RESEARCH ARTICLE

# Apigetrin induces erythroid differentiation of human leukemia cells K562: Proteomics approach

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**Scope:** Induction of cancer-cell differentiation is an alternative approach for cancer chemotherapy. There are numerous studies that diets containing an abundance of fruits and vegetables have protection against cancers, and the main agents thought to provide such protective effect are flavonoids. In this study we used apigetrin as a possible cell differentiation inducer and chronic leukemia cells K562 for their pluripotent differentiating potency.
**Methods and results:** Prolonged treatment with 75 μM apigetrin induced erythroid differentiation of K562 cells with specific marker glycophorin A expression and fetal hemoglobin synthesis in treated cells, which was accompanied with $G_2$/M arrest. Proteomics data revealed the downregulation of several proteins expression involved in cell cycle regulation, protein synthesis and nuclear import and export of signaling molecules.
**Conclusion:** This is the first evidence that natural compound apigetrin may induce cancer cell differentiation thus could be one of the possible explanations of its antitumor effects.

Received: December 21, 2010
Revised: January 18, 2011
Accepted: February 21, 2011

**Keywords:**
Apigetrin / Cell cycle / Differentiation / Leukemia / Proteomics

## 1 Introduction

Numerous studies have confirmed the anti-tumor effects of a variety of nutrients as well as non-nutritive compounds found in plants, with different cell culture systems and animal tumor models. The principle plant-derived agents thought to provide protection against cancer are flavonoids, which are the most common and widely distributed polyphenolic compounds [1, 3–5]. Apigenin is one of the representatives of such compounds; it abundantly presents in common fruits and vegetables such as parsley, onions, oranges, tea, wheat sprouts, olive oil and other foods. Since Birt et al. first demonstrated anti-tumor property of apigenin, this flavonoid has been intensively studied as a chemopreventive and/or chemotherapeutic agent [6]. Apigenin has been shown to possess anti-inflammatory effects, free radical scavenging properties and growth inhibitory activities in several cancer cell lines, including breast, colon, skin, thyroid, pancreatic and leukemia cells [6–10]. A broad range of molecular signaling pathways were shown to be involved in its anti-carcinogenic effects. Apigenin has been reported to inhibit protein kinase C activity, mitogen-activated protein kinase activity, modulate PI3K/AKT pathway and increase caspases release [1, 5]. Apigenin is well known for its non-mutagenic properties. There is very little evidence to date to suggest that apigenin promotes adverse metabolic reactions in vivo when consumed in nutritionally relevant quantities [10]. Study with the single oral administration of radiolabeled apigenin have shown that 24.8% of it would still present in rat body even 10 days after administration, indicating its slow excretion and possible accumulation in body tissues [11]. Chen et al. have also reported that apigenin has a slow elimination phase and potential to accumulate in the body [12]. The pure form of apigenin is unstable but in natural form in food it is present mostly as

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**Abbreviations:** **Ara-C,** cytosine arabinoside; **eEF-Tu,** elongation factor Tu; **glyA,** glycophorin A; **IPG,** immobilized pH gradient; **PE,** phycoerythrin; **RanBP1,** Ran-binding protein 1; **RanGAP1,** Ran GTPase-activating protein 1; **RCC1,** regulator of chromosome condensation 1; **RT-PCR,** real-time PCR

 

glucoside conjugate, which is called apigetrin. The conjugated form of apigenin could be an important determinant for its bioavailability and absorption. For instance, quercetin, another flavonoid, has been shown to have much better absorption in humans in its glycoside form rather than its pure one [1].

Leukemias represent a large proportion of hematological malignancies that occur as acute and chronic disease. Normally, the hematopoietic stem cells in bone marrow respond to stress such as the loss of blood, by giving rise to progenitor cells. These cells differentiate to mature circulating blood cells such as erythrocytes. Commitment of progenitor cells to differentiate is accompanied by cell growth arrest and irreversible maturation. In leukemias, the hematopoietic stem cells or progenitors do not respond to external signals to undergo differentiation to distinct blood cells; instead they grow in numbers in not matured form becoming the malignant disease. The failure of cells to mature makes leukemia the disorder of cell differentiation. If tumor cells can be forced to differentiate and to cease proliferation, their malignant potential can be controlled. So-called differentiation therapy was proposed and the paradigm of success of such approach was introduced with *all*-trans retinoic acid against acute myeloid leukemia [2]. Although a number of agents have been studied over years in most cases they are not targeted or they lack efficacy or possess toxic effects during the widespread long-term use.

We speculate that dietary compounds with slow elimination like apigenin may have chemopreventive property against carcinogenesis not only by inducing apoptosis but also in different ways such as turning abnormal cells back to differentiation. We used chronic leukemia K562 cells to elucidate our hypothesis; these cells are known for their pluripotent differentiating potency and are considered as a useful model to study cell differentiation mechanisms. To identify novel important players in the process of cell differentiation, we embraced the proteomics approach in this study.

## 2 Materials and methods

### 2.1 Cell culture and treatments

Human chronic leukemia cell line K562 was purchased from Riken Cell Bank (Tsukuba, Ibaraki, Japan) and maintained in RPMI 1640 medium (Gibco) supplemented with 10% v/v heat-inactivated fetal bovine serum (Hyclone) at 37°C in a humidified 5% $CO_2$ incubator. Erythroid differentiation was induced by the addition of 75 μM apigetrin (Sigma) to the cell suspension. Medium and apigetrin (75 μM) were renewed every 3 days of the cell culture. DMSO-dissolved apigetrin at 100 mM was stored at −20°C and was freshly diluted in culture medium immediately before use.

### 2.2 Determination of cell cycle distribution and apoptosis

To determine cell cycle phase distribution, the time-dependent change in K562 cell cycle kinetics was analyzed by flow cytometry (Guava Technologies). In total, $2.0 \times 10^4$ cells/mL were seeded in culture dish and treated with 25–100 μM apigetrin for indicated times. After treatment, control (0.075% vehicle-treated) and treated K562 cells were washed with PBS and fixed with 70% ethanol at 4°C for more than 12 h. The fixed cells were centrifuged at $500 \times g$, 25°C for 5 min, the supernatant was removed and the cells were washed with PBS. Cell cycle reagent (Guava Technologies) was added at 400 μL, followed by incubation in darkness for 30 min before measurement. Using this method, the population of cells exhibiting sub-$G_1$ DNA content representing the fraction of apoptotic cells in the culture can be determined.

### 2.3 Cell viability assay and observation of morphological changes

The viability of cells was determined by trypan blue staining. K562 cells were seeded into 100 mm dish ($2.0 \times 10^4$ cells/mL) and incubated with 75 μM apigetrin for indicated times. Cells were harvested, stained by trypan blue and viable and nonviable cells were quantified using Countess automatic cell counter (Invitrogen). Also, the average cell size was determined with the same equipment. Cellular morphology was observed by a phase contrast microscope (Leica Microsystems).

### 2.4 Determination of cell differentiation marker expression

Cell differentiation was scored by the expression of surface markers using flow cytometry as described before [13]. Briefly, $2.0 \times 10^4$ cells/mL in culture dishes were treated with the compound and incubated for 3, 6 and 9 days at 37°C in a humidified 5% $CO_2$ incubator. Cells were counted by Countess automatic cell counter and adjusted to the same number. After washing with PBS, $1.0 \times 10^5$ cells were labeled with R-Phycoerythrin (PE)-Cyanine 5 conjugated anti-CD11b (CD11b) (Coulter), PE-conjugated anti-CD14 (CD14) (Guava Technologies), PE-conjugated anti-CD41 (CD41) (Abcam) and PE-conjugated anti-glycophorin A (Abcam) antibodies for 30 min according to manufacturer's instructions, washed twice with ice-cold PBS, and finally resuspended in 600 μL PBS for measurement.

### 2.5 Total RNA isolation, cDNA synthesis and real-time PCR

DNA-free total RNA of cultured cells was isolated with Isogen kit (Molecular Research Center, Japan) according to

 

manufacturer's instructions. Briefly, cells were seeded at $2.0 \times 10^4$ cells/mL and treated with 75 μM apigetrin for indicated times. Isolated RNA was ethanol precipitated, quantified and quality assessed by Nanodrop ND-1000 spectrophotometer. About 1 μg of total RNA was submitted to reverse transcription with Superscript III (Invitrogen) using oligo(dT) primers according to manufacturer's protocol and cDNA was synthesized using thermal cycler (Applied Biosciences). Taqman master mix and GATA-1 taqman probe (Applied Biosystems) were used for real-time PCR (RT-PCR) and actin-b taqman probe (Applied Biosystems) was used as an internal control. cDNA amplification reactions were run on Applied Biosystems 7500/7500 fast RT-PCR system.

### 2.6 Protein isolation and quantification for proteomics

Treated and control cells were washed in cold PBS two times and lyzed in lysis buffer (7 M urea, 2 M thiourea, 4% w/v 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane sulfonate, 25 mM spermine base, 50 mM DTT, 1 mM EDTA) at room temperature for 1 h. The homogenate was centrifuged at $170\,000 \times g$ for 100 min at 4°C. The supernatant was assayed for protein content by Plus One 2D Quant kit (GE Healthcare) and stored at −80°C.

### 2.7 IEF and SDS-PAGE

First dimension was carried out using the Ettan IPGphor II (Amersham Biosciences). Aliquots of samples containing equal quantities of protein (400 μg) were diluted to 350 μL with rehydration buffer (7 M urea, 2 M thiourea, 2% w/v 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane sulfonate, 1% bromophenol blue stock (0.002%), 0.5% v/v immobilized pH gradient (IPG) buffer, 40 mM DTT) and subjected to IEF after overnight rehydration on Immobiline DryStrips (24 cm; pH 3–10 linear). Focusing was performed as follows: 1 h at 500 V, 1 h at 1000 V, 3 h at 10000 V and 2 h 45 min at 10 000 V for a total of 56 000 kVh. After IEF, IPG strips were equilibrated twice: first, 15 min in 10 mL of equilibration buffer supplemented with 100 mg of DTT and then for a further 15 min with 250 mg of iodoacetamide. Equilibrated strips were transferred onto 10% vertical slab gels and SDS-PAGE was run at 280 W × h on EttanDaltSix electrophoresis system (Amersham Biosciences).

### 2.8 Gel image analysis

Separated proteins were stained with Coomassie R-350 stain (GE Healthcare) in 30% methanol and 10% acetic acid. Gels were scanned at 600 dpi resolution and differential spot expression was performed using ImageMaster™ 2D Platinum 5.0 software (Amersham Biosciences). Experimental parameters were fixed for all the gels (smooth = 2; minimum area = 5; saliency = 50.000) and followed by automatic spot detection and matching. The authenticity and outline of each spot was validated by eye and edited manually where necessary. The pairs were labeled with annotations that define tie points for gel matching. Spot-normalized volume defines the volume of a given spot as a percentage of the volume of all spots in the gel. The normalized volume for each spot in the control gel was compared to the normalized volume of matched spot in the sample gels.

### 2.9 In-gel digestion and peptide analysis

Spots were excised and treated with the destaining solution (25 mM ammonium bicarbonate $NH_4HCO_3$ in 50% ACN). Gel pieces were washed in 100 μL ACN for 5 min and briefly dried at room temperature followed by reduction with 10 mM DTT in 25 mM $NH_4HCO_3$ and alkylation with 55 mM iodoacetamide in 25 mM $NH_4HCO_3$. Spots were digested overnight by 10 μg/mL trypsin in 50 mM $NH_4HCO_3$ at 37°C. Supernatants, which contain digested peptides, were carefully collected into a 1.5 mL tube. Twenty microliters of extraction buffer (50% v/v ACN, 5% v/v formic acid) was added to the digested spots and the samples were incubated for 10 min under gentle shaking. Supernatants were carefully removed from the tube and the process was repeated once again. Collected supernatants were concentrated using a vacuum centrifuge to a final volume of 20 μL.

Ten microliters of peptide mixtures were analyzed by online capillary UltiMate 3000 proteomics MDLC system (Dionex) coupled to a nanospray 3200QTrap MS/MS system (Applied Biosystems). Peptides mixtures were loaded in 0.1% formic acid onto a 300 μm id × 5 mm C18 PepMap100 trap column. The peptides were eluted and separated from the trap column using 0.1% formic acid in ACN on a 75 μm id × 5 mm C18 PepMap100 at a flow rate of 300 nL/min. Spray voltage was set to 2.0 kV to apply separated fractions to the mass spectrometer. The MS operated in positive ion mode using Analyst 1.4.1 software (Applied Biosystems) and data acquisition was set to cover a scan range of *m/z* 400–1200 followed by two MS/MS scans. Tandem mass spectrum WIFF files were converted to MGF files using Analyst 1.4.1 software and the peaks were searched using MASCOT search engine against Swiss-Prot database consisting of *Homo sapiens* sequences. The search settings were as follows: missed cleavage site value set to one, fixed carbamidomethylation of cysteine and variable oxidation of methionine. The proteins with ion scores greater than 34 were significant for the Swiss-Prot database ($p < 0.05$).

### 2.10 Statistical analysis

Data are expressed as the mean ± SD. Statistical analysis was performed by Student's *t*-test with significance of $p < 0.05$.

 

## 3 Results

### 3.1 Apigetrin induces $G_2$/M cell cycle arrest dose-dependently

Cell proliferation was inhibited with apigetrin treatment in a dose-dependant manner (data not shown), which was correlated with the induction of cell cycle arrest. Flow cytometric analysis of DNA content revealed that untreated K562 cells at $G_0/G_1$, S and $G_2$/M phase represented approximately 50.6, 16.0 and 31.4% of the total cell population, respectively (Fig. 1A). Exposure to different doses of apigetrin for 3 days resulted in marked accumulation of cells in $G_2$/M phase (approximately 78% of total cells at 75 μM) and this observation was dose-dependant. (Figs. 1A and B). During the prolonged treatment of K562 cells with 75 μM apigetrin cell cycle arrest pattern remained but a small population of cellular fragments at sub-$G_0$ become visible from day 6, indicating apoptosis in some cells. Nevertheless, viability of treated cells was still in the range of 70% after 9 days of treatment (Fig. 1C).

### 3.2 Apigetrin affect K562 cell morphology

The effect of apigetrin on K562 cells was also investigated by morphological analysis using light microscopy. Numerous large cells were visible after treatment, the average cell size reaching to approximately $15.7 \pm 0.7$, $15.3 \pm 1.0$ and $16.1 \pm 1.4$ μm after 3, 6 and 9 days, respectively (Figs. 2A and B), which is about 1.3–1.4 times bigger than that of control cells (11.5–12 μm). Careful morphological observation revealed that from 6 days of treatment some small cells were visualized among the numerous large cells (Fig. 2B). These cells had a distinct morphology and resembled erythroid cells.

### 3.3 Apigetrin induce erythroid differentiation of K562 cells

Morphological observation as well as cell proliferation arrest of K562 cells after treatment with apigetrin prompted us to investigate if the cells could be differentiating. K562 cells are

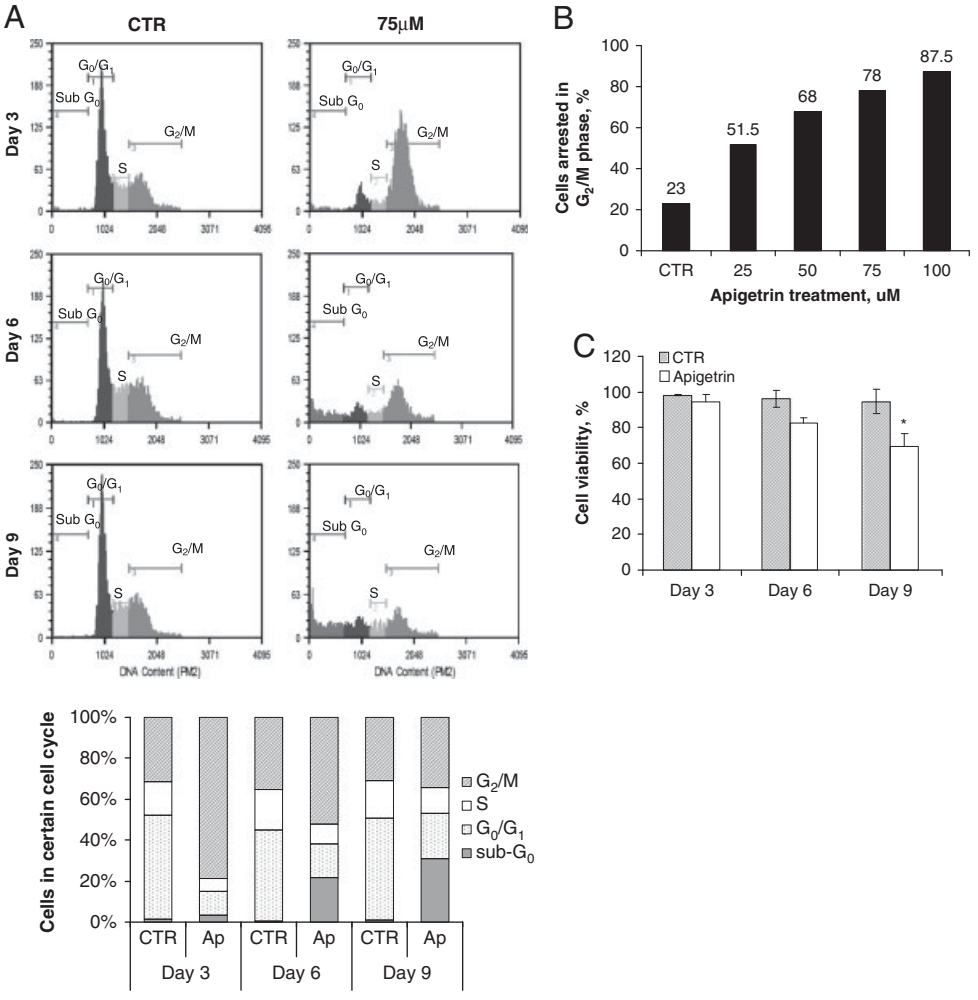


**Figure 1.** Cell viability and induction of cell cycle arrest of K562 cells after apigetrin treatment. (A) Apigetrin caused significant $G_2$/M arrest in treated cells (75 μM) compared to control cells (treated with 0.075% vehicle). No detectable sub-$G_0$ phase was observed after 3 days with apigetrin treatment but subcellular fragments become visible from day 6, indicating some cells were undergoing apoptosis. (B) $G_2$/M cell cycle arrest was concentration-dependent in apigetrin-treated cells (25–100 μM). (C) Cell viability was similar to control cells after 3 days with 75 μM apigetrin treatment but subsequent treatments with apigetrin induced reduction in viability of treated cells. Data of three independent experiments are presented.

 

well known for their pluripotency; blocked in their normal maturation at an early stage of differentiation they may exhibit granulocytic, megakaryocytic and erythroid features [14]. Many differentiating agents such as hemin, antracyclines, butyric acid and cytosine arabinoside (Ara-C) induce erythroid differentiation of K562 cell line [14, 15]. We used four markers for cell differentiation analysis: CD11b for granulocyte detection, CD14 for monocytes, CD41 for megakaryocytes and glycophorin A for erythroid cell detection. Even after 6 days of treatment with 75 μM apigetrin we could not detect the expression of granulocyte, monocyte and megakaryocyte markers (Figs. 3A–C). However, erythroid marker expression was clearly induced after 6 days, indicating that K562 cells were differentiating to erythroid lineage (Fig. 3D). Ara-C (25 μM) was used as a positive control to assess the extent of differentiation. Induction of differentiation with 75 μM of apigetrin treatment for 6 days was almost 50%, which is comparable with 3 days Ara-C treatment (60%). Also, it was evident by visual observation that pellet of treated cells turned to reddish pink color compared to the control (Fig. 3E). At 25 μM apigetrin we could not induce cell differentiation significantly even after longer incubation time.

### 3.4 Apigetrin upregulate mRNA expression of erythroid transcription factor GATA-1

During erythroid differentiation globin gene expression is upregulated, which induces synthesis of hemoglobin protein, the distinct marker of erythrocytes. Our results confirm this phenomenon with the change of cell pellet color and expression of fetal γ-hemoglobin as well as globin proteins as revealed by 2-D electrophoresis (Table 1) in apigetrin-treated cells. Since globin synthesis was promoted by apigetrin we investigated its effect on the major erythroid transcription factor GATA-1. It is well established that all known erythroid genes are regulated by GATA-1 and contain GATA-1-binding motifs in their promoters and/or enhancers [16]. As shown in Fig. 4, GATA-1 mRNA levels were transiently increased. Indeed, a 3.0-fold increase was observed after 3 days treatment whereas at 6 days mRNA expression reached 5.0-fold increase compared to control.

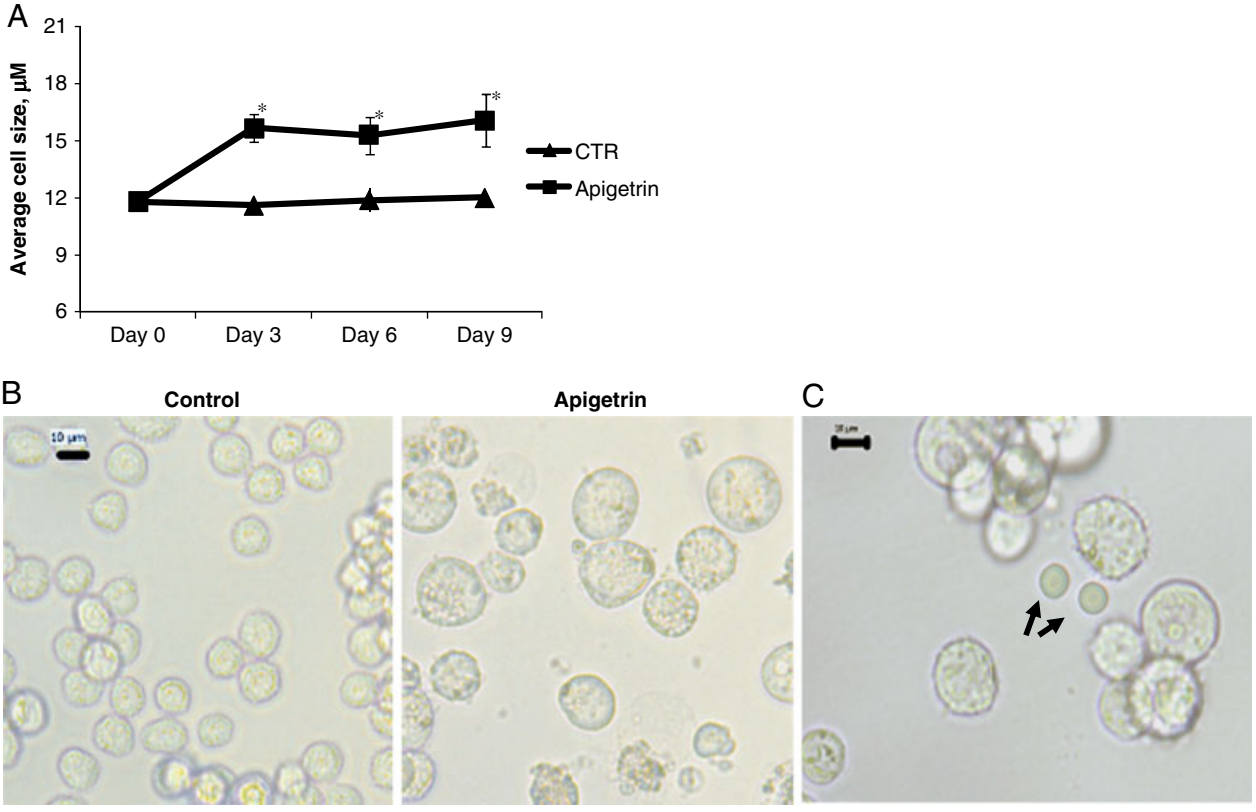


**Figure 2.** Morphological observations and change in cell size in K562 cells after 75 μM apigetrin treatment. (A) The average size of control cells were 11.6 ± 0.2 μm throughout the experimental period whereas treated cell size increased up to 15.7 ± 0.7, 15.3 ± 1.0 and 16.1 ± 1.4 μm after 3, 6 and 9 days treatment with apigetrin. (B) Average cell size increased from day 3. Scale bars represent 10 μm. (C) Morphological observation of K562 cells after 6 days apigetrin treatment. Some small cells (arrows) with distinct features were visualized among the numerous large cells. Scale bars represent 10 μm.

 

### 3.5 Proteomic analysis of induced erythroid differentiation

Despite the numerous important discoveries in the complex process of cell lineage commitment, our understanding of cellular differentiation is still very limited. To identify novel important factors in the process of induced erythroid differentiation we utilized proteomics approach and analyzed protein expression changes occurring in K562 cells after 2 days (as an early stage of differentiation) and 9 days (as the most cells are committed to differentiate) of induction with apigetrin.

Figure 4 shows CBB-stained 2-D gels of K562 cells at 0, 2, and 9 days after the cells were induced with apigetrin. The expression patterns of more than 300 protein spots were followed through the entire series of gels. Only those spots whose expression was constantly changed throughout the time of treatments were selected for analysis (Fig. 5).

The protein spots in the different gels were identified by LC/MS/MS and MALDI-TOF on the basis of peptide mass matching with the theoretical peptide masses in tryptic digests of all known proteins of human species. Seven differentially expressed proteins with their theoretical and observed p*I* and $M_r$ values, peptide sequence coverage and scores are presented in Table 1.

## 4 Discussion

Natural products, such as flavonoids, are emerging as potent cancer prevention and chemotherapeutic agents. The flavone apigenin broadly found in many fruits and vegetables has been previously shown to induce cell death in a number of human cancer cell lines. Apigenin has been reported to act via several mechanisms, including promotion of apoptosis, inhibition of cell transformation, inhibition of mutagenesis and suppression of signal transduction, gap junction function and angiogenesis [17]. Apigenin also causes $G_2$/M arrest in a number of cells such as murine keratinocytes through increased p53 protein stability and increased expression of p21 [18], human pancreatic cancer cell lines AsPC-1, CD 18, MIA PaCa2 and S2-013 by suppressing cyclin B-cdc2 activities [7]. In this study we used apigetrin, the naturally present glucoside form of apigenin, and confirmed that it induces $G_2$/M arrest in chronic leukemia cells K562. The glucoside forms of flavonoids are highly stable; however, there are few studies on their pharmacological effects. Nevertheless, Srivastava and Gupta demonstrated that human prostate cancer cells PC-3 converted apigetrin to apigenin with aglycone playing a major role in anticancer effect [19]. The aglycone release is

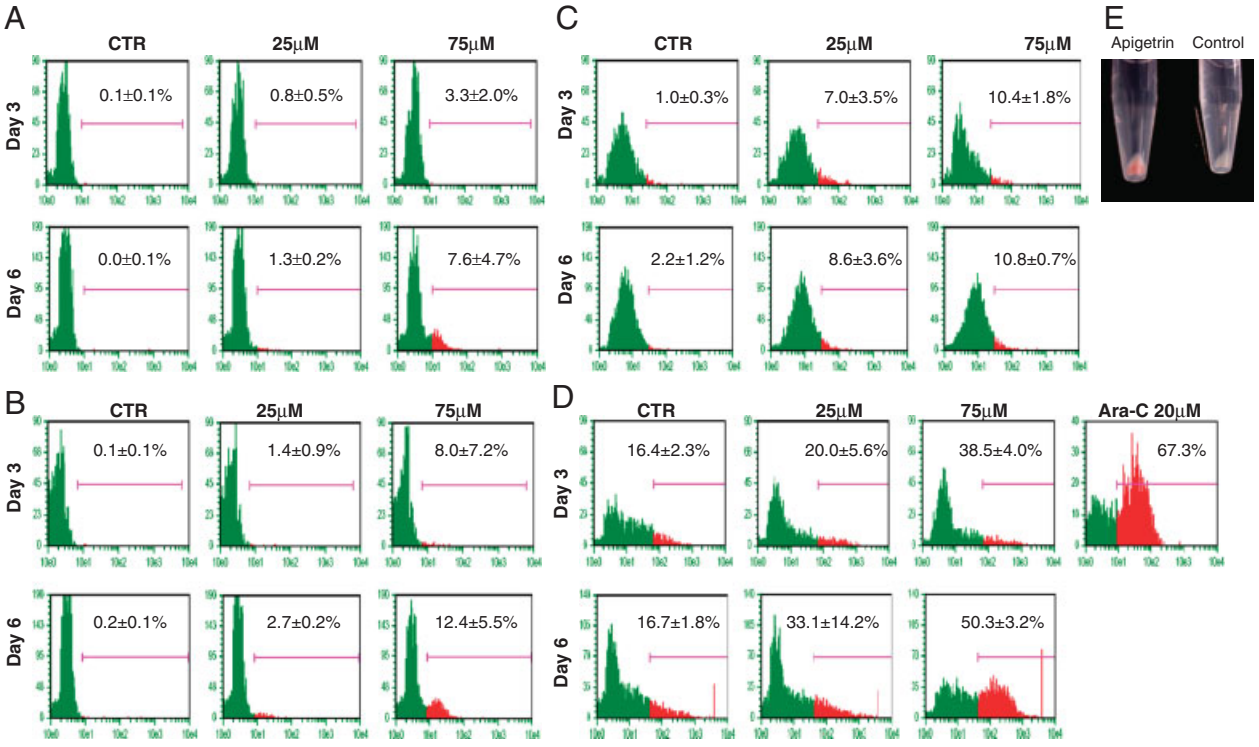


**Figure 3.** Differentiation marker analysis of K562 cells treated with apigetrin. Control cells were treated with vehicle (0.075% vehicle). Cells were incubated with 25 and 75 μM concentrations of apigetrin for indicated times. Granulocyte-specific cell surface markers CD11b (A), monocyte-specific marker CD14 (B) and megakaryocyte-specific marker CD41 (C) were not significantly expressed in control and treated cells. (D) Continuous induction with 75 μM apigetrin expressed erythroid specific marker glyA in K562 cells. Ara-C was used as a positive control for erythroid differentiation induction. (E) Cell pellet pictures after 6 days treatment with 75 M apigetrin. The total percentage of marker positive cells is indicated within each panel. Data representative of three similar experiments are shown.

 

**Table 1.** Summary of proteins identified from K562 cells after treatment with 75 μM apigetrin[a)]

| Relative expression change[b)] | Spot no. | Protein name | Observed pI/MW | Theoretical pI/MW | Seq.cov. % | Score |
|---|---|---|---|---|---|---|
| −2 | 1 | Elongation factor Tu | 8.0/48 | 7.4/47 | 47 | 325 |
| −2.5 | 2 | Multifunctional protein ADE2 | 8.2/48 | 7.0/48 | 25 | 220 |
| −3.3 | 3 | Peptidyl-prolyl *cis-trans* isomerase A | 8.0/16 | 7.7/18 | 32 | 152 |
| 2.8 | 4 | A-γ globin | 8.5/12 | 6.6/16 | 48 | 250 |
| 3.2 | 5 | Hemoglobin γ | 8.5/12 | 6.4/16.6 | 46 | 156 |
| −2.7 | 6 | Ran-binding protein 1 | 5.7/30 | 5.15/32 | 43 | 106 |
| 2.5 | 7 | 14-3-3 ζ/δ (YWHAZ protein) | 5.2/27 | 4.7/30 | 72 | 382 |

a) Identification was made by LC/MS/MS MS followed by database search with MASCOT MS/MS Ion Search (Matrix Science).
b) Protein expression changes are after 9 days of treatment.

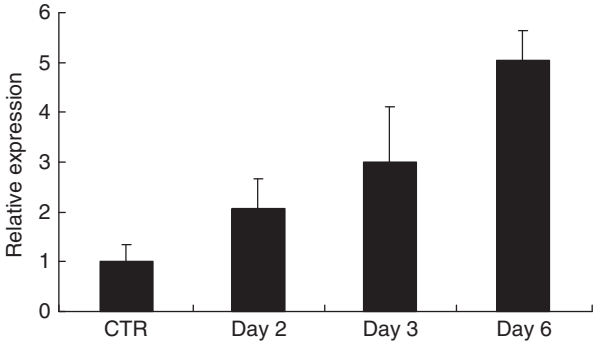


**Figure 4.** Relative expression of erythroid-specific transcriptional factor GATA-1 analyzed with RT-PCR. The bar graph plots the average expression values and standard deviations of GATA-1 mRNA levels normalized to internal control β-actin mRNA levels. Data representative of two independent experiments are shown.

explained by deglycosylation via β-glucosidase, an abundantly present enzyme in most of the cells [19, 20]. Therefore, we can assume the common pharmacological effect of apigetrin and apigenin.

Even though the majority of cells were arrested in $G_2$/M phase of cell cycle after 3 days with 75 μM apigetrin treatment, the cell viability still remained similar to control cells (Fig. 1C) and no detectable sub-$G_0$ phase was observed (Fig. 1A). Previously, several reports have shown that apigenin can induce antiproliferative activity with less cytotoxicity in some cancer cells [17, 21]. We speculated if continuous treatment with this compound may induce more pronounced effect on cancer cells. Besides, K562 cells display many similar characteristics as stem cells and are known for their strong resistance to chemical inducers as compared to other leukemia cell lines [22]. On the other hand, cell cycle arrest with less cytotoxicity could suggest cell differentiation, since it is well documented that cell differentiation is closely correlated with cell cycle arrest [22]. We continued apigetrin treatment up to 9 days with renewal of media and the compound for every 3 days. Morphological observations as well as cell differentiation marker analysis revealed that K562 cells were undergoing erythroid differentiation with continued apigetrin treatment (Fig. 2). Increase in cell size and cessation of cell proliferation was also observed during Ara-C and γ-irradiation induced erythroid differentiation in K562 cells [23]. The small proportion of apoptotic cells observed with prolonged treatment could be related to the differentiation-induced cell death. In fact, the cell viability of treated cells decreased in parallel with the expression of differentiation marker: the cells after 3 days incubation with apigetrin had less expression of marker protein and high viability whereas after day 6 significant expression of glyA was accompanied with reduced cell viability (Fig. 1C and Fig. 3D). We can also consider that differentiation-inducing effect evidenced after long time incubation with apigetrin could be due to its slow metabolizing properties. Vargo et al. have shown that in chronic leukemia cell line THP-1 apigenin was not converted to any other metabolites secreted to growth media or sequestered inside the cells. Instead, the significant fraction of apigenin was accumulated in these cells associated with cell membrane by reverse-phase HPLC experiments [8]. It is speculated that the increased accumulation of apigenin in cellular membrane might explain the slow metabolism and elimination reported for apigenin in pharmacokinetic as well as with the high $IC_{50}$ values observed with different cell lines. We continued our investigations to find out what could be the possible mechanisms of apigetrin-induced erythroid differentiation in K562 cells.

Even though there are numerous important discoveries in the complex process of cell lineage commitment, our understanding of cellular differentiation is still very limited. Classical methods such as using specific antibodies to detect known proteins by immunolabeling, observation of changes due to gene expression for specific mRNAs by PCRs have some limitation due to their difficulty in identifying proteins with no previously described function in cell signal transduction. To identify novel important factors in the process of induced erythroid differentiation by apigetrin we utilized proteomics approach and analyzed protein expression changes occurring in K562 cells.

 

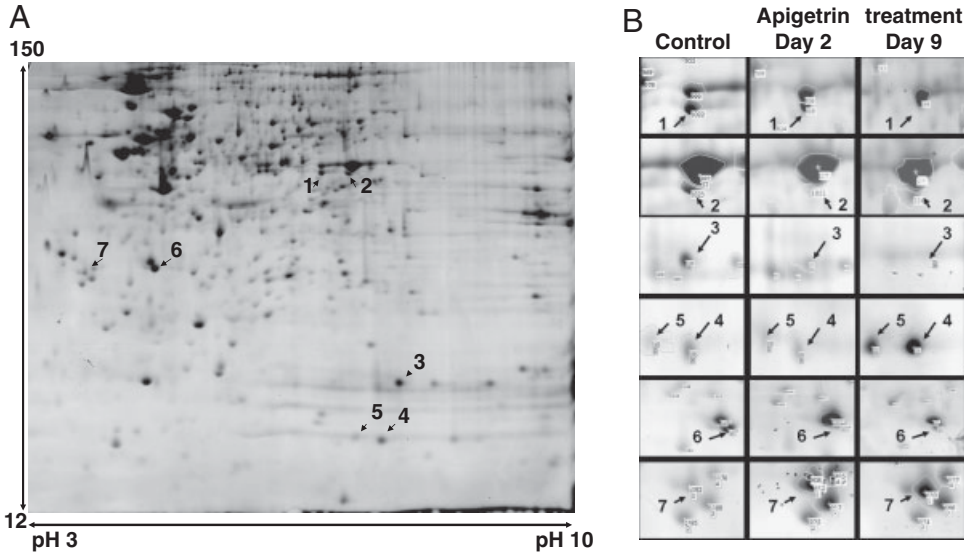


**Figure 5.** Two-dimensional electrophoretic separation of cell lysates from control and treated with 75 μM apigetrin cells. (A) IEF performed on IPG strips (pH 3–10) was followed by second dimension separation on 10% SDS-PAGE in Tris/glycine buffer system. Gels were stained with CBB. Spots having a significant difference at ratio above 2 or under 0.5 are numbered. (B) Enlarged image of differentially expressed proteins. Data representative of two independent experiments are shown.

Differentially expressed proteins found in this study were known to be associated with important cellular activities such as cell cycle control, protein synthesis and folding and nuclear transport of signaling molecules.

The 14-3-3 proteins are composed of at least seven mammalian isoforms and have a large number of binding partners. Little is known about the consequences of these binding interactions and thus are the subjects of ongoing studies [24]. 14-3-3s act as an adaptor or chaperone molecules, which are able to move freely from cytoplasm to nucleus and vice versa and interact with various cellular proteins such as protein kinases, receptor proteins, enzymes, structural and cytoskeletal proteins, proteins involved in cell cycle and transcriptional control. 14-3-3 plays a major role in cell cycle regulation: its overexpression can cause $G_2$/M cell cycle arrest [25]. We can explain $G_2$/M arrest observed in K562 with apigetrin by the expression of this protein in treated cells. Also, we cannot eliminate the fact that 14-3-3 proteins acting as chaperones could be involved in sequestration of proteins engaged in cell proliferation. Moreover, in a recent study, gene expression of 14-3-3 sigma in breast cancer cells was 7–10-fold lower than in normal breast cells, suggesting that its high level in normal cells may block the mitogenic effect of growth factors, thus controlling cell proliferation [26].

During protein synthesis in eukaryotic cells the binding of tRNA to ribosomes is prompted by elongation factor Tu (eEF-Tu) in the presence of GTP [25]. Level of this protein is shown to be decreased during erythroid differentiation with apigetrin in K562 cells in our proteomic results. Hexamethylene-bisacetamide-induced erythroid differentiation of murine leukemia cells also showed a dramatic reduction in the steady-state level of eEF-Tu mRNA as differentiation proceeded [27]. The general reduction of total cellular protein synthesis is thought to be the hallmark of cell differentiation process although the synthesis of some proteins remains unchanged, the others increase or decrease. Thus, induced to differentiate with apigetrin, cells could downregulate proteins involved in proliferation during the mitotic delay and reduce total protein synthesis, which could be the possible reason of eEF-Tu decline in differentiated K562 cells.

Cyclophilin A is a cytosolic protein abundantly expressed in all tissues. It is an enzyme catalyzing the *cis-trans* isomerization of prolyl peptide bonds and initially it was found to be the intracellular receptor protein for cyclosporin A, widely employed immunosuppressant drug in organ and tissue transplants [28]. Recently, it has been shown that cyclophillin A was overexpressed in hematological malignancies up to tenfold compared with normal peripheral blood mononuclear cells [29]. Proteomics analysis of sodium butyrate-induced erythroid differentiation of K562 cells revealed the reduction of cyclophilin A expression in treated cells [30]. This is consistent with our observations with decreased levels of cyclophilin A during apigetrin-induced erythroid differentiation of K562 cells.

Another protein significantly downregulated during K562 cell differentiation was multifunctional protein ADE2. The main function of this protein seems to be involved in nucleotide synthesis. During mouse embryonic stem cell differentiation this protein was found in the list of downregulated proteins [31] but the specific role in the process of differentiation is unknown. In our proteomics results it is downregulated in K562 cells. Study on anticancer effect of several synthetic casein kinase 2 inhibitors have shown that multifunctional protein ADE2 was one of the targets of some inhibitors in HeLa and U2OS cells [32]. Since apigenin is well demonstrated to be an inhibitor of casein kinase 2 our data may support the possibility that signaling pathway in differentiation initiation induced by apigetrin could be through this protein kinase. It is also worth to note here that maturation of erythroid cells involves elimination of

 

nucleus and thus synthesis of purine and pyrimidine bases are terminated.

The Ran GTPase system controls several cellular processes, including nucleocytoplasmic transport and cell cycle progression. Ran-binding protein 1 (RanBP1) is one of the major regulators of the Ran GTPase together with Ran GTPase-activating protein 1 (RanGAP1) and regulator of chromosome condensation 1 (RCC1) proteins. RanBP1 and RanGAP1 are cytoplasmic proteins that stimulate conversion of RanGTP to the Guanosine diphosphate-bound form and thereby depletes RanGTP from the cytoplasm whereas RCC1 functions by generating RanGTP in the nucleus. Thus, Ran GTPase system maintains a steep RanGTP gradient across the nuclear envelope with a high nuclear concentration and a very low level in the cytoplasm, which drives the nuclear import and export of signaling molecules [33]. Collapse of the RanGTP gradient was observed by inducing imbalance in these protein ratios with nuclear injection of either RanGAP1, RanBP1 or a Ran mutant that could not stably bind GTP. These treatments blocked major export and import pathways across the nuclear envelope [34]. Recent study suggests that casein kinase 2 can phosphorylate RanGAP1 protein at $S^{358}$ increasing its stabilization and favoring proper formation of RanGTPase complex [35]. With this respect apigetrin can be a good candidate to interfere with Ran cycle through inhibition of casein kinase 2 protein. Our proteomics data suggest that apigetrin may also be involved in this pathway by reducing expression of RanBP1. In addition, mRNA levels for RanBP1 was shown to be declined during Mouse promyelocytic cell line murine cell line differentiation treated with *all*-trans retinoic acid [36]. Moreover, evidence shows that both RanBP1 and RCC1 are required for $G_2$/M transition of cells [37], and since apigenin induce $G_2$/M arrest in the number of different cancer cells the probability to hinder this pathway is also possible. If that is true, the imbalance in the GTP gradient may cause the change of some molecules transport in and out of the nucleus, which may induce the changes in cell signaling leading cancer cells to cell cycle arrest and differentiation program. Surprisingly, we found an interesting report on GTP-mediated erythroid differentiation of K562 cells [37]. The authors have shown that only GTP but not ATP, CTP and UTP induced cell differentiation in this cell line; besides, the cell differentiation pattern was imitated closely to ours with the late expression of markers and hemoglobin synthesis. Furthermore, the study of Morceau et al. showed that GTP induces erythroid differentiation of K562 cells by transient overexpression of GATA-1 transcriptional factor [14].

In summary, we have shown that prolonged treatment with apigetrin induces erythroid differentiation in K562 cells. The changes in identified proteins expression from proteomics results may indicate the overall anti-tumor effect of apigetrin accompanied by cell differentiation. We propose that this cell differentiation activity of apigetrin could be due to regulating Ran cycle proteins and changing nuclear and cytoplasmic GTP distribution, thus affecting nuclear transport and localization of signaling molecules such as GATA-1. The change in the expression of proteins such as eEF-Tu, which use GTP for its functions, and decline of multifunctional protein ADE2 may indicate the decrease in total protein synthesis as a consequence of diminished nuclear transport. Also, upregulation of 14-3-3 proteins may indicate the sequestration of unnecessary cytoplasmic proteins, which again well correlates with reduced protein synthesis. Our study may give insight into how apigetrin may interfere with nuclear transport system playing important roles in cell decisions such as cell differentiation. Further studies on this issue will provide more information to understand the exact role of apigetrin in tumor prevention.

*The authors have declared no conflict of interest.*

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