract cancers. Thus, the discovery of novel natural products and their components that block MDR1 is a goal of cancer researchers. Since the first MDR1 inhibitor, verapamil, was found in 1981, a variety of agents including cyclosporin A and others have been reported as agents for overcoming MDR [37, 38]. However, due to dose-limiting toxicity, the clinical trial results of these drugs are very disappointing so far. It is widely known that natural compounds found in fruits, vegetables, oilseeds, and herbs have anticancer, antiviral, and antioxidant properties have been defined as a new catalog of chemopreventive agents [18, 19, 20, 25]. The aim of this study was to find an effective MDR-reversing agent from natural products that might have fewer side effects and to gain insight into the reversal effect and molecular mechanism. Previous studies reported some flavonoids and natural phytochemicals inhibited MDR1 expression [19, 21, 22]. In the present study, we demonstrated that puerarin, an isoflavonoid from *P. lobata*, is a traditional oriental herbal medicine [23] that reversed MDR1 in MCF-7/adr cells.

Adriamycin was selected for development of MDR1 in MCF-7/adr cells, based on the suggestion of some studies reporting that adriamycin caused MDR1 in breast cancer cells [31, 32]. Puerarin was not toxic at concentrations from 20–200 μM (Fig. 2A) and it enhanced the cytotoxicity of adriamycin in MCF-7/adr cells by MTT assay and LDH assay (Figs. 2B and C). MDR1 increased at both the mRNA and protein levels when MCF-7/adr cells developed resistance but decreased significantly upon puerarin treatment. To further explore the reduction of MDR1 mRNA by puerarin, MCF-7/adr cells were transfected with MDR1 promoter. The activity of MDR1 promoter was

dramatically inhibited by puerarin, as estimated by luciferase assay (Fig. 5A). Reduction of the expression of MDR1 at both the transcriptional and translational levels may be one of the mechanisms by which certain modulators or agents reverse MDR phenotype [6]. Furthermore, increased intracellular accumulation of Rh-123 by puerarin through inhibition of the drug efflux function of MDR1 was observed. The efflux of fluorescent Rh-123 is known to be MDR1-dependent and has consequently been used extensively to assess efflux from drug-resistant cell lines expressing MDR1 [33, 34].

MDR expression has been studied in cancer cells such as MCF-7/adr cells [19, 39], K562/ADM [21], A549 [22], HepG2/ADM [40], Caco-2 [41] and *in vivo* [9, 10]. The molecular mechanisms of induction of MDR expression were reported to correlate with NF-κB activation [9–11], Cyclooxygenases-2 [42], CYP3A4 [43], reactive oxygen species [9], mitogen-activated protein kinase pathway [44], phosphatidylinositol-3-kinase (PI3k) extracellular signal-regulated kinase kinase (MEK) -extracellular signal-regulated kinase (ERK) -ribosomal S6 kinase (RSK) pathway [11, 44], and protein kinase C [45]. Among them, NF-κB activation is the molecular mechanism of induction of multidrug resistance expression mentioned most frequently.

Previous studies demostrated that a NF-κB site located at position -159 of the MDR1 promoter is involved in the activation of MDR1 by growth factors [46] and the generation of reactive oxygen species, activation of IκB kinase, and degradation of IκB-αIκB-βresult in activation of NF-κB [9]. Furthermore, the induction of MDR1 expression is mediated by an NF-κB activating signal that requires a NF-κB binding site located distal to the MDR1 promoter [11]. The current experiments were designed to determine a possible role of NF-κB activation in puerarin-suppressed MDR1 expression, and puerarin was found to inhibit NF-κB activation through degradation of IκB-α (Fig. 5). Our results suggest that puerarin inhibited MDR1 in MCF-7/adr cells through inhibition of NF-κB activation.

Until now, no evidence had been reported of a correlation between MDR1 expression and AMPK pathway. Several reports have demonstrated that MDR1 expression can be modulated by PKA [13, 14]. Moreover, PKA inhibitor (H89) increased phosphorylation of AMPK (Thr 172) [47] and inhibited MDR1 [48]. The ubiquitously expressed CREB and closely related factors represent transcription factors were activated by PKA [49]. Previous study showed that GSK-3β phosphorylates CREB [36, 50] and Lim *et al.* 2008 reported that GSK-3β inhibitor increased MDR1 [51]. However, MDR1 expression by GSK-3β remains unclear until now. We considered the possibility that GSK-3β contributes to AMPK-induced MDR1 gene suppression. In our study, treatment of MCF-7/adr cells with puerarin increased phosphorylation of AMPK, ACC and GSK-3β but inhibited CREB (Fig. 6D). To address the role of AMPK, PKA, and CRE transcriptional activity in the effect of puerarin on MDR1 expression in MCF-7/adr cells, we examined the effect of compound C, H89, and forskolin on MDR1 expression and activity. The data showed that inhibitor of AMPK (compound C) blocked puerarin-induced phosphorylation of AMPK. Similarly, transient transfection assay and Western blotting analysis also demonstrated that the decrease of MDR1 activity by puerarin involved AMPK (Figs. 7A and B). Furthermore, MDR1 expression and CRE activity were inhibited by puerarin and H89 (Fig. 8C). These data suggested that puerarin inhibited MDR1 in MCF-7/adr cells through AMPK pathway and CRE transcriptional activity. As indicated, we identified a new molecular mechanism for puerarin reversal of MDR in MCF7-adr cells *via* AMPK activation and CRE transcriptional activity through GSK-3β, apart from inhibition of NF-κB activation.

In conclusion, our data strongly imply that puerarin inhibited MDR1 in MCF-7/adr cells. First, puerarin suppressed MDR1 *via* inhibition of NF-κB activation. Second, puerarin suppressed MDR1 *via* CRE transcriptional activity through up-regulation of AMPK. And third, these regulations may trigger puerarin to block MDR1 expression. Thus, this study has provided a natural potent inhibitor of human MDR1, *i.e.* puerarin and this finding may bring direct and immediate clinical benefits *via* more effective and safer cancer chemotherapy treatments in human breast cancer MCF-7/adr cells.

The authors have declared no conflict of interest.

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