BIOCHEMICAL PHARMACOLOGY 77 (2009) 384–396

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/biochempharm

Regulation of genistein-induced differentiation in human acute myeloid leukaemia cells (HL60, NB4) Protein kinase modulation and reactive oxygen species generation

Yolanda Sánchez, Donna Amrán, Elena de Blas, Patricio Aller *

Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, Ramiro de Maeztu 9, 28040 Madrid, Spain

ARTICLE INFO

Article history:
Received 12 September 2008
Accepted 29 October 2008

Keywords:
Genistein
Differentiation
Protein kinases
Oxidative stress
Myeloid leukaemia cells

ABSTRACT

While it has been reported that genistein induces differentiation in multiple tumour cell models, the signalling and regulation of isoflavone-provoked differentiation are poorly known. We here demonstrate that genistein causes G_2/M cycle arrest and expression of differentiation markers in human acute myeloid leukaemia cells (HL60, NB4), and cooperates with all-trans retinoic acid (ATRA) in inducing differentiation, while ATRA attenuates the isoflavone-provoked toxicity. Genistein rapidly stimulates Raf-1, MEK1/2 and ERK1/2 phosphorylation/activation, but does not stimulate and instead causes a late decrease in Akt phosphorylation/activation which is attenuated by ATRA. Both differentiation and G_2/M arrest are attenuated by MEK/ERK inhibitors (PD98059, U0126) and ERK1-/ERK2-directed small interfering RNAs (siRNAs), and by the PI3K inhibitor LY294002, but not by the p38-MAPK inhibitor SB203580. Genistein stimulates $p21^{waf1/cip1}$ and cyclin B1 expression, phosphorylation/activation of ATM and Chk2 kinases, and Tyr15-phosphorylation/inactivation of Cdc2 (Cdk1) kinase, and these effects are attenuated by MEK/ERK inhibitors, while LY294002 also attenuates ERK and ATM phosphorylation. Caffeine abrogates the genistein-provoked G_2/M blockade and alterations in cell cycle regulatory proteins, and also suppresses differentiation. Finally, genistein causes reactive oxygen species (ROS) overaccumulation, but the antioxidant *N*-acetyl-L-cysteine fails to prevent ERK activation, G_2/M arrest, and differentiation induction. By contrast, *N*-acetyl-L-cysteine and p38-MAPK inhibitor attenuate the apoptosis-sensitizing (pro-apoptotic) action of genistein when combined with the antileukaemic agent arsenic trioxide. In summary, genistein-induced differentiation in acute myeloid leukaemia cells is a ROS-independent, Raf-1/MEK/ERK-mediated and PI3K-dependent response, which is coupled and co-regulated with G_2/M arrest, but uncoupled to the pro-apoptotic action of the drug.

* *Corresponding author*. Tel.: +34 918373112x4247; fax: +34 915360432.
E-mail address: aller@cib.csic.es (P. Aller).

Abbreviations: AMPK, AMP-activated kinase; ATM, ataxia telangiectasia mutated kinase; ATRA, all-trans retinoic acid; DAPI, 4,6-diamino-2-phenylindole; EGCG, epigallocatechin-3-gallate; ERK, extracellular signal-regulated kinase; FITC, fluorescein isothiocyanate; H_2DCFDA, dichlorodihydrofluorescein diacetate; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; NAC, *N*-acetyl-L-cysteine; NBT, nitroblue tetrazolium; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol 3-kinase; ROS, reactive oxygen species.

0006-2952/$ – see front matter
doi:10.1016/j.bcp.2008.10.035

1. Introduction

Flavonoids represent a class of phenolic compounds widely distributed in the plant kingdom and present in the daily diet. These agents display beneficial chemopreventive effects, generally attributed to their antioxidative and anti-inflammatory properties [1]. One of the most studied phytochemicals is genistein, an isoflavone present in soybeans and other legumes. Genistein is mainly known as a strong tyrosine kinase inhibitor, but exhibits other biochemical effects such as estrogen receptor binding activation, DNA topoisomerase II inhibition, and alteration of cell cycle regulatory proteins leading to G_2/M arrest [2]; down-regulation of the PI3K/Akt and NF-κB signalling pathways [3]; and activation [4–6] or inhibition [4,7,8] of MAP kinases. Recent reports demonstrated that genistein possesses antitumour activity. This property was attributed to the capacity of the isoflavone to provoke apoptosis *per se* and specially to potentiate apoptosis induction by radiation and other anticancer agents, an effect which was in turn explained by the down-regulation of defensive, anti-apoptotic signals (NF-κB, Akt, ERK) (reviewed in Refs. [3,9]). In addition, genistein was also reported to induce *in vitro* differentiation in several tumour cell models, including leukaemia cells [10–13]. However, the regulation and signalling of genistein-induced leukaemia cell differentiation are poorly known.

Although genistein was formerly described as a reactive oxygen species (ROS)-scavenging agent, recent studies proved that this isoflavone disrupts the respiratory chain, which causes ROS generation in isolated mitochondria [14] and intact cells [15]. In this regard, we recently demonstrated that genistein exerted a rapid pro-apoptotic effect in leukaemia cells, measured by the capacity to sensitize apoptosis induction by the antileukaemic drug arsenic trioxide, and that this response was mediated by the capacity of the isoflavone to cause intracellular ROS over-accumulation and ROS-dependent activation of p38-MAPK and AMPK [16]. Also importantly, other reports indicated that intracellular ROS mediate myeloid leukaemia cell differentiation in response to specific agents [17,18].

With these precedents in mind, we were now interested in investigating the regulation of genistein-provoked differentiation in human acute myeloid leukaemia cell models (HL60, NB4), with particular interest on the signalling by protein kinases and the possible influence of intracellular oxidation. The obtained results indicate that genistein rapidly activates the expression of differentiation markers, and cooperates with all-trans retinoic acid (ATRA) in inducing differentiation. Differentiation is a ROS-independent response, which is mediated by the activation of the Raf-1/MEK/ERK pathway, and also requires the PI3K/Akt pathway. Finally, differentiation is coupled and co-regulated with G_2/M cycle arrest, but uncoupled to the pro-apoptotic action of the drug.

2. Materials and methods

2.1. Reagents and antibodies

All components for cell culture were obtained from Invitrogen Inc. (Carlsbad, CA, USA). Dichlorodihydrofluorescein diacetate (H_2DCFDA) was obtained from Molecular Probes (Eugene, OR, USA). 4,6-diamino-2-phenylindole (DAPI) was obtained from Serva (Heidelberg, Germany). The kinase inhibitors PD98059, U0126, SB203580 and LY294002 were obtained from Calbiochem (Darmstad, Germany). Adaphostin was obtained from Biaffin GmbH & CoKG (Kassel, Germany). Rabbit polyclonal antibodies against p44/42 MAPK, phospho-p44/42 MAPK (Thr^{202}/Tyr^{204}), MEK1/2, phospho-MEK1/2 ($Ser^{217/221}$), phospho-Chk2 (Thr^{68}), phospho-cdc2 (Tyr^{15}), Akt and phospho-Akt (Ser^{473}), were obtained from Cell Signaling Technology Inc. (Danvers, MA, USA). Rabbit polyclonal antibodies against phospho-Raf-1 (Ser^{338}/Tyr^{341}) and p21 (C-19), and mouse monoclonal antibody against cyclin B1 (D-11) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Mouse monoclonal antibody against phospho-ATM (Ser^{1981}) was from Upstate Cell Signaling Solutions (Temecula, CA, USA). Mouse anti-human CD11b monoclonal antibody, and peroxidase- and fluorescein isothiocianate (FITC)-conjugated immunoglobulin G, were obtained from DAKO Diagnostics, S.A. (Barcelona, Spain). Small interfering RNAs (siRNAs) against ERK1 and ERK2, and control scramble siRNAs, were obtained from Santa Cruz. All other reagents were from Sigma (Madrid, Spain).

2.2. Cells and treatments

The human leukaemia cells lines HL60 (myelomonocytic) and NB4 (acute promyelocytic) were grown as earlier indicated [19]. For experiments, the cell number was adjusted at 10^5 cells/ml before initiation of the treatments. To prevent long-term cultures from reaching plateau densities, at the end of the second day of treatment the cultures were supplemented with an equal volume of fresh medium containing, when pertinent, the corresponding amount of drugs.

Stock solutions of genistein and epigallocatechin-3-gallate (EGCG) (50 mM), adaphostin (10 mM), SB203580, PD98059 and LY294002 (20 mM), U0126 (2,63 mM), *N*-acetyl-L-cysteine (NAC, 3 M) and 12-*O*-tetradecanoylphorbol 13-acetate (20 mM) were prepared in dimethyl sulfoxide; stock solutions of all-trans retinoic acid (10 mM) and H_2DCFDA (5 mM) were prepared in ethanol, and stock solutions of siRNAs (10 μM) were prepared in distilled water. All these solutions were stored at −20 °C. Stock solutions of DAPI (10 μg/ml) and propidium iodide (1 mg/ml) were prepared in phosphate-buffered saline (PBS). Arsenic trioxide was initially dissolved in a small amount of 1 M NaOH, after which PBS was added to give a final solution of 100 mM. These solutions were stored at 4 °C. Stock solutions of nitroblue tetrazolium (NBT, 2 mg/ml in PBS) and caffeine (250 mM in distilled water) were freshly prepared.

2.3. Nucleofection of siRNAs

HL60 cells were nucleofected with siRNAs using the optimized protocol for this cell line developed by the manufacturer (Amaxa Biosystems, Cologne, Germany). Namely, 2×10^6 cells were centrifuged and re-suspended in 100 μl of Nucleofector solution (Cell line Nucleofector kit V, Amaxa Biosystems), after which the siRNAs were added to a final concentration of 9 nM. The samples were nucleofected using the program T-019 (Amaxa Nucleofector v.2.1., Amaxa Biosystems), and then transferred to 4 ml of fresh culture medium. The levels of total

and phosphorylated ERKs were analyzed at different incubation times by means of immunoblot.

2.4. Determination of cell cycle distribution and apoptosis

To determine cell cycle phase distribution, the cells were permeabilized, stained with propidium iodide, and the fluorescence determined by flow cytometry. Using this method, the subpopulation of cells exhibiting sub-G_1 fluorescence (as an indication of sub-G_1 DNA content) represented the fraction of apoptotic cells in the culture. An additional criterion for apoptosis was the presence of chromatin condensation/fragmentation, as determined by cell permeabilization followed by DAPI staining and microscopy examination. A detailed description of all these procedures was presented in a preceding publication [20], and hence is omitted here.

2.5. Determination of cell differentiation

To detect the surface expression of the differentiation-specific CD11b antigen, cells were incubated with anti-CD11b antibody for 30 min at 4 °C, then extensively washed with RPMI-1640 medium, and incubated for 30 min more at 4 °C with FITC-labelled goat anti-mouse immunoglobulin G. The background of non-specific fluorescence was obtained by incubating cells only with this secondary antibody. After extensive washing with RPMI-1640 medium, the fluorescence was estimated by flow cytometry.

To measure NBT reduction, the cells were centrifuged, resuspended in PBS containing 0.2% NBT, and seeded by triplicate in 96 well flat bottom plates (200 μl per well). After addition of 12-*O*-tetradecanoylphorbol 13-acetate to a final concentration of 100 μM, the cells were incubated for 30 min at 37 °C. SDS was then added to a final concentration of 2% to disrupt the cells and dissolve the formazan crystals, and dye absorbance was measured by spectrometry at 595 nm. The absorption of samples containing NBT and 12-*O*-tetradecanoylphorbol 13-acetate without cells was also measured as the background signal.

2.6. Measurement of intracellular ROS accumulation

Intracellular ROS accumulation was measured by flow cytometry using the fluorescent probe H_2DCFDA, as previously described [20].

2.7. Flow cytometry

The analysis of samples was carried out using an EPICS XL flow cytometer (Coulter, Hialeah, FL) equipped with an air-cooled argon laser tuned to 488 nm. The specific fluorescence signals corresponding to FITC and H_2DCFDA were collected with a 525-nm band pass filter, and the signal corresponding to propidium iodide with a 620-nm band pass filter. As a rule, 10^4cells were counted in each determination.

2.8. Immunoblot assays

To obtain total cellular protein extracts, cells were collected by centrifugation, washed with PBS, and lysed for 20 min at 4 °C in a buffer consisting of 20 mM Tris–HCl, pH 7.5, containing 137 mM NaCl, 10% (v/v) glycerol and 1% (v/v) Nonidet P-40, supplemented with a protease inhibitor cocktail, 1 mM sodium orthovanadate and 10 mM NaF. After centrifugation at 10,000 × *g* for 15 min at 4 °C, the supernatants were collected. Fractions containing equal protein amounts were analyzed by SDS-polyacrylamide gel electrophoresis, blotted onto membranes and immunodetected, as previously described [21].

2.9. Statistical analysis

When required, the significance of differences between experimental conditions was examined, using the Student's *t*-test, and when positive indicated by asterisks ($^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

3. Results

3.1. Cell growth and differentiation

We firstly examined the efficacy of genistein to induce differentiation in HL60 and NB4 cells, as determined by the increased cell surface expression of CD11b antigen. Genistein was initially assayed at 10–50 μM, which is in the range of concentrations currently used to study apoptosis in leukaemia cells and other tumour cell models [16, and references therein]. Some of the obtained results are indicated in Fig. 1A. Increased CD11b expression was already detected at 24 h of treatment with 25–50 μM genistein, and at 96 h with 10 μM genistein. Differentiation induction by 25 μM genistein was corroborated by measuring NBT reduction (see Fig. 2B). As expected, the isoflavone caused cell accumulation at G_2/M in HL60 cell cultures, and this effect was already observed at 24 and 96 h of treatment with 25 and 10 μM genistein, respectively (Fig. 1B), thus paralleling the initiation of differentiation marker expression. On the other hand, within the assayed time periods 10 μM genistein did not cause significant apoptosis, while at the concentration of 25 μM apoptosis was detected at 72 h, as revealed by accumulation of cells with sub-G_1 DNA content (Fig. 1B). This conclusion was corroborated by examining chromatin condensation/fragmentation, which is also a characteristic of apoptosis (frequency of cells with condensed/fragmented chromatin in HL60 cells: 3, 3, 7 and 32% at 0, 24, 48 and 72 h of treatment with 25 μM genistein, respectively). On the ground of the obtained results, the concentration of 25 μM genistein was normally adopted for further mechanistic studies.

ATRA is the main differentiation inducer used in clinical settings against acute myeloid leukaemia. For this reason, we wanted to analyze the capacity of genistein to interact with ATRA. Some of the obtained results are indicated in Fig. 2. Within the assayed time period (24–72 h), treatment with 1 μM ATRA induced CD11b expression (Fig. 2A); caused cell accumulation at G_1 (Fig. 2C), and failed to cause significant apoptosis (Fig. 2D). When used together, genistein plus ATRA cooperated to induce CD11b cell surface expression in additive or even more than additive manner (Fig. 2A). This cooperative effect was corroborated by measuring NBT reduction (Fig. 2B). On the other hand, ATRA slightly reduced the G_2/M blockade

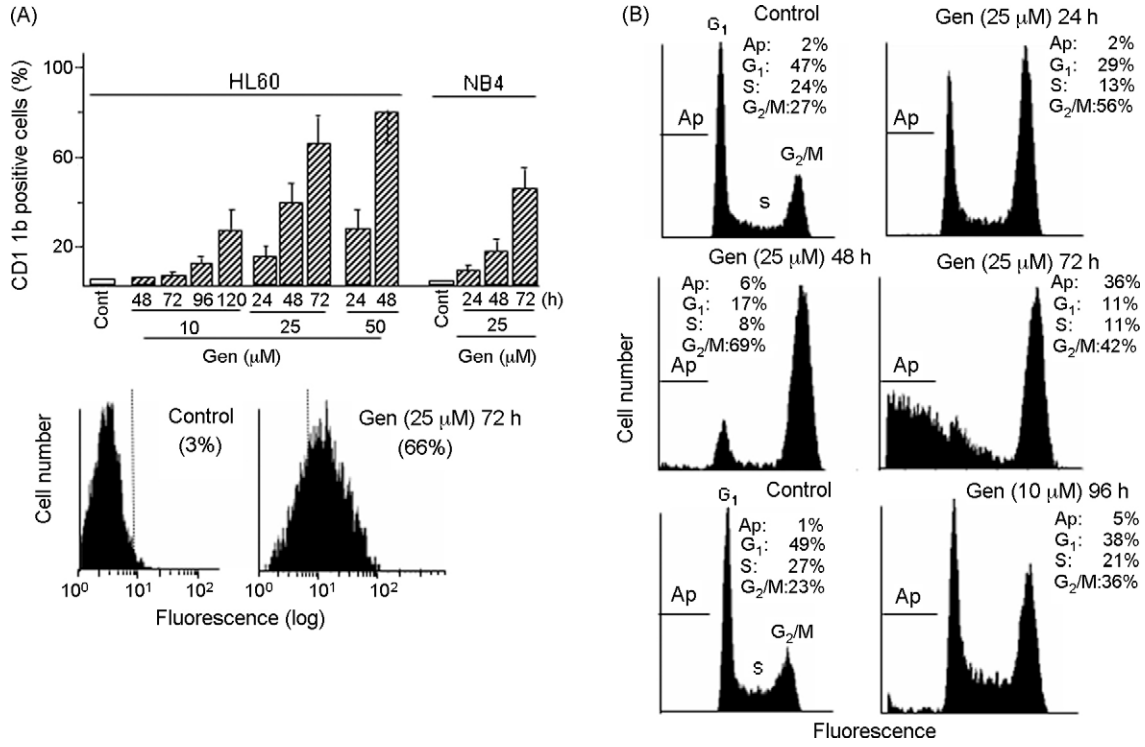

Fig. 1 – Differentiation induction and cell cycle alterations by genistein. (A) Frequency (mean ± S.D. of three determinations) of cells exhibiting CD11b surface expression, as determined by indirect immunofluorescence and flow cytometry, in untreated (Cont) HL60 and NB4 cell cultures and cultures treated for the indicated time periods with the indicated concentrations of genistein (Gen). An example of the obtained flow cytometric profiles using HL60 cells is shown at the bottom. The vertical dotted line delimits the region of positive cells (above the background fluorescence). (B) Cell cycle distribution according to their DNA content, as revealed by propidium iodide-derived fluorescence and flow cytometry, in untreated HL60 cell cultures and cultures treated for the indicated time periods with the indicated concentrations of genistein. The presence of sub-G_1 DNA content is considered as a criterion of apoptosis (Ap). The insets indicate the frequency of apoptotic cells and cells in the different phases of the cell cycle. The profiles are representative of one of two experiments with similar results. Ten thousand cells were scored in each cytometric profile.

caused by 25 μM genistein (Fig. 2C), and what is more important suppressed or greatly reduced the isoflavone-provoked apoptosis, as determined by chromatin condensation/fragmentation and frequency of cells with sub-G_1 DNA content (Fig. 2D, and results not shown).

For comparative purposes experiments were carried using the tyrphostin derivative adaphostin and the flavonol EGCG, two antitumour agents which as genistein cause tyrosine kinase inhibition [22,23] and ROS-mediated apoptosis in leukaemia cells [24,25 and our unpublished observations]. Adaphostin and EGCG were used at the concentrations of 0.25 and 25 μM, respectively, which according to preliminary determinations caused a moderate toxicity at 72 h of treatment, as in the case of 25 μM genistein. By contrast to genistein, these agents did not cause G_2/M blockade or stimulation of CD11b surface expression, and also failed to potentiate the stimulation produced by ATRA (not shown results).

3.2. *Protein kinase modulation and effects of protein kinase inhibitors*

Earlier studies indicated that ERK activation is involved in the regulation of myeloid cell differentiation [26]. However, genistein was reported to inhibit the MEK/ERK pathway in some cell systems [4,7–8]. For these reasons, we examined the capacity of the isoflavone to modulate the phosphorylation/activation of ERK and upstream kinases (MEK, Raf-1) in HL60 cells. It was observed that 25 μM genistein caused a rapid and stable increase in the phosphorylation state of these kinases, which was already detected at 6 h of treatment (Fig. 3A). The increase in ERK phosphorylation was corroborated at 24 h in NB4 cells, and still persisted at 48 h in both cell lines (results not shown). Treatment for 24 h with ATRA alone slightly increased Raf-1, MEK and ERK phosphorylation, but the effect of genistein plus ATRA was not higher than that caused by genistein alone (Fig. 3B). The cause–effect relationship between ERK activation and differentiation induction was then examined using the MEK/ERK inhibitors PD98059 (20 μM) and U0126 (2 μM), as well as specific siRNAs directed against ERK1 and ERK2. Under the used conditions both the pharmacologic inhibitors and siRNAs efficaciously attenuated ERK phosphorylation (see Fig. 4A (inset) and C). It was observed that co-treatment with PD98059 and U0126 attenuated CD11b surface expression (Fig. 4A, left bar chart) and NBT reduction (Fig. 4B, left bar chart) in genistein-treated HL60 and NB4 cells, and that ERK-directed siRNAs also attenuated antigen expres-

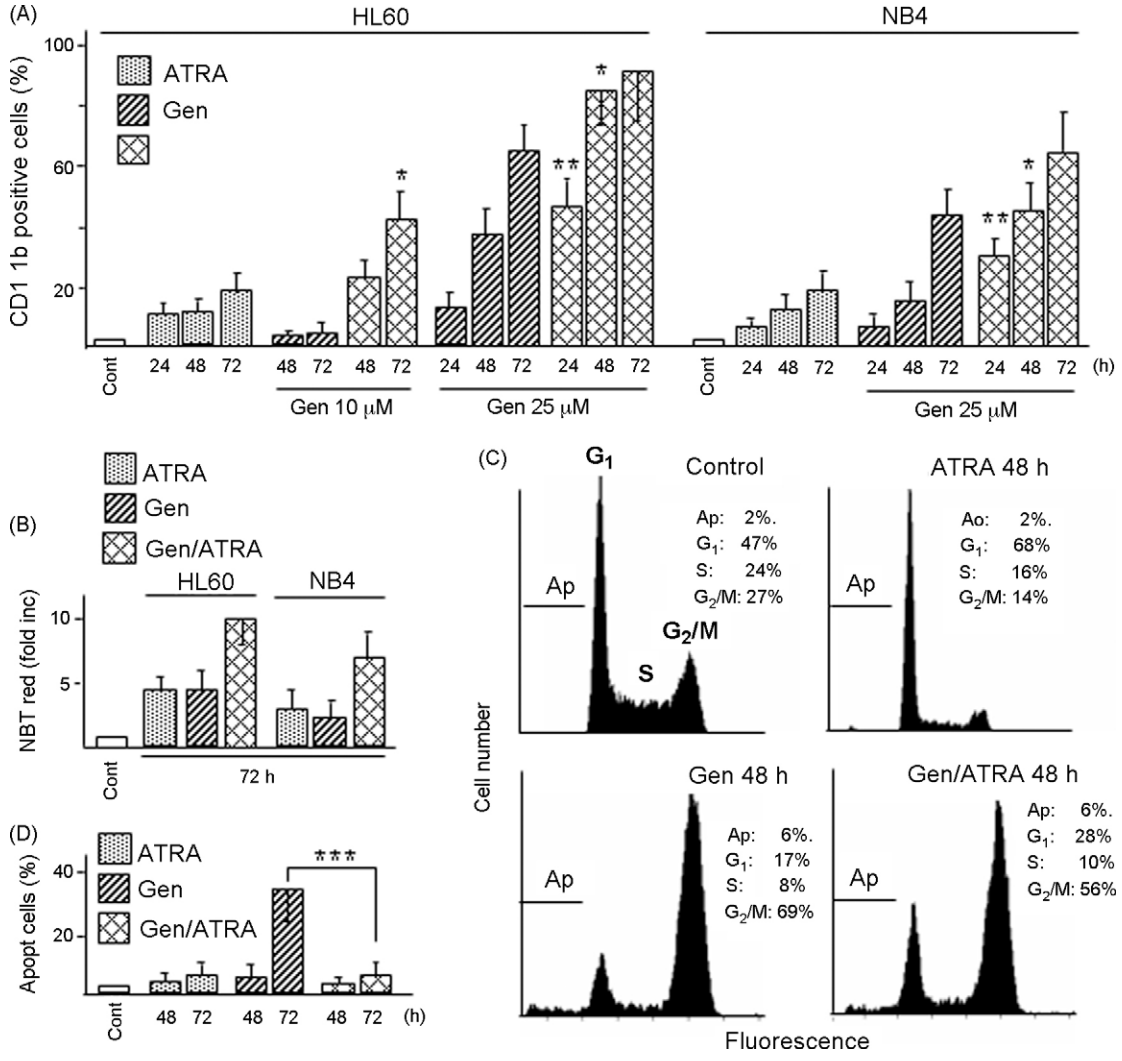

Fig. 2 – Effects of the combination of genistein and ATRA. (A) Frequency of HL60 and NB4 cells exhibiting CD11b surface expression upon treatment for the indicated time periods with ATRA and the indicated concentrations of genistein, alone and in combination. Asterisks indicate that the value in the combined treatment (Gen/ATRA) is significantly higher than the sum of values in the corresponding isolated treatments (Gen alone and ATRA alone). (B) NBT reduction in HL60 and NB4 cell cultures treated with ATRA and genistein, alone and in combination. The results (absorbance at 595 nm) are expressed as fold increase in relation to untreated (Cont) cells, which received the arbitrary value of one. (C) Cell cycle distribution in HL60 cell cultures subjected to same treatments as in (B). (D) Frequency of apoptotic cells, as determined by chromatin condensation/fragmentation, in untreated HL60 cell cultures subjected to the same treatments as in (B). Asterisks indicate significant differences between the indicated pair of values. ATRA was always used at 1 μM, and genistein at 25 μM except when otherwise indicated. All other conditions were as in Fig. 1.

sion in HL60 cells (Fig. 4C), proving that genistein-provoked differentiation is in fact regulated by the MEK/ERK pathway. The kinase inhibitors also reduced differentiation marker expression in cells treated with genistein plus ATRA (Fig. 4A, B, right bar charts). Finally, the effect of PD98059 was also examined in relation to cell cycle regulation. It was observed that treatment with the inhibitor alone slightly caused cell accumulation at G_1 and reduced the accumulation at G_2/M in genistein-treated HL60 (Fig. 5A) and NB4 (result not shown) cell cultures. The analysis of proteins responsible for the regulation of G_2 to M transition indicated that genistein cause phosphorylation/activation of ATM and Chk2 kinases, Tyr15-phosphorylation/inactivation of Cdc2 kinase, and increased expression of $p21^{waf1/cif1}$ and cyclin B1, measured at 24 h of treatment, and that these effects are prevented or reduced by co-treatment with PD98059 (Fig. 5B) and U0126 (result not shown). Moreover, significant ATM phosphorylation was only detected at 14–24 h of treatment with genistein (Fig. 3A), suggesting that ATM activation is downstream ERK activation.

For comparison we also examined the effect of the p38-MAPK inhibitor SB203580, since p38-MAPK was earlier reported to regulate myeloid cell differentiation [26]. The concentration of 10 μM SB203580 was adopted on the ground of our preceding studies on apoptosis [16, and references therein]. It was observed that this kinase inhibitor failed to affect CD11b expression (Fig. 4A) and G_2/M cell accumulation

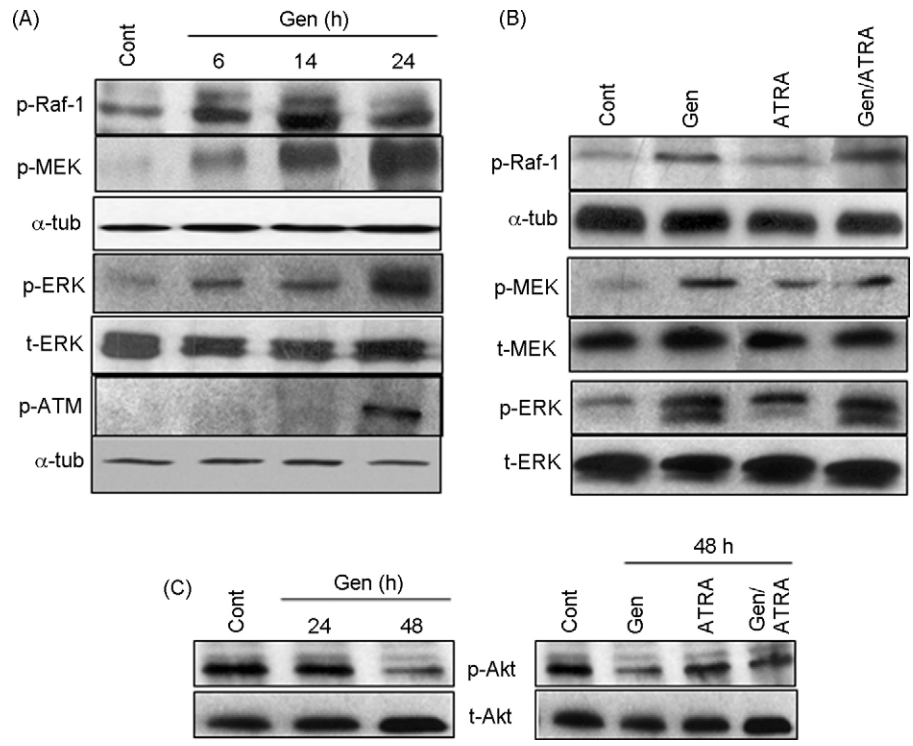

Fig. 3 – Protein kinase phosphorylation. (A) Relative levels of phosphorylated (p-) Raf-1, MEK1/2, ERK1/2, and ATM, in untreated (Cont) HL60 cells and cells treated for the indicated time periods with genistein, as measured by immunoblot. The level of α-tubulin (α-tub) or the corresponding total (t-) protein kinase were measured as loading controls. (B) Relative levels of total and phosphorylated protein kinases in HL60 cells treated for 24 h with genistein and ATRA, alone and in combination. (C) Relative levels of total and phosphorylated Akt in untreated HL60 cells and cells treated for the indicated time periods with genistein (left blot), and in cells treated for 48 h with genistein and ATRA, alone and in combination (right blot). ATRA was always used at 1 μM, and genistein at 25 μM. All other conditions were as in Figs. 1 and 2.

(Fig. 5A) in response to genistein, suggesting that p38-MAPK does not regulate isoflavone-promoted differentiation and cycle arrest.

It was recently reported that Akt is activated by ATRA, and regulates ATRA-provoked differentiation of acute myeloid leukaemia cells [27]. On the other hand, genistein was commonly observed to down-regulate the PI3K/Akt pathway in different cell systems [3]. To investigate the importance of this signalling pathway for differentiation and cell cycle arrest, we examined Akt phosphorylation in HL60 cells upon treatment with 25 μM genistein. As indicated in Fig. 3C, the isoflavone decreased Akt phosphorylation, but the decrease was only detected at 48 h of treatment, following the initiation (24 h) of G_2/M arrest and differentiation (see Fig. 1A, B). On the other hand, co-treatment with ATRA, which as indicated above prevented the late (72 h) genistein-provoked apoptosis (see Fig. 2), also prevented the decrease in Akt phosphorylation (Fig. 3C). A second group of experiments was carried out using the PI3K inhibitor LY294002. It was observed that the administration of 20 μM LY294002, which efficaciously decreased the basal Akt phosphorylation level (Fig. 6C), prevented the isoflavone-provoked stimulation of CD11b expression (Fig. 6A) and G_2/M cell accumulation (Fig. 6B), as well as the increase in ERK and ATM phosphorylation (Fig. 6C). Thus, while the experiments with PI3K inhibitor indicate that Akt is important for proper execution of both differentiation and G_2/M arrest, the kinetic assays indicate that the trigger of these processes by genistein may not be adequately explained by changes in Akt phosphorylation. On the other hand, kinase down-regulation might facilitate the late apoptotic action of the isoflavone.

To strengthen the relationship between cell cycle blockade and differentiation induction, a complementary set of experiments was carried out using caffeine. This methylxanthine is known to override the G_2 check-point by inhibiting ATM phosphorylation, although other studies indicate that caffeine also delays G_1 to S phase transition [28]. In agreement with this, treatment of HL60 cells with 5 mM caffeine caused cell accumulation at G_1, suppressed the G_2/M arrest caused by genistein (Fig. 7A), and accordingly abrogated the isoflavone-provoked increase in ATM, Chk2 and Cdc2-Tyr15-phosphorylation, and the increase in cyclin B1 expression (Fig. 7B). Interestingly, caffeine also inhibited the isoflavone-induced increase in CD11b expression and NBT reduction (Fig. 7C). Taken together, these results strongly indicate that genistein-provoked G_2/M arrest and differentiation are closely associated events, which share similar regulatory mechanisms.

3.3. *ROS production*

Earlier reports indicated that intracellular ROS over-accumulation may mediate drug-induced differentiation in leukaemia

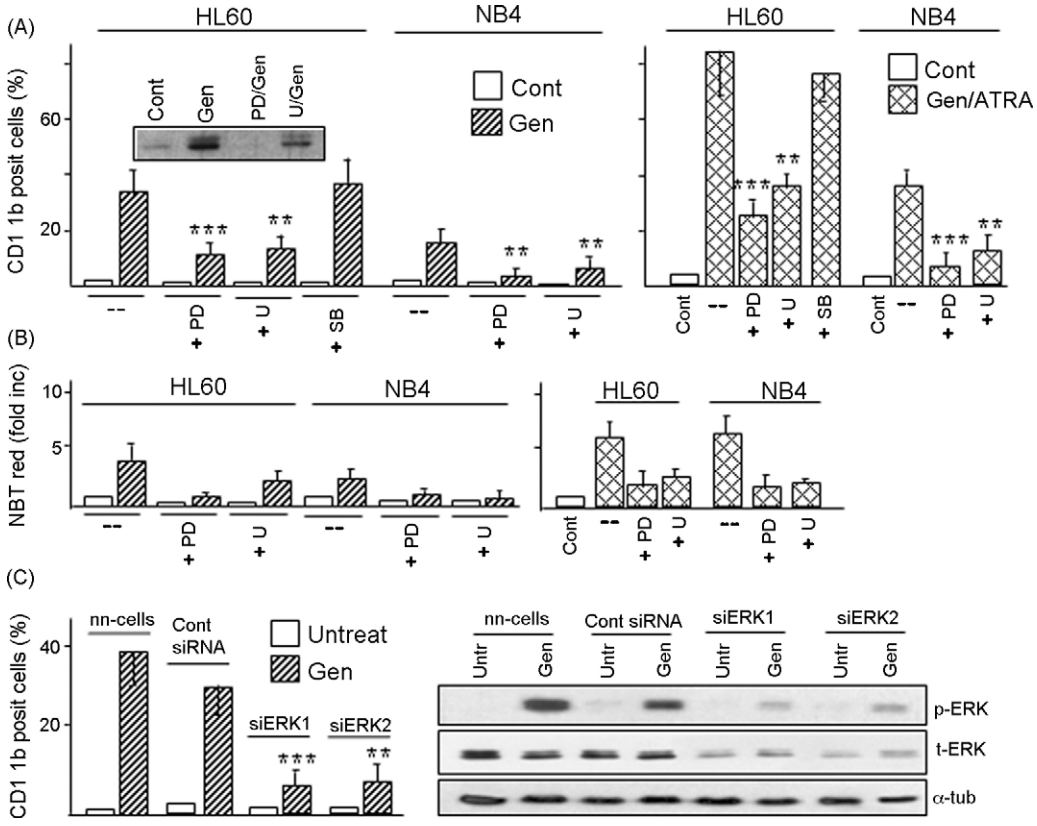

Fig. 4 – Effect of MAPK inhibition on genistein-induced differentiation. (A, B) CD11b surface expression at 48 h (A) and NBT reduction at 72 h (B) in untreated (Cont) HL60 and NB4 cell cultures and cultures treated with genistein alone (left bar charts) or genistein plus ATRA (right bar charts), either in the absence (–) or the presence of the MEK/ERK inhibitors PD98059 (PD, 20 μM) and U0126 (U, 2.5 μM) or the p38-MAPK inhibitor SB203580 (SB, 10 μM). The inset in (A) shows inhibition by PD98050 and U0126 of genistein-provoked ERK phosphorylation. The kinase inhibitors were added 30 min in advance to the other drugs. (C) CD11b surface expression (bar chart) and total (t-) and phosphorylated (p-) ERK levels (immunoblot) in non-nucleofected HL60 cells (nn-cells) and cells nucleofected with irrelevant scrambled (Cont) and ERK1- and ERK2-directed siRNAs, either without treatment or after genistein. Genistein was added at 24 h post-nucleofection, and the determinations performed at 24 h (ERK levels) or 48 h (CD11b expression) of treatment. In all experiments, ATRA was used at 1 μM and genistein at 25 μM. The asterisks indicate significant differences in relation to the control. All other conditions were as in Figs. 1–3.

cells [17,18] as well as ERK activation and ERK-regulated G_2/M arrest in other cell systems [29]. For these reasons, we wanted to examine the possible implication of ROS in the regulation of genistein-promoted differentiation and cell cycle blockade. In agreement with our preceding observation in another leukaemia cell line [16], treatment for 3–16 h with 25 μM genistein caused a moderate (approximately three- to four-fold) increase in intracellular ROS accumulation in HL60 cells, as measured by the increase in H_2DCFDA-derived fluorescence (Fig. 8A, and results not shown), and accordingly the antioxidant NAC attenuated the capacity of genistein to potentiate apoptosis induction by the ROS-sensitive drug arsenic trioxide (Fig. 8B). By, contrast NAC failed to prevent the genistein-provoked G_2/M blockade (Fig. 8C), CD11b induction (Fig. 8D), and ERK activation (Fig. 8E), indicating that these processes are independent of intracellular oxidation. Noteworthy, while the pro-apoptotic action of genistein was attenuated by the p38-MAPK inhibitor SB203580, it was potentiated by the MEK/ERK inhibitors PD98059 and U0126 (Fig. 8B), indicating an opposite mode of regulation by these kinases.

4. Discussion

The present results corroborate the capacity of genistein to induce differentiation followed by apoptosis, and also indicate that the isoflavone cooperates with ATRA in inducing differentiation, in acute myeloid leukaemia cells. On the ground of kinetic assays the expression of differentiation markers was a rapid response (24 h, at 25 μM genistein), while apoptosis execution was a delayed event (72 h). This late apoptosis might represent a secondary response derived from differentiation and/or sustained cell cycle disruption, which clearly contrasts with the capacity of genistein to rapidly sensitize to apoptosis induction by radiation and chemotherapeutic drugs (here defined as "pro-apoptotic" effect) in leukaemia [16] and other tumour cell models [3,9]. The capacity of genistein to interact with other differentiation inducers was examined in preceding works, with discrepant results. For instance, the isoflavone was observed either to potentiate [30] or prevent [31] the expression of differentiation markers in ATRA-treated HL60 and other leukaemia cell lines.

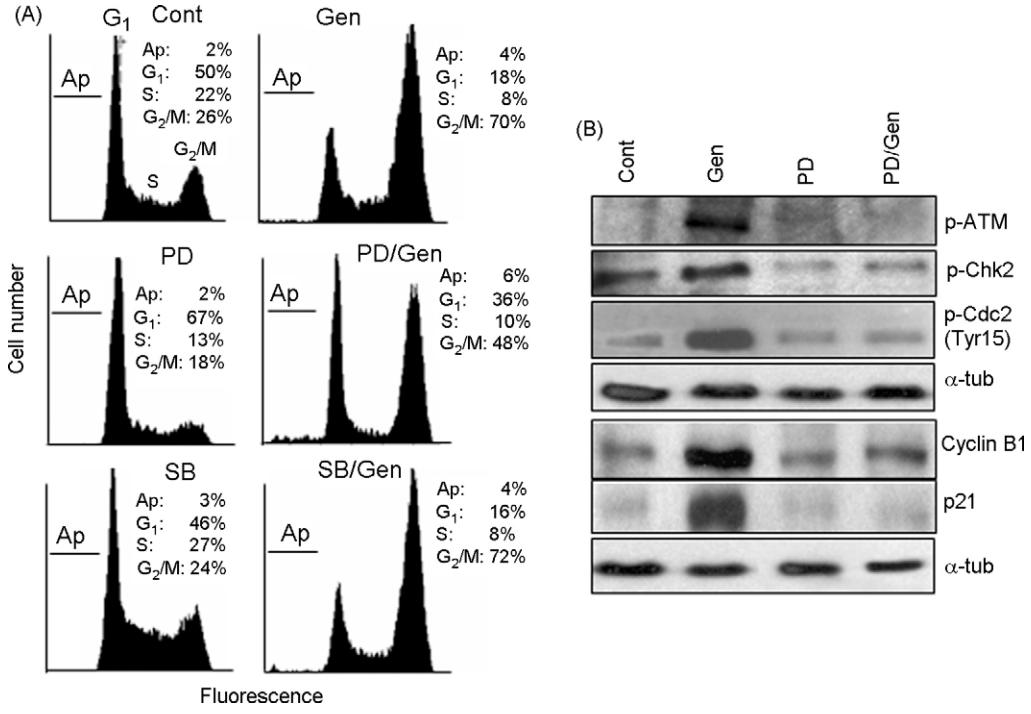

Fig. 5 – Effects of MEK/ERK and p38-MAPK inhibitors on genistein-provoked cell cycle arrest and cell cycle-related protein alterations. (A) Changes in cell cycle distribution in HL60 cell cultures treated for 48 h with 25 μM genistein and 20 μM PD98059 or 10 μM SB203580, alone and in combination. (B) Relative levels of total cyclin B1 and p21$^{waf1/cif1}$ and of phosphorylated (p-) ATM, Chk2 and Cdc2 (Tyr15) proteins in cells subjected for 24 h to the same treatments as above. All other conditions were as in Figs. 1, 3 and 4.

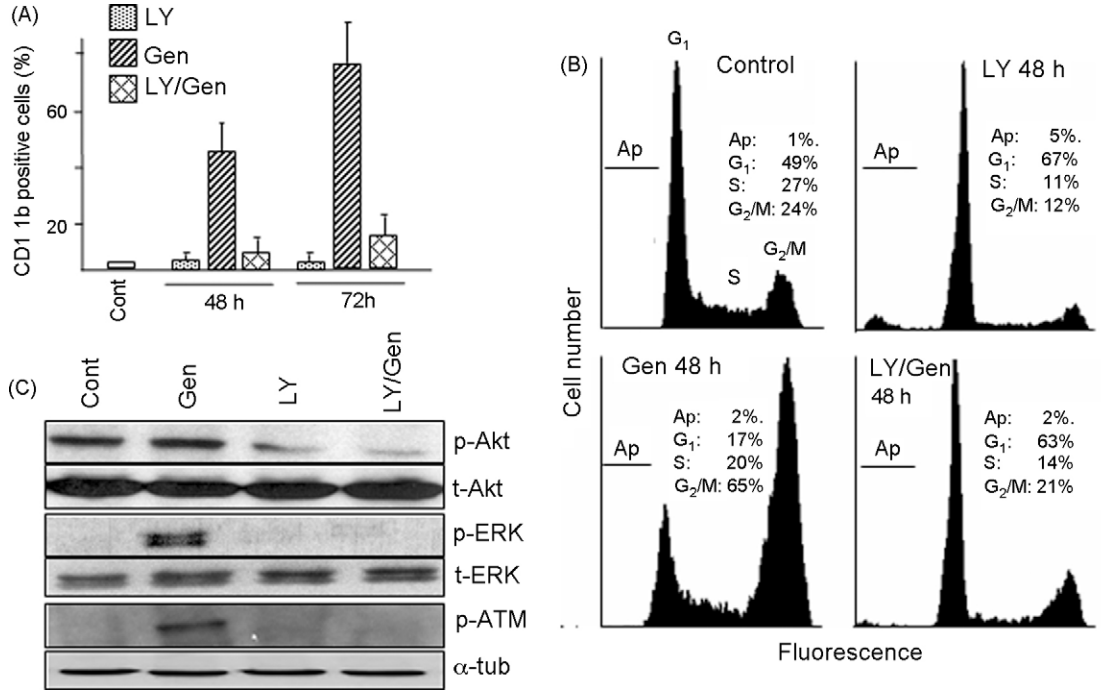

Fig. 6 – Effects of PI3K/Akt inhibitor. (A, B) CD11b surface expression (A) and cell cycle distribution (B) in untreated HL60 cell cultures (Cont) and cultures treated for the indicated time periods with LY294002 (LY, 20 μM) and 25 μM genistein, alone and in combination. (C) Relative levels of total (t-) and phosphorylated (p-) Akt, ERK and ATM, in untreated HL60 cells and cells treated for 24 h with 25 μM genistein and 20 μM LY294002. All other conditions were as in Figs. 1 and 3.

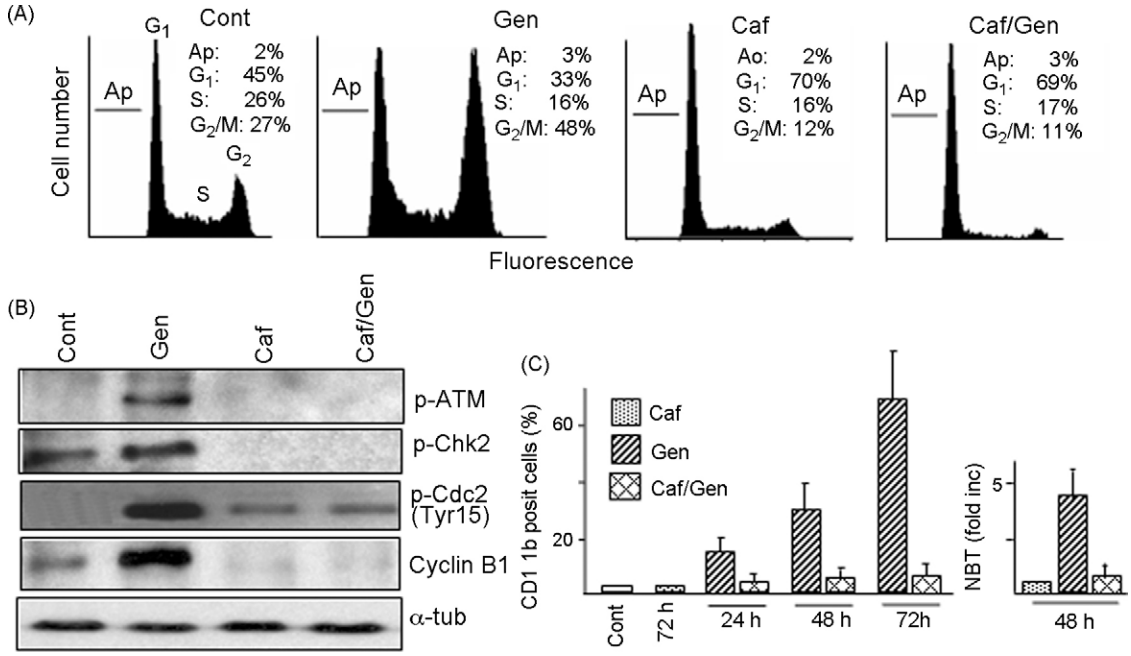

Fig. 7 – Modulation by caffeine of genistein-provoked effects. (A) Cell cycle distribution in HL60 cell cultures treated for 24 h with 25 μM genistein and 5 mM caffeine, alone or in combination. (B) Relative levels of total cyclin B1 and phosphorylated (p-) ATM, Chk2 and Cdc2 (Tyr15) in cells subjected to the same treatments as above. (C) CD11b surface expression and NBT reduction in cells subjected for the indicated time periods to the same treatments as above. All other conditions were as in Figs. 1–3.

In another study, genistein caused premature cell death when used in combination with ATRA in HL60 cells [32]. However, in our present experiments genistein and ATRA cooperated to induce differentiation in HL60 and NB4 cells while, what is equally important, ATRA attenuated the isoflavone-provoked apoptosis. Noteworthy, recent studies indicated that dietary achievable doses of genistein caused tumour reduction and elicited molecular events in animal models, similar to those elicited in tumour cell lines *in vitro* by apoptosis-inducing isoflavone concentrations [33,34]. Taking everything into consideration, one should not exclude the possibility that genistein (and perhaps other flavonoids) might also be used to improve the differentiation capacity of ATRA under *in vivo* conditions, and its clinical efficacy as antileukaemic agent.

As indicated above, genistein was reported to cause either ERK activation [5,6] or inhibition [4,7,8] in tumour cell models. Our present results demonstrate that the Raf-1/MEK/ERK pathway is strictly required for differentiation induction by genistein in acute myeloid leukaemia cells. Thus, the isoflavone rapidly stimulated the phosphorylation of these kinases (6 h), and the expression of differentiation markers was reduced by pharmacologic MEK inhibitors and ERK-directed siRNAs. By contrast, the failure of SB203580 to affect CD11b expression suggests that p38-MAPK does not participate in the regulation of genistein-provoked differentiation in this cell model. Finally, our observations indicate that both ERK activation and subsequent differentiation induction are dependent on the proper functioning of the PI3K/Akt pathway, since these processes were prevented by the PI3K inhibitor LY294002. Nonetheless, differentiation could be initiated (24 h) in the absence of Akt activation, and differentiation progression tolerated the partial Akt down-regulation caused by genistein (48 h). On the other hand, it is conceivable that down-regulation of Akt, which is a protective kinase, may favour the late genistein-provoked apoptosis (72 h). Moreover, we may speculate that the attenuation of Akt de-phosphorylation by ATRA might explain at least in part the protective (anti-apoptotic) action of the retinoid, and also perhaps the cooperation between genistein and ATRA in inducing differentiation.

In addition, the present results corroborate the capacity of genistein to cause G_2/M cell cycle blockade, and indicate that cycle arrest and differentiation induction are closely associated and co-regulated events in isoflavone-treated myeloid leukaemia cells. Thus, the initiation of G_2/M cell accumulation was coincident in time with the expression of differentiation markers, and both cycle arrest and differentiation were prevented by MEK/ERK and PI3K inhibitors, but not by p38-MAPK inhibitor. Our observations are congruent with a previous study indicating that genistein causes G_2/M blockade via stable ERK activation in MDA-MB-231 breast cancer cells [35], but contrast with another report indicating regulation of this blockade by p38-MAPK inT47D breast cancer cells [36]. The genistein-provoked G_2/M arrest is regulated by ATM and Chk2 activation, Cdc2 inactivation, and increased $p21^{waf1/cif1}$ expression, which essentially agrees with earlier observations in other cell types [37], but the isoflavone also increased the levels of cyclin B1. Whether this increase plays a regulatory role, or it merely reflects a failure of protein degradation resulting in progressive accumulation due to sustained pre-mitotic arrest, is presently unknown. Importantly, in our experiments the genistein-provoked ATM activation was a relatively late event (14–24 h) in comparison to ERK activation (6 h), and was also regulated by both Akt and ERK, as proved by

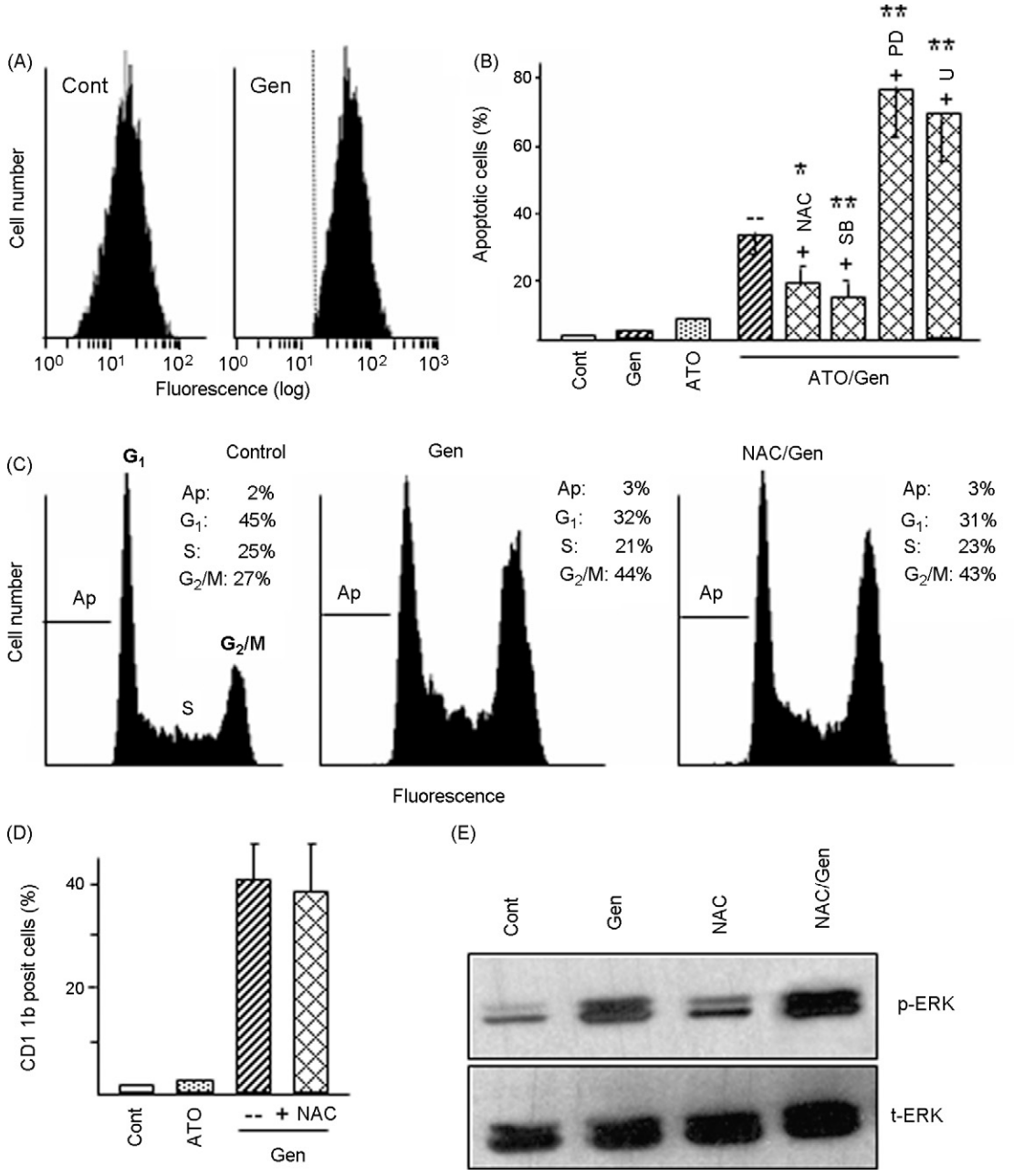

Fig. 8 – Oxidative stress. (A) Relative intracellular peroxide accumulation in untreated HL60 cells (Cont) and cells treated for 8 h with genistein, as determined by flow cytometry after cell loading with H_2DCFDA. The vertical dotted line indicates the main fluorescence in the control, to better discern the displacement caused by genistein. (B) Frequency of apoptotic cells, as determined by chromatin condensation/fragmentation, in HL60 cell cultures treated for 24 h with genistein, arsenic trioxide (ATO), and genistein plus ATO (ATO/Gen), either alone (-) or with NAC, SB203580, PD98059 and U0126. The asterisks indicate significant differences in relation to treatment with ATO plus genistein alone. (C) Cell cycle distribution at 24 h, (D) CD11b surface expression at 48 h, and (E) relative levels of total (t-) and phosphorylated (p-) ERK1/2 at 24 h, in HL60 cells treated with genistein, alone and in combination with NAC. Genistein was used at 25 μM, NAC at 10 mM, and ATO at 2 μM. NAC was added 1 h in advance to the other agents. All other conditions were as in Figs. 1, 3 and 4.

the capacity of PI3K and MEK/ERK inhibitors to prevent ATM phosphorylation. These results contrast with observations in other cell models, which characterized ERK and Akt as downstream targets of ATM in etoposide-treated murine embryonic fibroblasts [38] and insulin-stimulated murine muscle cells [39], respectively. Finally, an intriguing aspect is the actual relationship between cell cycle blockade and differentiation induction. One of the multiple biochemical effects of genistein is the generation of topoisomerase II-mediated DNA breakage, and it is well known that DNA-damaging agents prevent cell progression from G_2 into mitosis. In addition, we and others demonstrated that

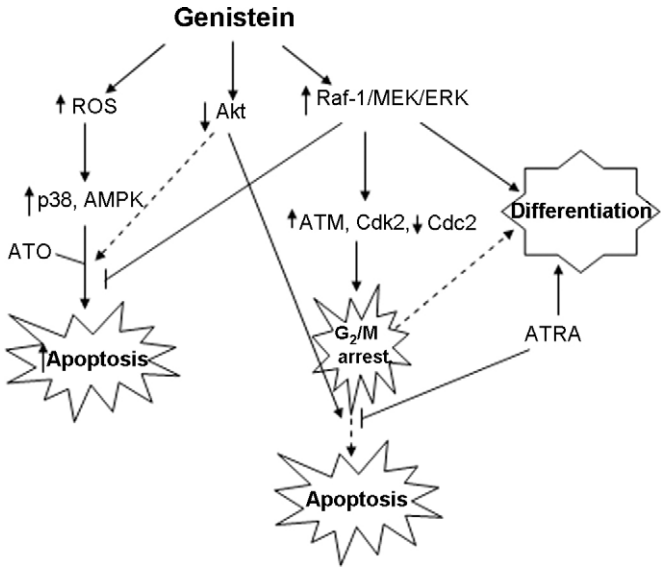

Fig. 9 – Effects of genistein in myeloid leukaemia cells. The scheme summarizes the results obtained in the present work and in a preceding publication [16]. Genistein causes ROS over-production, leading to the activation of the ROS-inducible kinases p38-MAPK and AMPK. This facilitates apoptosis induction by the ROS-sensitive drug ATO, which on the other hand is restrained by ERK activation. Genistein also activates the Raf-1/MEK/ERK pathway, leading to ATM and Cdk2 activation and Cdc2 de-activation, which cause blockade of G_2 to M phase transition. In this situation, persistent cycle blockade might lead to apoptosis. In addition, ERK activation signals differentiation induction, which might be also facilitated by the unbalanced cell growth resulting from cell cycle arrest. Genistein also causes late down-regulation of the PI3K/Akt pathway, which facilitates apoptosis. Co-treatment with ATRA cooperates with genistein in inducing differentiation but restrains apoptosis, possible by preventing PI3K/Akt down-regulation. Broken lines indicate that these pathways are only speculative.

topoisomerase poisons and other DNA-damaging agents which cause S or G_2/M phase arrest efficaciously induce myeloid cell differentiation [40, and references therein]. Thus, it is tempting to speculate that differentiation induction by genistein might in part represent a response to cell cycle disturbance. The observation that the ATM inhibitor caffeine, which overrides the G_2/M arrest, also prevents differentiation might support this hypothesis. Nonetheless, further studies would be required to conclude the existence of a true cause–effect relationship between these phenomena.

Intracellular ROS mediate multiple cellular responses, including protein kinase activation [41], cell cycle progression [42], myeloid cell differentiation [17,18], and apoptotic and necrotic cell death [43]. Moreover, the level of intracellular ROS may modulate the toxicity of antitumour drugs [44]. In this regard our preceding publication demonstrated that genistein sensitizes leukaemia cells to arsenic trioxide-provoked apoptosis via ROS-mediated activation of p38-MAPK and AMPK, while these kinases were irrelevant for G_2/M cycle blockade [16]. As a complement, the present work demonstrates that ROS accumulation does not mediate genistein-provoked ERK activation or G_2/M blockade; that ROS and p38-MAPK are also irrelevant for differentiation induction; and that MEK/ERK inhibitors exacerbate the pro-apoptotic action of genistein. Taken together, our results indicate that genistein elicits opposite signalling pathways in myeloid leukaemia cells, which are tentatively schematized in Fig. 9. On the one hand, Raf-1/MEK/ERK activation, which in turn triggers ATM-mediated G_2/M blockade, and also mediates differentiation induction. This response is not dependent on ROS production or activation of ROS-regulated kinases (e.g., p38-MAPK). On the other hand, ROS over-production and activation of ROS-regulated kinases (p38-MAPK, AMPK), which probably in conjunction with other factors (e.g., NF-κB inhibition and PI3K/Akt down-regulation [3]), override the protective action of ERK activation and facilitate the pro-apoptotic action of the isoflavone.

Acknowledgements

This work was supported by the Ministerio de Educación y Ciencia, Spain, Grant SAF2007-64721 (Plan Nacional de Investigación Científica, Desarrollo e Innovación Tecnológica, Dirección General de Investigación) and Grant CSD2007-00020 (Programa Consolider-Ingenio 2010). Y.S. and D.A. were recipients of pre-doctoral fellowships from the Plan de Formación de Personal Investigador, Ministerio de Educación y Ciencia, and Universidad Complutense de Madrid, respectively.

REFERENCES

[1] Rahman I, Biswas SK, Kirkham PA. Regulation of inflammation and redox signaling by dietary polyphenols. Biochem Pharmacol 2006;72:1439–52.

[2] Klein CB, King AA. Genistein genotoxicity: critical considerations of in vitro exposure dose. Toxicol Appl Pharmacol 2007;224:1–11.
[3] Banerjee S, Li Y, Wang Z, Sarkar FH. Multi-targeted therapy of cancer by genistein. Cancer Lett 2008;269:226–42.
[4] Frey RS, Singletary KW. Genistein activates p38 mitogen-activated protein kinase, inactivates ERK1/ERK2 and decreases Cdc25C expression in immortalized human mammary epithelial cells. J Nutr 2003;133:226–31.
[5] Sagara Y, Vanhnasy J, Maher P. Induction of PC12 cell differentiation by flavonoids is dependent upon extracellular signal-regulated kinase activation. J Neurochem 2004;90:1144–55.
[6] Hung SP, Hsu JR, Lo CP, Huang HJ, Wang JP, Chen ST. Genistein-induced neuronal differentiation is associated with activation of extracellular signal-regulated kinases and upregulation of p21 and N-cadherin. J Cell Biochem 2005;96:1061–70.
[7] Akimoto T, Nonaka T, Ishikawa H, Sakurai H, Saitoh JI, Takahashi T, et al. Genistein, a tyrosine kinase inhibitor, enhanced radiosensitivity in human esophageal cancer cell lines in vitro: possible involvement of inhibition of survival signal transduction pathways. Int J Radiat Oncol Biol Phys 2001;50:195–201.
[8] Vanden Berghe W. Attenuation of mitogen and stress activated protein kinase-1-driven nuclear factor-kappaB gene expression by soy isoflavones does not require estrogenic activity. Cancer Res 2006;66:4852–62.
[9] Garg AK, Buchholz TA, Aggarwal BB. Chemosensitization and radiosensitization of tumours by plant polyphenols. Antioxid Redox Signal 2005;7:1630–47.
[10] Constantinou A, Kiguchi K, Huberman E. Induction of differentiation and DNA strand breakage in human HL-60 and K-562 leukemia cells by genistein. Cancer Res 1990;50:2618–24.
[11] Jing Y, Waxman S. Structural requirements for differentiation-induction and growth-inhibition of mouse erythroleukemia cells by isoflavones. Anticancer Res 1995;15:1147–52.
[12] Li W. Synergistic action of tiazofurin and genistein on growth inhibition and differentiation of K-562 human leukaemic cells. Life Sci 1998;63:1975–81.
[13] Ng AP, Nin DS, Fong JH, Venkataraman D, Chen CS, Khan M. Therapeutic targeting of nuclear receptor corepressor misfolding in acute promyelocytic leukemia cells with genistein. Mol Cancer Ther 2007;6:2240–8.
[14] Salvi M, Brunati AM, Clari G, Toninello A. Interaction of genistein with the mitochondrial electron transport chain results in opening of the membrane transition pore. Biochim Biophys Acta 2002;1556:187–96.
[15] Hwang JT, Park IJ, Shin JI, Lee SK, Baik HW, Ha J, et al. Genistein, EGCG, and capsaicin inhibit adipocyte differentiation process via activating AMP-activated protein kinase. Biochem Biophys Res Commun 2005;338:694–9.
[16] Sanchez Y, Amrán D, Fernandez C, de Blas E, Aller P. Genistein selectively potentiates arsenic trioxide-induced apoptosis in human leukaemia cells via reactive oxygen species generation and activation of reactive oxygen-species inducible protein kinases (p38-MAPK, AMPK). Int J Cancer 2008;123:1205–14.
[17] Richard D, Hollender P, Chenais B. Involvement of reactive oxygen species in aclarubicin-induced differentiation and invasiveness of HL-60 leukemia cells. Int J Oncol 2002;21:393–9.
[18] Batista Jr EL, Warbington M, Badwey JA, Van Dyke TE. Differentiation of HL-60 cells to granulocytes involves regulation of select diacylglycerol kinases (DGKs). J Cell Biochem 2005;94:774–93.
[19] Ramos AM, Fernandez C, Amran D, Sancho P, de Blas E, Aller P. Pharmacologic inhibitors of PI3K/Akt potentiate the apoptotic action of the antileukemic drug arsenic trioxide via glutathione depletion and increased peroxide accumulation in myeloid leukemia cells. Blood 2005;105:4013–20.
[20] Troyano A, Fernandez C, Sancho P, de Blas E, Aller P. Effect of glutathione depletion on antitumor drug toxicity (apoptosis and necrosis) in U-937 human promonocytic cells. The role of intracellular oxidation. J Biol Chem 2001;276:47107–15.
[21] Galan A, Garcia-Bermejo ML, Troyano A, Vilaboa NE, de Blas E, Kazanietz MG, et al. Stimulation of p38 mitogen-activated protein kinase is an early regulatory event for the cadmium-induced apoptosis in human promonocytic cells. J Biol Chem 2000;275:11418–24.
[22] Wang YC, Bachrach U. The specific anti-cancer activity of green tea (-) epigallocatechin-3-gallate (EGCG). Amino Acids 2002;22:131–43.
[23] Kaur G, Narayanan VL, Risbood PA, Hollingshead MG, Stinson SF, Varma RK, et al. Synthesis, structure-activity relationship, and p210(bcr-abl) protein tyrosine kinase activity of novel AG 957 analogs. Bioorg Med Chem 2005;13:1749–61.
[24] Chandra J, Hackbarth J, Le S, Logering D, Bone N, Bruzek LM, et al. Involvement of reactive oxygen species in adaphostin-induced cytotoxicity in human leukemia cells. Blood 2003;102:4512–9.
[25] Nakazato T, Ito K, Ikeda Y, Kizaki M. Green tea component, catechin, induces apoptosis of human malignant B cells via production of reactive oxygen species. Clin Cancer Res 2005;11:6040–9.
[26] You A. Differentiation, apoptosis and function of human immature and mature myeloid cells: intracellular signaling mechanism. Int J Hematol 2001;73:438–52.
[27] Matkovic K, Brugnoli F, Bertagnolo V, Banfic H, Visnjic D. The role of the nuclear Akt activation and Akt inhibitors in all-trans-retinoic acid-differentiated HL-60 cells. Leukemia 2006;20:941–51.
[28] Bode AM, Dong Z. The enigmatic effects of caffeine in cell cycle and cancer. Cancer Lett 2007;247:26–39.
[29] Zhang Z, Leonard SS, Huang C, Vallyathan V, Castranova V, Shi X. Role of reactive oxygen species and MAPKs in vanadate-induced G(2)/M phase arrest. Free Radic Biol Med 2003;34:1333–42.
[30] Makishima M, Honma Y, Hozumi M, Sampi K, Hattori M, Umezawas K, et al. Effects of inhibitors of protein tyrosine kinase activity and/or phosphatidylinositol turnover on differentiation of some human myelomonocytic leukemia cells. Leuk Res 1991;15:701–8.
[31] Hughes PJ, Zhao Y, Chandraratna RA, Brown G. Retinoid-mediated stimulation of steroid sulfatase activity in myeloid leukemic cell lines requires RARalpha and RXR and involves the phosphoinositide 3-kinase and ERK-MAP kinase pathways. J Cell Biochem 2006;97:327–50.
[32] Katagiri K, Yokoyama KK, Yamamoto T, Omura S, Irie S, Katagiri T. Lyn and Fgr protein-tyrosine kinases prevent apoptosis during retinoic acid-induced granulocytic differentiation of HL-60 cells. J Biol Chem 1996;271: 11557–62.
[33] Li Y, Che M, Bhagat S, Ellis KL, Kucuk O, Doerge DR, et al. Regulation of gene expression and inhibition of experimental prostate cancer bone metastasis by dietary genistein. Neoplasia 2004;6:354–63.
[34] Li Y, Ahmed F, Ali S, Philip PA, Kucuk O, Sarkar FH. Inactivation of nuclear factor κB by soy isoflavone genistein contributes to increased apoptosis induced by chemotherapeutic agents in human cancer cells. Cancer Res 2005;65:6934–42.

[35] Li Z, Li J, Mo B, Hu C, Liu H, Qi H, et al. Genistein induces G2/M cell cycle arrest via stable activation of ERK1/2 pathway in MDA-MB-231 breast cancer cells. Cell Biol Toxicol 2008;24:401–9.
[36] Nguyen DT, Hernandez-Montes E, Vauzour D, Schöntal AH, Rice-Evans C, Cadenas E, et al. The intracellular genistein metabolite 5,7,3′,4′-tetrahydroxyisoflavone mediates G2-M cell cycle arrest in cancer cells via modulation of the p38 signaling pathway. Free Radic Biol Med 2006;41:1225–39.
[37] Chang KL, Kung ML, Chow NH, Su SJ. Genistein arrests hepatoma cells at G2/M phase: involvement of ATM activation and upregulation of p21waf1/cip1 and Wee1. Biochem Pharmacol 2004;67:717–26.
[38] Tang D, Wu D, Hirao A, Lahti JM, Liu L, Mazza B, et al. ERK activation mediates cell cycle arrest and apoptosis after DNA damage independently of p53. J Biol Chem 2002;277:12710–7.
[39] Halaby MJ, Hibma JC, He J, Yang DQ. ATM protein kinase mediates full activation of Akt and regulates glucose transporter 4 translocation by insulin in muscle cells. Cell Signal 2008;20:1555–63.
[40] Perez C, Vilaboa NE, Garcia-Bermejo L, de Blas E, Creighton AM, Aller P. Differentiation of U-937 promonocytic cells by etoposide and ICRF-193, two antitumour DNA topoisomerase II inhibitors with different mechanisms of action. J Cell Sci 1997;110(Part 3):337–43.
[41] Torres M, Forman HJ. Redox signaling and the MAP kinase pathways. Biofactors 2003;17:287–96.
[42] Boonstra J, Post JA. Molecular events associated with reactive oxygen species and cell cycle progression in mammalian cells. Gene 2004;337:1–13.
[43] Orrenius S, Gogvadze V, Zhivotovsky B. Mitochondrial oxidative stress: implications for cell death. Annu Rev Pharmacol Toxicol 2007;47:143–83.
[44] Pelicano H, Feng L, Zhou Y, Carew JS, Hileman EO, Plunkett W, et al. Inhibition of mitochondrial respiration: a novel strategy to enhance drug-induced apoptosis in human leukemia cells by a reactive oxygen species-mediated mechanism. J Biol Chem 2003;278:37832–9.