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

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*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<sub>2</sub>DCFDA, 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.

*Category:* Antibiotics and Chemotherapeutics.

## ABSTRACT

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4 While it has been reported that genistein induces differentiation in multiple tumour cell  
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6 models, the signalling and regulation of isoflavone-provoked differentiation are poorly  
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8 known. We here demonstrate that genistein causes G<sub>2</sub>/M cycle arrest and expression of  
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10 differentiation markers in human acute myeloid leukaemia cells (HL60, NB4), and  
11  
12 cooperates with all-trans retinoic acid (ATRA) in inducing differentiation, while ATRA  
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14 attenuates the isoflavone-provoked toxicity. Genistein rapidly stimulates Raf-1, MEK1/2  
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16 and ERK1/2 phosphorylation/activation, but does not stimulate and instead causes a late  
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18 decrease in Akt phosphorylation/activation which is attenuated by ATRA. Both  
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20 differentiation and G<sub>2</sub>/M arrest are attenuated by MEK/ERK inhibitors (PD98059, U0126)  
21  
22 and ERK1-/ERK2-directed siRNAs, and by the PI3K inhibitor LY294002, but not by the  
23  
24 p38-MAPK inhibitor SB203580. Genistein stimulates p21<sup>waf1/cip1</sup> and cyclin B1 expression,  
25  
26 phosphorylation/activation of ATM and Chk2 kinases, and Tyr15-  
27  
28 phosphorylation/inactivation of Cdc2 (Cdk1) kinase, and these effects are attenuated by  
29  
30 MEK/ERK inhibitors, while LY294002 also attenuates ERK and ATM phosphorylation.  
31  
32 Caffeine abrogates the genistein-provoked G<sub>2</sub>/M blockade and alterations in cell cycle  
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34 regulatory proteins, and also suppresses differentiation. Finally, genistein causes reactive  
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36 oxygen species (ROS) over-accumulation, but the antioxidant N-acetyl-L-cysteine fails to  
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38 prevent ERK activation, G<sub>2</sub>/M arrest, and differentiation induction. By contrast, N-acetyl-L-  
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40 cysteine and p38-MAPK inhibitor attenuate the apoptosis-sensitizing (pro-apoptotic) action  
41  
42 of genistein when combined with the antileukaemic agent arsenic trioxide. In summary,  
43  
44 genistein-induced differentiation in acute myeloid leukaemia cells is a ROS-independent,  
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46 Raf-1/MEK/ERK-mediated and PI3K-dependent response, which is coupled and co-  
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48 regulated with G<sub>2</sub>/M arrest, but uncoupled to the pro-apoptotic action of the drug.  
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*Keywords:* Genistein; Differentiation; Protein kinases; Oxidative stress; Myeloid leukaemia

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## 1. Introduction

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

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

4 With these precedents in mind, we were now interested in investigating the regulation of  
5 genistein-provoked differentiation in human acute myeloid leukaemia cell models (HL60,  
6 NB4), with particular interest on the signalling by protein kinases and the possible  
7 influence of intracellular oxidation. The obtained results indicate that genistein rapidly  
8 activates the expression of differentiation markers, and cooperates with all-trans retinoic  
9 acid (ATRA) in inducing differentiation. Differentiation is a ROS-independent response,  
10 which is mediated by the activation of the Raf-1/MEK/ERK pathway, and also requires the  
11 PI3K/Akt pathway. Finally, differentiation is coupled and co-regulated with G<sub>2</sub>/M cycle  
12 arrest, but uncoupled to the pro-apoptotic action of the drug.  
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## 27 **2. Materials and methods**

### 28 *2.1. Reagents and antibodies*

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32 All components for cell culture were obtained from Invitrogen, Inc. (Carlsbad, CA, USA).  
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34 Dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCFDA) was obtained from Molecular Probes  
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36 (Eugene, OR, USA). 4,6-diamino-2-phenylindole (DAPI) was obtained from Serva  
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38 (Heidelberg, Germany). The kinase inhibitors PD98059, U0126, SB203580 and LY294002  
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40 were obtained from Calbiochem (Darmstadt, Germany). Adaphostin was obtained from  
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42 Biaffin GmbH & CoKG (Kassel, Germany). Rabbit polyclonal antibodies against p44/42  
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44 MAPK, phospho-p44/42 MAPK (Thr<sup>202</sup>/Tyr<sup>204</sup>), MEK1/2, phospho-MEK1/2 (Ser<sup>217/221</sup>),  
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46 phospho-Chk2 (Thr<sup>68</sup>), phospho-cdc2 (Tyr<sup>15</sup>), Akt and phospho-Akt (Ser<sup>473</sup>), were  
47  
48 obtained from Cell Signaling Technology Inc (Danvers, MA, USA). Rabbit polyclonal  
49  
50 antibodies against phospho-Raf-1 (Ser<sup>338</sup>/Tyr<sup>341</sup>) and p21 (C-19), and mouse monoclonal  
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52 antibody against cyclin B1 (D-11) were from Santa Cruz Biotechnology, Inc. (Santa Cruz,  
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1 CA, USA). Mouse monoclonal antibody against phospho-ATM (Ser<sup>1981</sup>) was from Upstate  
2 Cell Signaling Solutions (Temecula, CA, USA). Mouse anti-human CD11b monoclonal  
3 antibody, and peroxidase- and fluorescein isothiocyanate (FITC)-conjugated  
4 immunoglobulin G, were obtained from DAKO Diagnostics, S.A. (Barcelona, Spain). Small  
5 interfering RNAs (siRNAs) against ERK1 and ERK2, and control scramble siRNAs, were  
6 obtained from Santa Cruz. All other reagents were from Sigma (Madrid, Spain).  
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## 16 2.2. Cells and treatments

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20 The human leukaemia cells lines HL60 (myelomonocytic) and NB4 (acute promyelocytic)  
21 were grown as earlier indicated [19]. For experiments, the cell number was adjusted at 10<sup>5</sup>  
22 cells/ml before initiation of the treatments. To prevent long-term cultures from reaching  
23 plateau densities, at the end of the second day of treatment the cultures were  
24 supplemented with an equal volume of fresh medium containing, when pertinent, the  
25 corresponding amount of drugs.  
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33 Stock solutions of genistein and epigallocatechin-3-gallate (EGCG) (50 mM),  
34 adaphostin (10 mM), SB203580, PD98059 and LY294002 (20 mM), U0126 (2,63 mM), N-  
35 acetyl-L-cysteine (NAC, 3 M) and 12-O-tetradecanoylphorbol 13-acetate (20 mM) were  
36 prepared in dimethyl sulfoxide; stock solutions of all-trans retinoic acid (ATRA, 10 mM)  
37 and H<sub>2</sub>DCFDA (5 mM) were prepared in ethanol, and stock solutions of siRNAs (10 μM)  
38 were prepared in distilled water. All these solutions were stored at -20°C. Stock solutions  
39 of DAPI (10 μg/ml) and propidium iodide (1 mg/ml) were prepared in phosphate-buffered  
40 saline (PBS). Arsenic trioxide was initially dissolved in a small amount of 1 M NaOH, after  
41 which PBS was added to give a final solution of 100 mM. These solutions were stored at  
42 4°C. Stock solutions of nitroblue tetrazolium (NBT, 2 mg/ml in PBS) and caffeine (250 mM  
43 in distilled water) were freshly prepared.  
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### 2.3. *Nucleofection of siRNAs*

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4 HL60 cells were nucleofected with siRNAs using the optimized protocol for this cell line  
5 developed by the manufacturer (Amaxa Biosystems, Cologne, Germany). Namely,  $2 \times 10^6$   
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7 cells were centrifuged and resuspended in 100  $\mu$ l of Nucleofector solution (Cell line  
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9 Nucleofector kit V, Amaxa Biosystems), after which the siRNAs were added to a final  
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11 concentration of 9 nM. The samples were nucleofected using the program T-019 (Amaxa  
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13 Nucleofector v.2.1., Amaxa Biosystems), and then transferred to 4 ml of fresh culture  
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15 medium. The levels of total and phosphorylated ERKs were analyzed at different  
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17 incubation times by means of immunoblot.  
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### 2.4. *Determination of cell cycle distribution and apoptosis*

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29 To determine cell cycle phase distribution, the cells were permeabilized, stained with  
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31 propidium iodide, and the fluorescence determined by flow cytometry. Using this method,  
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33 the subpopulation of cells exhibiting sub-G<sub>1</sub> fluorescence (as an indication of sub-G<sub>1</sub> DNA  
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35 content) represented the fraction of apoptotic cells in the culture. An additional criterion for  
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37 apoptosis was the presence of chromatin condensation/fragmentation, as determined by  
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39 cell permeabilization followed by DAPI staining and microscopy examination. A detailed  
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41 description of all these procedures was presented in a preceding publication [20], and  
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43 hence is omitted here.  
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### 2.5. *Determination of cell differentiation*

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54 To detect the surface expression of the differentiation-specific CD11b antigen, cells were  
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56 incubated with anti-CD11b antibody for 30 min at 4°, then extensively washed with RPMI-  
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58 1640 medium, and incubated for 30 min more at 4°C with FITC-labelled goat anti-mouse  
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1 immunoglobulin G. The background of non-specific fluorescence was obtained by  
2 incubating cells only with this secondary antibody. After extensive washing with RPMI-  
3 1640 medium, the fluorescence was estimated by flow cytometry.  
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6 To measure NBT reduction, the cells were centrifuged, re-suspended in PBS  
7 containing 0.2% NBT, and seeded by triplicate in 96 well flat bottom plates (200  $\mu$ l per  
8 well). After addition of 12-O-tetradecanoylphorbol 13-acetate to a final concentration of  
9 100  $\mu$ M, the cells were incubated for 30 min at 37°C. SDS was then added to a final  
10 concentration of 2% to disrupt the cells and dissolve the formazan crystals, and dye  
11 absorbance was measured by spectrometry at 595 nm. The absorption of samples  
12 containing NBT and 12-O-tetradecanoylphorbol 13-acetate without cells was also  
13 measured as the background signal.  
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#### 27 *2.6. Measurement of intracellular ROS accumulation*

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29 Intracellular ROS accumulation was measured by flow cytometry using the fluorescent  
30 probe H<sub>2</sub>DCFDA, as previously described [20].  
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#### 38 *2.7. Flow cytometry*

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40 The analysis of samples was carried out using an EPICS XL flow cytometer (Coulter,  
41 Hialeah, FL) equipped with an air-cooled argon laser tuned to 488 nm. The specific  
42 fluorescence signals corresponding to FITC and H<sub>2</sub>DCFDA were collected with a 525-nm  
43 band pass filter, and the signal corresponding to propidium iodide with a 620-nm band  
44 pass filter. As a rule, 10<sup>4</sup> cells were counted in each determination.  
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#### 57 *2.8. Immunoblot assays*

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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 x 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 to 50  $\mu$ M, 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  $\mu$ M genistein, and at 96 h with 10  $\mu$ M genistein. Differentiation induction by 25  $\mu$ M genistein was corroborated by

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measuring NBT reduction (see Fig. 2B). As expected, the isoflavone caused cell  
accumulation at G<sub>2</sub>/M in HL60 cell cultures, and this effect was already observed at 24  
and 96 h of treatment with 25 μM 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 hour 72, as revealed by accumulation of  
cells with sub-G<sub>1</sub> 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.

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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<sub>1</sub> (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<sub>2</sub>/M blockade 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<sub>1</sub> DNA content (Fig. 2D, and results not shown).

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For comparative purposes experiments were carried using the tyrphostin derivative  
adaphostin and the flavonol EGCG, two antitumour agents which as genistein cause

1 tyrosine kinase inhibition [22,23] and ROS-mediated apoptosis in leukaemia cells [24,25  
2 and our unpublished observations]. Adaphostin and EGCG were used at the  
3 concentrations of 0.25  $\mu$ M and 25  $\mu$ M, respectively, which according to preliminary  
4 determinations caused a moderate toxicity at 72 h of treatment, as in the case of 25  $\mu$ M  
5 genistein. By contrast to genistein, these agents did not cause G<sub>2</sub>/M blockade or  
6 stimulation of CD11b surface expression, and also failed to potentiate the stimulation  
7 produced by ATRA (not shown results).  
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### 10 3.2. *Protein kinase modulation and effects of protein kinase inhibitors*

11 Earlier studies indicated that ERK activation is involved in the regulation of myeloid cell  
12 differentiation [26]. However, genistein was reported to inhibit the MEK/ERK pathway in  
13 some cell systems [4,7-8]. For these reasons, we examined the capacity of the isoflavone  
14 to modulate the phosphorylation/activation of ERK and upstream kinases (MEK, Raf-1) in  
15 HL60 cells. It was observed that 25  $\mu$ M genistein caused a rapid and stable increase in the  
16 phosphorylation state of these kinases, which was already detected at 6 h of treatment  
17 (Fig. 3A). The increase in ERK phosphorylation was corroborated at 24 h in NB4 cells, and  
18 still persisted at 48 h in both cell lines (results not shown). Treatment for 24 h with ATRA  
19 alone slightly increased Raf-1, MEK and ERK phosphorylation, but the effect of genistein  
20 plus ATRA was not higher than that caused by genistein alone (Fig. 3B). The cause-effect  
21 relationship between ERK activation and differentiation induction was then examined using  
22 the MEK/ERK inhibitors PD98059 (20  $\mu$ M) and U0126 (2  $\mu$ M), as well as specific siRNAs  
23 directed against ERK1 and ERK2. Under the used conditions both the pharmacologic  
24 inhibitors and siRNAs efficaciously attenuated ERK phosphorylation (see Fig. 4A (inset)  
25 and C). It was observed that co-treatment with PD98059 and U0126 attenuated CD11b  
26 surface expression (Fig. 4A, left bar chart) and NBT reduction (Fig. 4B, left bar chart) in  
27 genistein-treated HL60 and NB4 cells, and that ERK-directed siRNAs also attenuated  
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antigen expression 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<sub>1</sub> and reduced the accumulation at G<sub>2</sub>/M in genistein-treated HL60 (Fig. 5A) and NB4 (result not shown) cell cultures. The analysis of proteins responsible for the regulation of G<sub>2</sub> 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<sup>waf1/cif1</sup> 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  $\mu$ M 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<sub>2</sub>/M cell accumulation (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  $\mu$ M

1 genistein. As indicated in Fig. 3C, the isoflavone decreased Akt phosphorylation, but the  
2 decrease was only detected at 48 h of treatment, following the initiation (hour 24) of G<sub>2</sub>/M  
3 arrest and differentiation (see Fig. 1A,B). On the other hand, co-treatment with ATRA,  
4 which as indicated above prevented the late (hour 72) genistein-provoked apoptosis (see  
5 Fig. 2), also prevented the decrease in Akt phosphorylation (Fig. 3C). A second group of  
6 experiments was carried out using the PI3K inhibitor LY294002. It was observed that the  
7 administration of 20 μM LY294002, which efficaciously decreased the basal Akt  
8 phosphorylation level (Fig. 6C), prevented the isoflavone-provoked stimulation of CD11b  
9 expression (Fig. 6A) and G<sub>2</sub>/M cell accumulation (Fig. 6B), as well as the increase in ERK  
10 and ATM phosphorylation (Fig. 6C). Thus, while the experiments with PI3K inhibitor  
11 indicate that Akt is important for proper execution of both differentiation and G<sub>2</sub>/M arrest,  
12 the kinetic assays indicate the trigger of these processes by genistein may not be  
13 adequately explained by changes in Akt phosphorylation. On the other hand, kinase down-  
14 regulation might facilitate the late apoptotic action of the isoflavone.  
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32 To strengthen the relationship between cell cycle blockade and differentiation induction,  
33 a complementary set of experiments was carried out using caffeine. This methylxanthine is  
34 known to override the G<sub>2</sub> check-point by inhibiting ATM phosphorylation, although other  
35 studies indicate that caffeine also delays G<sub>1</sub> to S phase transition [28]. In agreement with  
36 this, treatment of HL60 cells with 5 mM caffeine caused cell accumulation at G<sub>1</sub>,  
37 suppressed the G<sub>2</sub>/M arrest caused by genistein (Fig. 7A), and accordingly abrogated the  
38 isoflavone-provoked increase in ATM, Chk2 and Cdc2-Tyr15 phosphorylation, and the  
39 increase in cyclin B1 expression (Fig. 7B). Interestingly, caffeine also inhibited the  
40 isoflavone-induced increase in CD11b expression and NBT reduction (Fig. 7C). Taken  
41 together, these results strongly indicate that genistein-provoked G<sub>2</sub>/M arrest and  
42 differentiation are closely associated events, which share similar regulatory mechanisms.  
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### 3.3. ROS production

Earlier reports indicated that intracellular ROS over-accumulation may mediate drug-induced differentiation in leukaemia cells [17,18] as well as ERK activation and ERK-regulated G<sub>2</sub>/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  $\mu$ M genistein caused a moderate (approximately three- to four-fold) increase in intracellular ROS accumulation in HL60 cells, as measured by the increase in H<sub>2</sub>DCFDA-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<sub>2</sub>/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  $\mu$ M genistein), while apoptosis execution was a delayed event (72 h). This late apoptosis might represent



1 a secondary response derived from differentiation and/or sustained cell cycle disruption,  
2 which clearly contrasts with the capacity of genistein to rapidly sensitize to apoptosis  
3 induction by radiation and chemotherapeutic drugs (here defined as “pro-apoptotic” effect)  
4 in leukaemia [16] and other tumour cell models [3,9]. The capacity of genistein to interact  
5 with other differentiation inducers was examined in preceding works, with discrepant  
6 results. For instance, the isoflavone was observed either to potentiate [30] or prevent [31]  
7 the expression of differentiation markers in ATRA-treated HL60 and other leukaemia cell  
8 lines. In another study, genistein caused premature cell death when used in combination  
9 with ATRA in HL60 cells [32]. However, in our present experiments genistein and ATRA  
10 cooperated to induce differentiation in HL60 and NB4 cells while, what is equally important,  
11 ATRA attenuated the isoflavone-provoked apoptosis. Noteworthy, recent studies indicated  
12 that dietary achievable doses of genistein caused tumour reduction and elicited molecular  
13 events in animal models, similar to those elicited in tumour cell lines *in vitro* by apoptosis-  
14 inducing isoflavone concentrations [33,34]. Taking everything into consideration, one  
15 should not exclude the possibility that genistein (and perhaps other flavonoides) might also  
16 be used to improve the differentiation capacity of ATRA under *in vivo* conditions, and its  
17 clinical efficacy as antileukaemic agent.

18 As indicated above, genistein was reported cause either ERK activation [5,6] or  
19 inhibition [4,7,8] in tumour cell models. Our present results demonstrate that the Raf-  
20 1/MEK/ERK pathway is strictly required for differentiation induction by genistein in acute  
21 myeloid leukaemia cells. Thus, the isoflavone rapidly stimulated the phosphorylation of  
22 these kinases (6 h), and the expression of differentiation markers was reduced by  
23 pharmacologic MEK inhibitors and ERK-directed siRNAs. By contrast, the failure of  
24 SB203580 to affect CD11b expression suggests that p38-MAPK does not participate in the  
25 regulation of genistein-provoked differentiation in this cell model. Finally, our observations  
26 indicate that both ERK activation and subsequent differentiation induction are dependent  
27 on the proper functioning of the PI3K/Akt pathway, since these processes were prevented  
28 by the PI3K inhibitor LY294002. Nonetheless, differentiation could be initiated (24 h) in the

1 absence of Akt activation, and differentiation progression tolerated the partial Akt down-  
2 regulation caused by genistein (48 h). On the other hand it is conceivable that down-  
3 regulation of Akt, which is a protective kinase, may favour the late genistein-provoked  
4 apoptosis (72 h). Moreover, we may speculate that the attenuation of Akt de-  
5 phosphorylation by ATRA might explain at least in part the protective (anti-apoptotic)  
6 action of the retinoid, and also perhaps the cooperation between genistein and ATRA in  
7 inducing differentiation.  
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10 In addition, the present results corroborate the capacity of genistein to cause G<sub>2</sub>/M cell  
11 cycle blockade, and indicate that cycle arrest and differentiation induction are closely  
12 associated and co-regulated events in isoflavone-treated myeloid leukaemia cells. Thus,  
13 the initiation of G<sub>2</sub>/M cell accumulation was coincident in time with the expression of  
14 differentiation markers, and both cycle arrest and differentiation were prevented by  
15 MEK/ERK and PI3K inhibitors, but not by p38-MAPK inhibitor. Our observations are  
16 congruent with a previous study indicating that genistein causes G<sub>2</sub>/M blockade via stable  
17 ERK activation in MDA-MB-231 breast cancer cells [35], but contrast with another report  
18 indicating regulation of this blockade by p38-MAPK in T47D breast cancer cells [36]. The  
19 genistein-provoked G<sub>2</sub>/M arrest is regulated by ATM and Chk2 activation, Cdc2  
20 inactivation, and increased p21<sup>waf1/cif1</sup> expression, which essentially agrees with earlier  
21 observations in other cell types [37], but the isoflavone also increased the levels of cyclin  
22 B1. Whether this increase plays a regulatory role, or it merely reflects a failure of protein  
23 degradation resulting in progressive accumulation due to sustained pre-mitotic arrest, is  
24 presently unknown. Importantly, in our experiments the genistein-provoked ATM activation  
25 was a relatively late event (14-24 h) in comparison to ERK activation (6 h), and was also  
26 regulated by both Akt and ERK, as proved by the capacity of PI3K and MEK/ERK  
27 inhibitors to prevent ATM phosphorylation. These results contrast with observations in  
28 other cell models, which characterized ERK and Akt as downstream targets of ATM in  
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1 etoposide-treated murine embryonic fibroblasts [38] and insulin-stimulated murine muscle  
2 cells [39], respectively. Finally, an intriguing aspect is the actual relationship between cell  
3 cycle blockade and differentiation induction. One of the multiple biochemical effects of  
4 genistein is the generation of topoisomerase II-mediated DNA breakage, and it is well  
5 known that DNA-damaging agents prevent cell progression from G<sub>2</sub> into mitosis. In  
6 addition, we and others demonstrated that topoisomerase poisons and other DNA-  
7 damaging agents which cause S or G<sub>2</sub>/M phase arrest efficaciously induce myeloid cell  
8 differentiation [40, and references therein]. Thus, it is tempting to speculate that  
9 differentiation induction by genistein might in part represent a response to cell cycle  
10 disturbance. The observation that the ATM inhibitor caffeine, which overrides the G<sub>2</sub>/M  
11 arrest, also prevents differentiation might support this hypothesis. Nonetheless, further  
12 studies would be required to conclude the existence of a true cause-effect relationship  
13 between these phenomena.  
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30 Intracellular ROS mediate multiple cellular responses, including protein kinase  
31 activation [41], cell cycle progression [42], myeloid cell differentiation [17,18], and  
32 apoptotic and necrotic cell death [43]. Moreover, the level of intracellular ROS may  
33 modulate the toxicity of antitumour drugs [44]. In this regard our preceding publication  
34 demonstrated that genistein sensitizes leukaemia cells to arsenic trioxide-provoked  
35 apoptosis via ROS-mediated activation of p38-MAPK and AMPK, while these kinases  
36 were irrelevant for G<sub>2</sub>/M cycle blockade [16]. As a complement, the present work  
37 demonstrates that ROS accumulation does not mediate genistein-provoked ERK  
38 activation or G<sub>2</sub>/M blockade; that ROS and p38-MAPK are also irrelevant for differentiation  
39 induction; and that MEK/ERK inhibitors exacerbate the pro-apoptotic action of genistein.  
40 Taken together, our results indicate that genistein elicits opposite signalling pathways in  
41 myeloid leukaemia cells, which are tentatively schematized in Fig. 9. On the one hand Raf-  
42 1/MEK/ERK activation, which in turn triggers ATM-mediated G<sub>2</sub>/M blockade, and also  
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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- $\kappa$ B inhibition and PI3K/Akt down-regulation [3], override the protective action of ERK activation and facilitate the pro-apoptotic action of the isoflavone.

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## LEGENDS FOR FIGURES

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4 Fig. 1 - Differentiation induction and cell cycle alterations by genistein. (A) Frequency  
5 (mean  $\pm$  SD of three determinations) of cells exhibiting CD11b surface expression, as  
6 determined by indirect immunofluorescence and flow cytometry, in untreated (Cont) HL60  
7 and NB4 cell cultures and cultures treated for the indicated time periods with the indicated  
8 concentrations of genistein (Gen). An example of the obtained flow cytometric profiles  
9 using HL60 cells is shown at the bottom. The vertical dotted line delimits the region of  
10 positive cells (above the background fluorescence). (B) Cell cycle distribution according to  
11 their DNA content, as revealed by propidium iodide-derived fluorescence and flow  
12 cytometry, in untreated HL60 cell cultures and cultures treated for the indicated time  
13 periods with the indicated concentrations of genistein. The presence of sub-G<sub>1</sub> DNA  
14 content is considered as a criterion of apoptosis (Ap). The insets indicate the frequency of  
15 apoptotic cells and cells in the different phases of the cell cycle. The profiles are  
16 representative of one of two experiments with similar results. Ten thousand cells were  
17 scored in each cytometric profile.

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38 Fig. 2 - Effects of the combination of genistein and ATRA. (A) Frequency of HL60 and NB4  
39 cells exhibiting CD11b surface expression upon treatment for the indicated time periods  
40 with ATRA and the indicated concentrations of genistein, alone and in combination.  
41 Asterisks indicate that the value in the combined treatment (Gen/ATRA) is significantly  
42 higher than the sum of values in the corresponding isolated treatments (Gen alone and  
43 ATRA alone). (B) NBT reduction in HL60 and NB4 cell cultures treated with ATRA and  
44 genistein, alone and in combination. The results (absorbance at 595 nm) are expressed as  
45 fold increase in relation to untreated (Cont) cells, which received the arbitrary value of one.  
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56 (C) Cell cycle distribution in HL60 cell cultures subjected to same treatments as in (B). (D)  
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1 untreated HL60 cell cultures subjected to the same treatments as in (B). Asterisks  
2 indicate significant differences between the indicated pair of values. ATRA was always  
3 used at 1  $\mu$ M, and genistein at 25  $\mu$ M except when otherwise indicated. All other  
4 conditions were as in Fig. 1.  
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10 Fig. 3 – Protein kinase phosphorylation. (A) Relative levels of phosphorylated (p-) Raf-1,  
11 MEK1/2, ERK1/2, and ATM, in untreated (Cont) HL60 cells and cells treated for the  
12 indicated time periods with genistein, as measured by immunoblot. The level of  $\alpha$ -tubulin  
13 ( $\alpha$ -tub) or the corresponding total (t-) protein kinase were measured as loading controls. (B)  
14 Relative levels of total and phosphorylated protein kinases in HL60 cells treated for 24 h  
15 with genistein and ATRA, alone and in combination. (C) Relative levels of total and  
16 phosphorylated Akt in untreated HL60 cells and cells treated for the indicated time periods  
17 with genistein (left blot), and in cells treated for 48 h with genistein and ATRA, alone and in  
18 combination (right blot). ATRA was always used at 1  $\mu$ M, and genistein at 25  $\mu$ M. All other  
19 conditions were as in Figs. 1 and 2.  
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36 Fig. 4 - Effect of MAPK inhibition on genistein-induced differentiation. (A,B) CD11b surface  
37 expression at hour 48 (A) and NBT reduction at hour 72 (B) in untreated (Cont) HL60 and  
38 NB4 cell cultures and cultures treated with genistein alone (left bar charts) or genistein  
39 plus ATRA (right bar charts), either in the absence (--) or the presence of the MEK/ERK  
40 inhibitors PD98059 (PD, 20  $\mu$ M) and U0126 (U, 2.5  $\mu$ M) or the p38-MAPK inhibitor  
41 SB203580 (SB, 10  $\mu$ M). The inset in (A) shows inhibition by PD98059 and U0126 of  
42 genistein-provoked ERK phosphorylation. The kinase inhibitors were added 30 min in  
43 advance to the other drugs. (C) CD11b surface expression (bar chart) and total (t-) and  
44 phosphorylated (p-) ERK levels (immunoblot) in non-nucleofected HL60 cells (nn-cells)  
45 and cells nucleofected with irrelevant scrambled (Cont) and ERK1- and ERK2-directed  
46 siRNAs, either without treatment or after genistein. Genistein was added at 24 h post-  
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1 nucleofection, and the determinations performed at 24 h (ERK levels) or 48 h (CD11b  
2 expression) of treatment. In all experiments, ATRA was used at 1  $\mu$ M and genistein at 25  
3  $\mu$ M. The asterisks indicate significant differences in relation to the control. All other  
4  $\mu$ M. The asterisks indicate significant differences in relation to the control. All other  
5 conditions were as in Figs. 1-3.  
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10 Fig. 5 - Effects of MEK/ERK and p38-MAPK inhibitor on genistein-provoked cell cycle  
11 arrest and cell cycle-related protein alterations. (A) Changes in cell cycle distribution in  
12 HL60 cell cultures treated for 48 h with 25  $\mu$ M genistein and 20  $\mu$ M PD98059 or 10  $\mu$ M  
13 SB203580, alone and in combination. (B) Relative levels of total cyclin B1 and p21<sup>waf1/cif1</sup>  
14 and of phosphorylated (p-) ATM, Chk2 and Cdc2 (Tyr15) proteins in cells subjected for 24  
15 h to the same treatments as above. All other conditions were as in Figs. 1, 3 and 4.  
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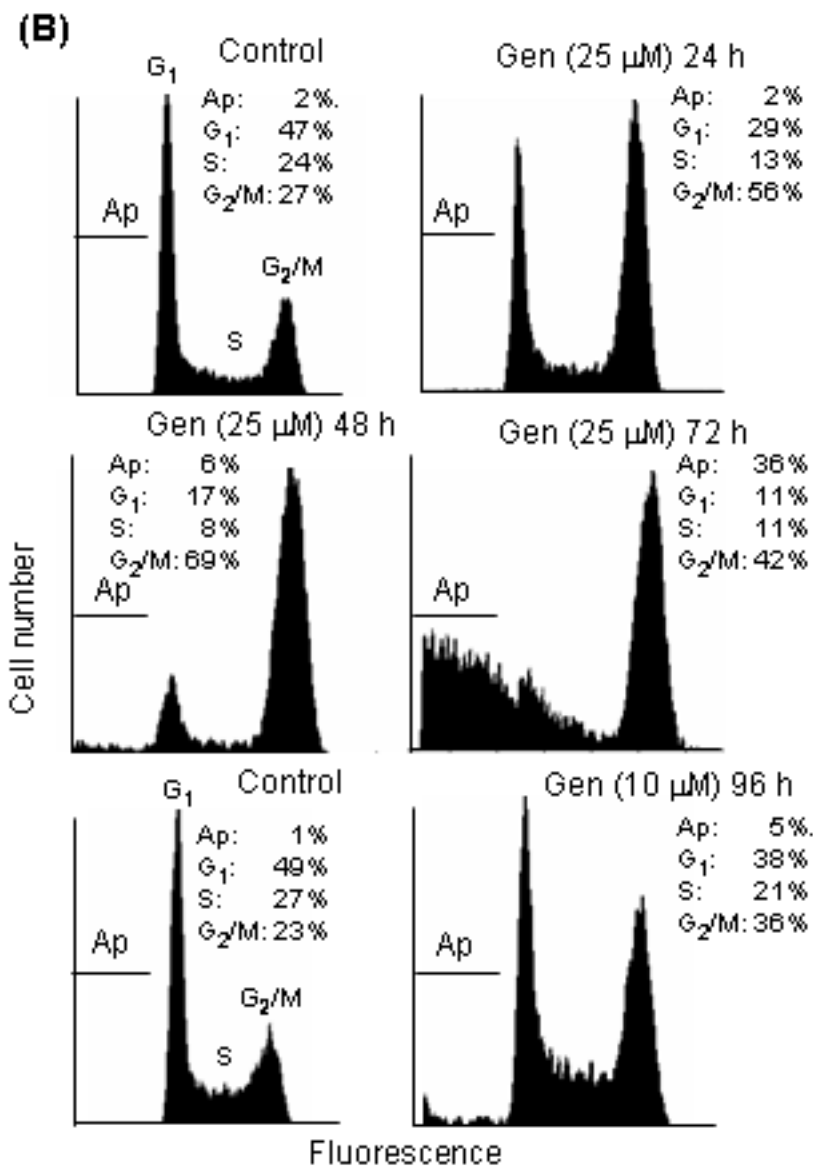
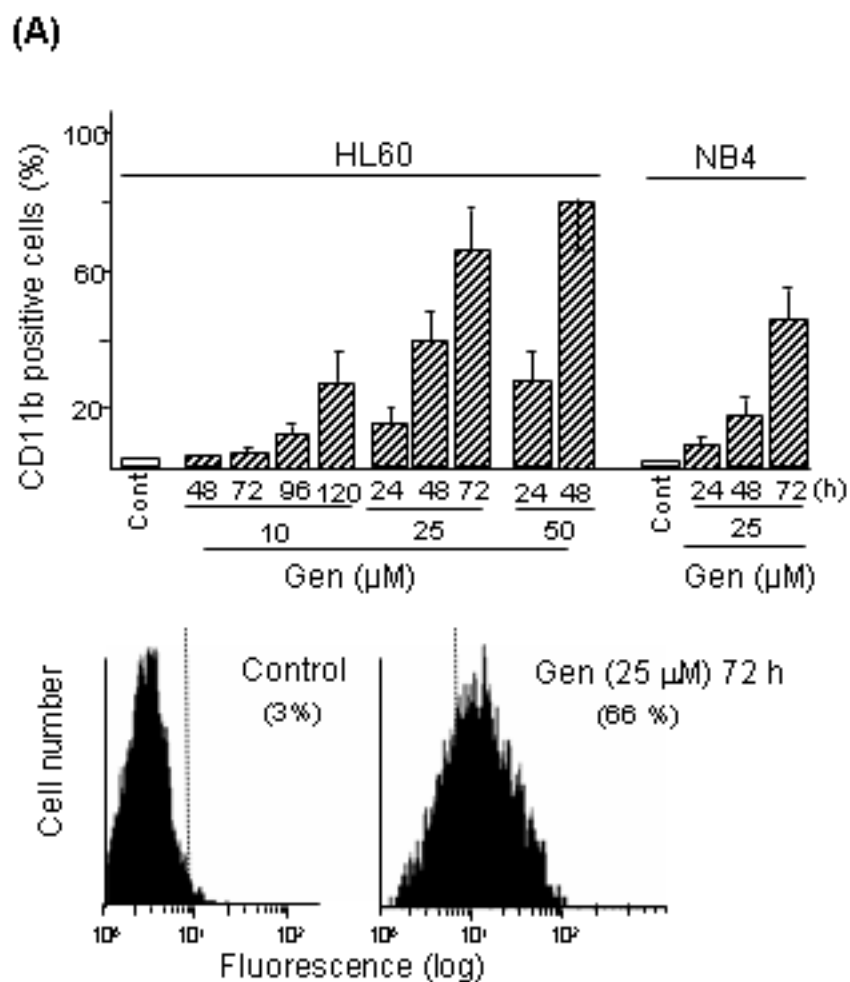
27 Fig. 6 - Effects of PI3K/Akt inhibitor. (A,B) CD11b surface expression (A) and cell cycle  
28 distribution (B) in untreated HL60 cell cultures (Cont) and cultures treated for the indicated  
29 time periods with LY294002 (LY, 20  $\mu$ M) and 25  $\mu$ M genistein, alone and in combination.  
30 (C) Relative levels of total (t-) and phosphorylated (p-) Akt, ERK and ATM, in untreated  
31 HL60 cells and cells treated for 24 h with 25  $\mu$ M genistein and 20  $\mu$ M LY294002. All other  
32 conditions were as in Figs. 1 and 3.  
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43 Fig. 7 - Modulation by caffeine of genistein-provoked effects. (A) Cell cycle distribution in  
44 HL60 cell cultures treated for 24 h with 25  $\mu$ M genistein and 5 mM caffeine, alone or in  
45 combination. (B) Relative levels of total cyclin B1 and phosphorylated (p-) ATM, Chk2 and  
46 Cdc2 (Tyr15) in cells subjected to the same treatments as above. (C) CD11b surface  
47 expression and NBT reduction in cells subjected for the indicated time periods to the same  
48 treatments as above. All other conditions were as in Figs. 1, 2 and 3.  
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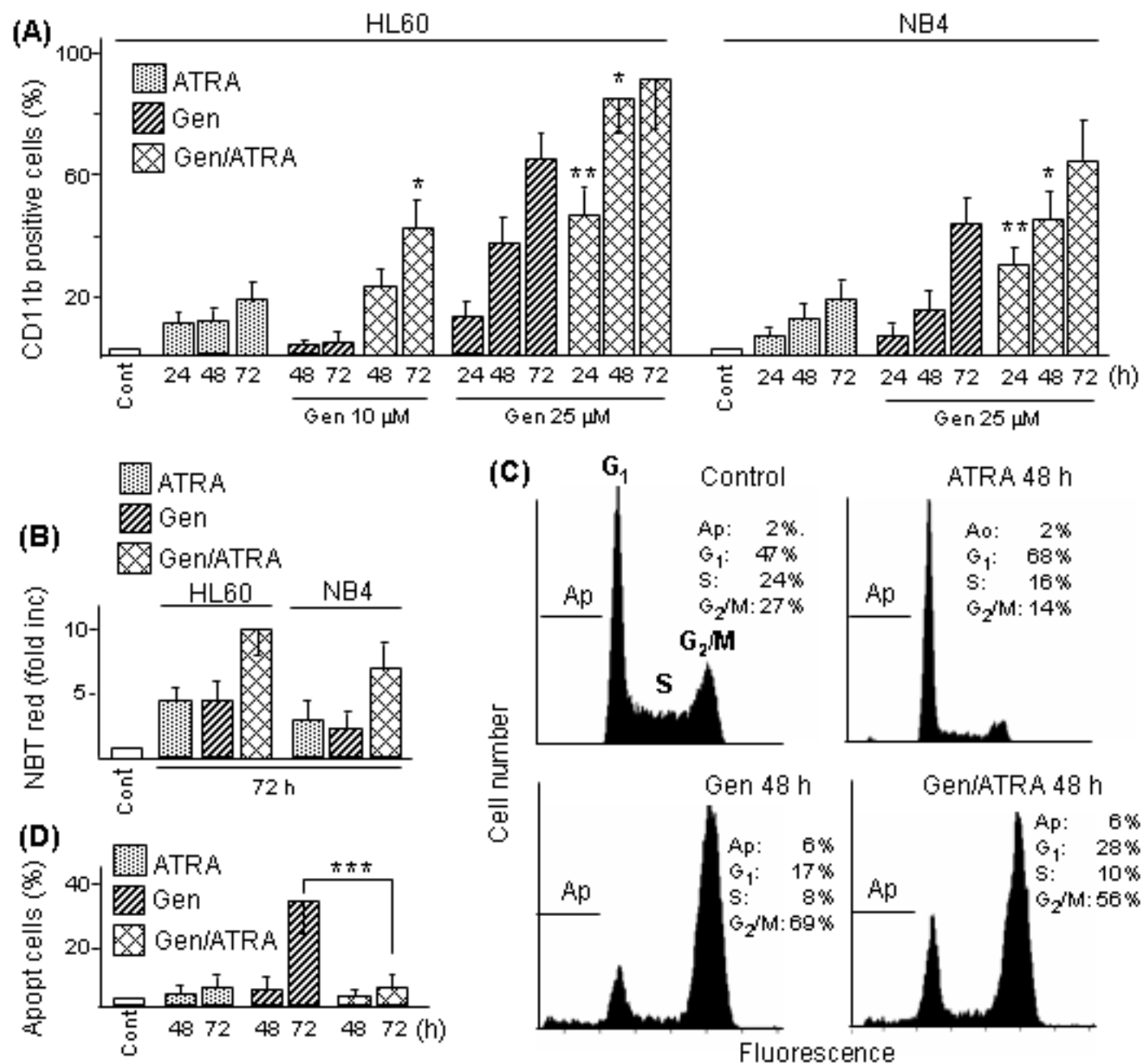
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Fig. 8 - Oxidative stress. (A) Relative intracellular peroxide accumulation in untreated HL60 cells (Cont) and cells treated for 8 h with genistein, as determined by flow cytometry after cell loading with H<sub>2</sub>DCFDA. The vertical dotted line 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 hour 24, (D) CD11b surface expression at hour 48, and (E) relative levels of total (t-) and phosphorylated (p-) ERK1/2 at hour 24, in HL60 cells treated with genistein, alone and in combination with NAC. Genistein was used at 25  $\mu$ M, NAC at 10 mM, and ATO at 2  $\mu$ M. NAC was added 1 h in advance to the other agents. All other conditions were as in Figs. 1, 3 and 4.

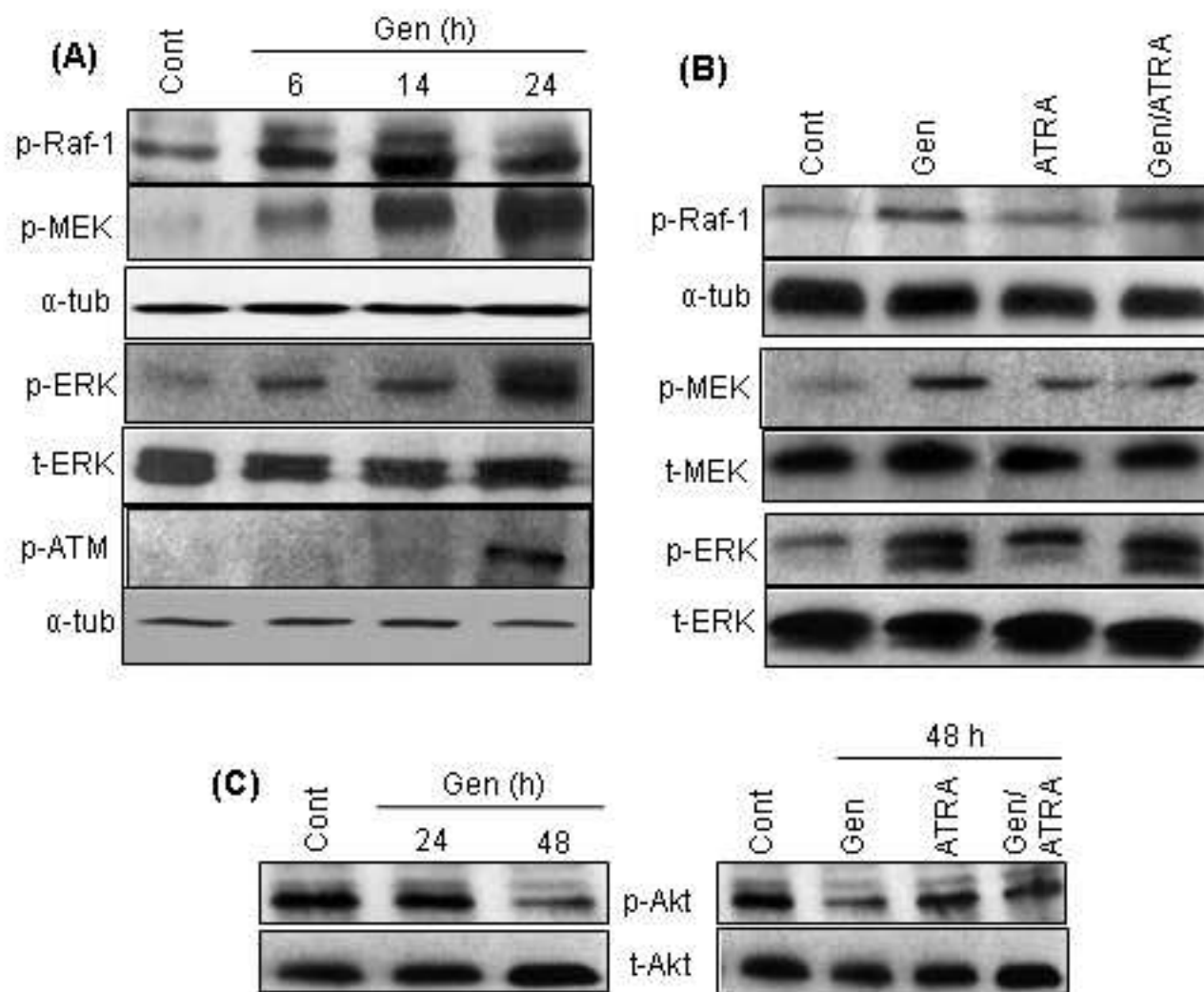
Fig. 9 - Effects of genisten in myeloid leukaemia cells. The scheme summarizes the results obtained in the present work and in a preceding publication [Ref. 16]. Genistein causes ROS over-production, leading to the activation of the ROS-inducible kinases p38-MAPK and AMPK. This facilitates apoptosis 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<sub>2</sub> 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.



**Fig 1**

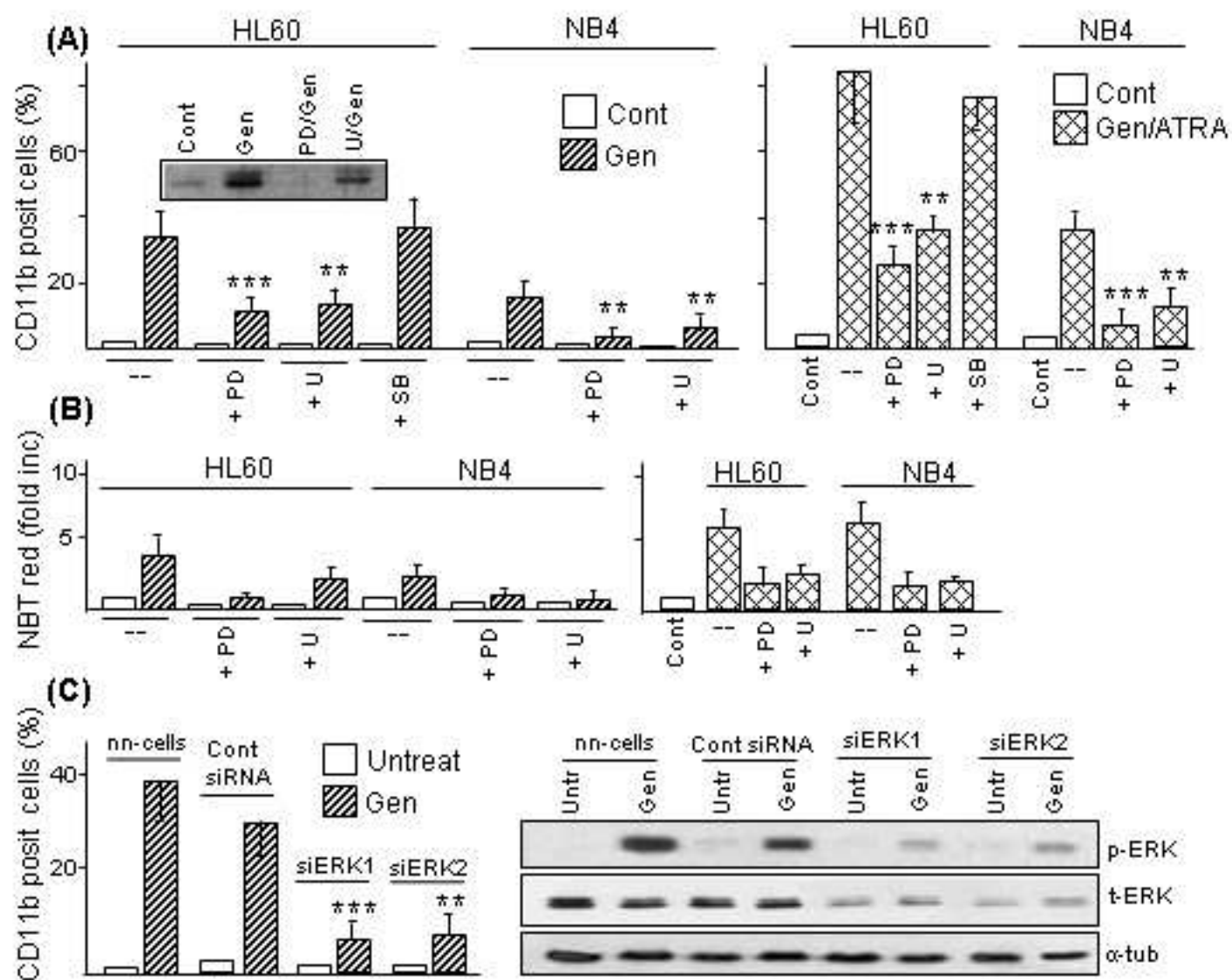


**Fig 2**

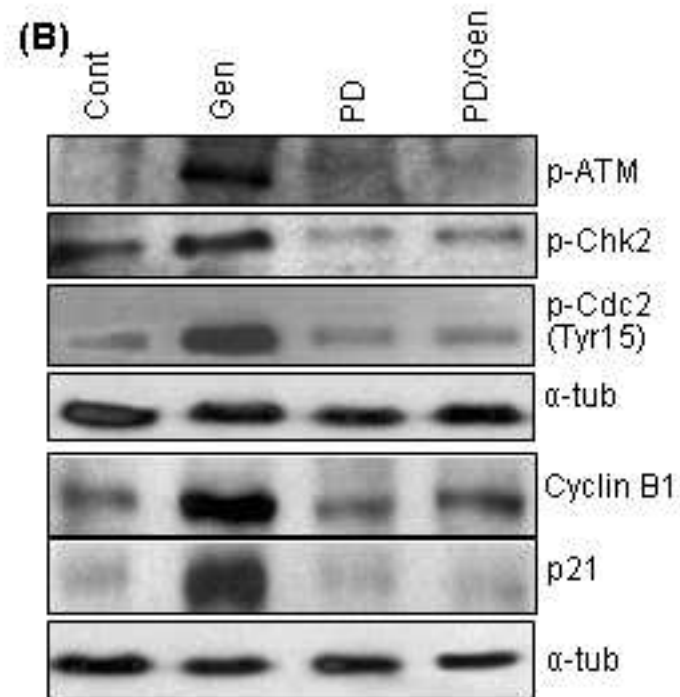
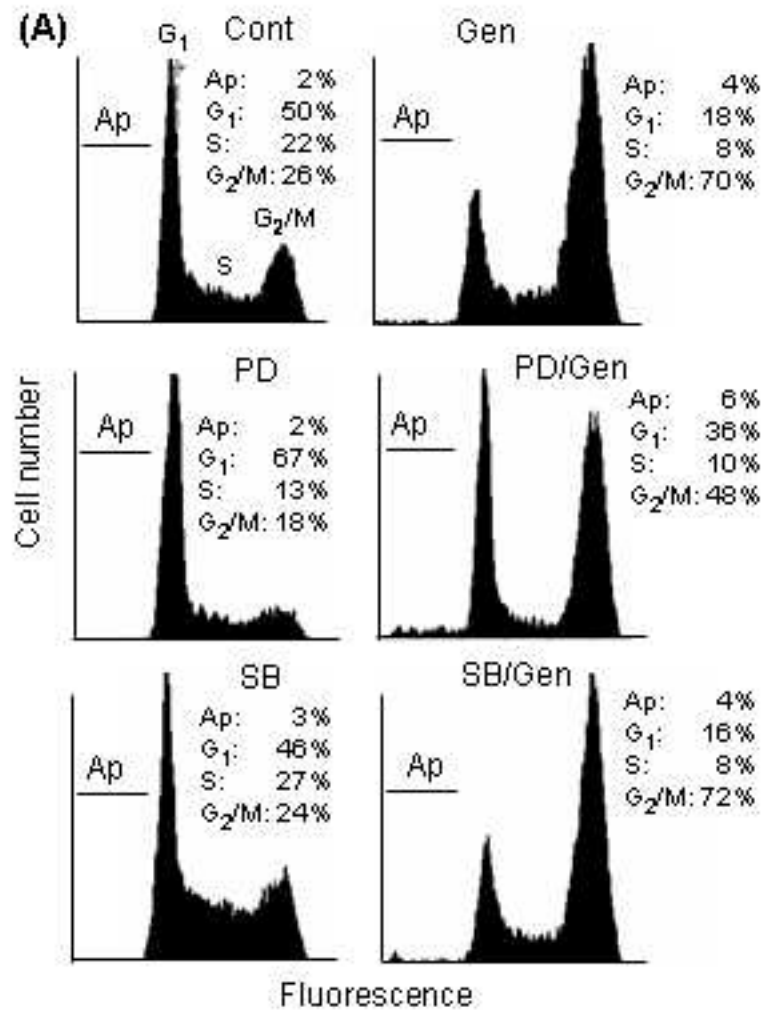


**Fig 3**

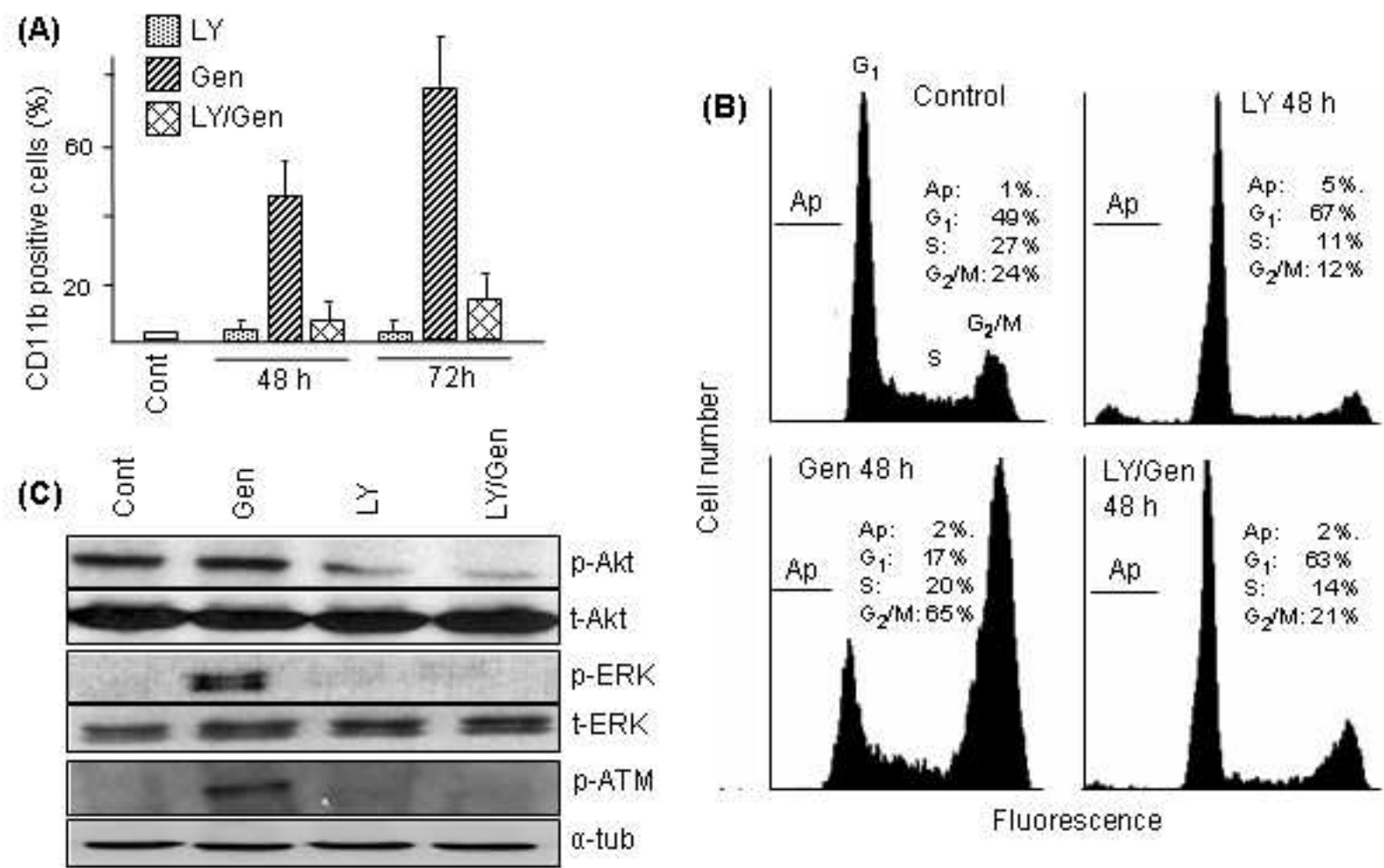




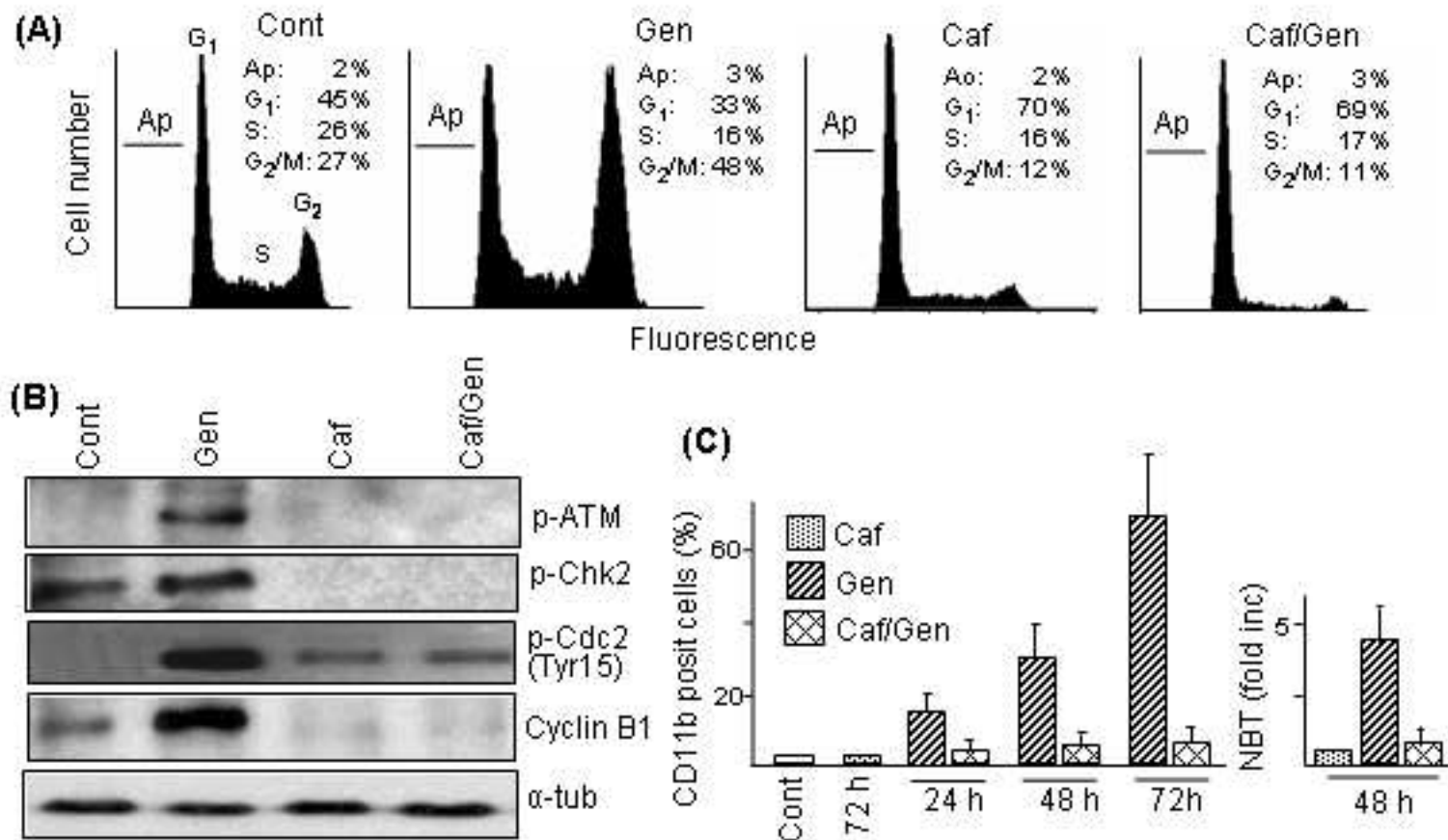
**Fig 4**



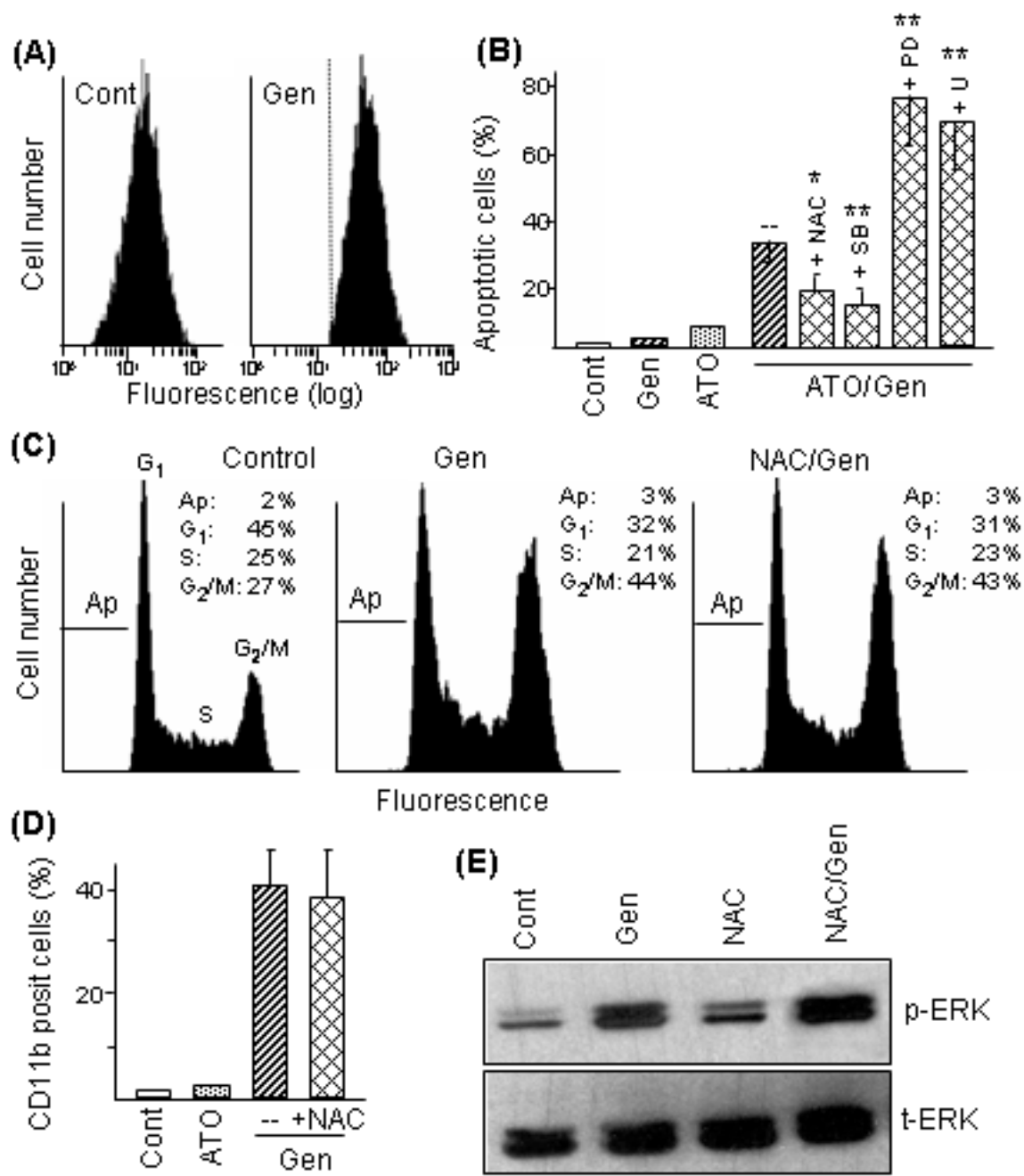
**Fig 5**



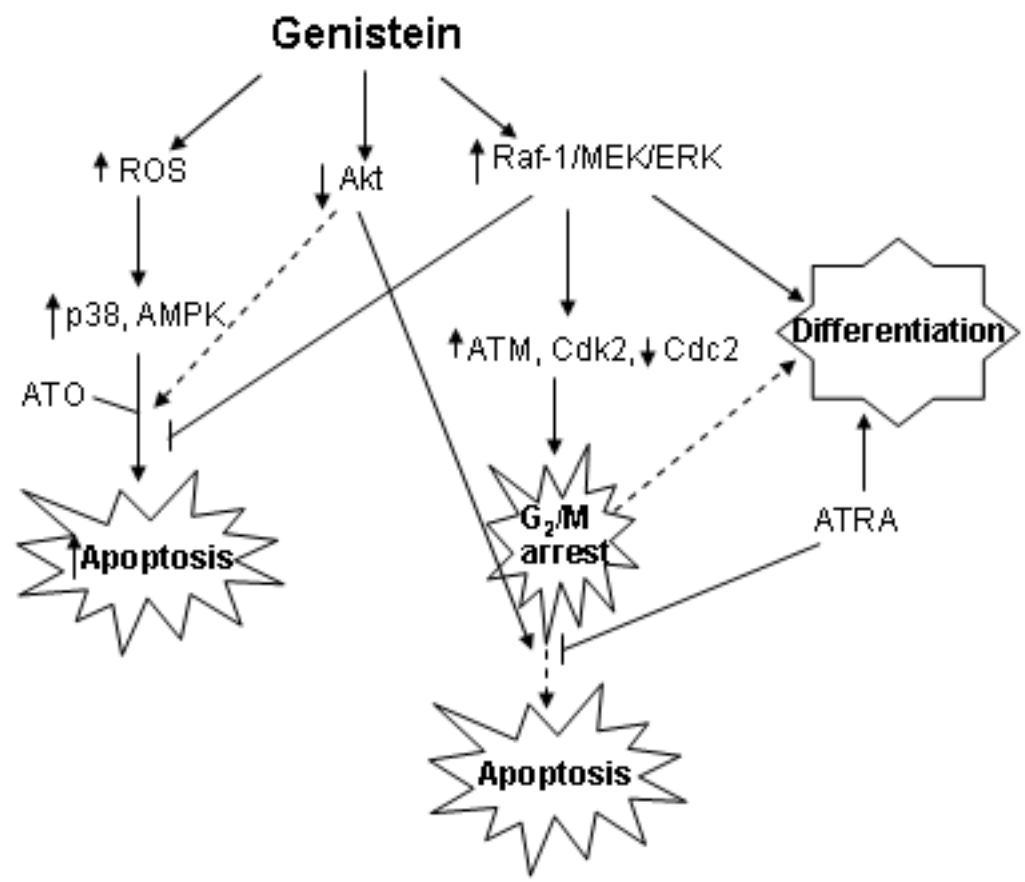
**Fig 6**



**Fig 7**



**Fig 8**



**Fig 9**