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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; H₂DCFDA, 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 G₂/M cycle arrest and expression of
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10 differentiation markers in human acute myeloid leukaemia cells (HL60, NB4), and
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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 G₂/M arrest are attenuated by MEK/ERK inhibitors (PD98059, U0126)
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22 and ERK1-/ERK2-directed siRNAs, and by the PI3K inhibitor LY294002, but not by the
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24 p38-MAPK inhibitor SB203580. Genistein stimulates p21^{waf1/cip1} and cyclin B1 expression,
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26 phosphorylation/activation of ATM and Chk2 kinases, and Tyr15-
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28 phosphorylation/inactivation of Cdc2 (Cdk1) kinase, and these effects are attenuated by
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30 MEK/ERK inhibitors, while LY294002 also attenuates ERK and ATM phosphorylation.
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32 Caffeine abrogates the genistein-provoked G₂/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, G₂/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
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42 of genistein when combined with the antileukaemic agent arsenic trioxide. In summary,
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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 G₂/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 G₂/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 G₂/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 (H₂DCFDA) 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 (Thr²⁰²/Tyr²⁰⁴), MEK1/2, phospho-MEK1/2 (Ser^{217/221}),
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46 phospho-Chk2 (Thr⁶⁸), phospho-cdc2 (Tyr¹⁵), Akt and phospho-Akt (Ser⁴⁷³), were
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48 obtained from Cell Signaling Technology Inc (Danvers, MA, USA). Rabbit polyclonal
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50 antibodies against phospho-Raf-1 (Ser³³⁸/Tyr³⁴¹) 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 (Ser¹⁹⁸¹) 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 10⁵
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 H₂DCFDA (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, 2×10^6
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7 cells were centrifuged and resuspended in 100 μ 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-G₁ fluorescence (as an indication of sub-G₁ 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 μ l per
8 well). After addition of 12-O-tetradecanoylphorbol 13-acetate to a final concentration of
9 100 μ 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 H₂DCFDA, 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 H₂DCFDA 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, 10⁴ cells were counted in each determination.
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57 *2.8. Immunoblot assays*

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2 To obtain total cellular protein extracts, cells were collected by centrifugacion, washed with
3 PBS, and lysed for 20 min at 4°C in a buffer consisting of 20 mM Tris-HCl, pH 7.5,
4 containing 137 mM NaCl, 10% (v/v) glycerol and 1% (v/v) Nonidet P-40, supplemented
5 with a protease inhibitor cocktail, 1 mM sodium orthovanadate and 10 mM NaF. After
6 centrifugation at 10,000 x g for 15 min at 4°C, the supernatants were collected. Fractions
7 containing equal protein amounts were analyzed by SDS-polyacrylamide gel
8 electrophoresis, blotted onto membranes and immunodetected, as previously described
9 [21].
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20 2.9. Statistical analysis

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

3. Results

3.1. Cell growth and differentiation

44 We firstly examined the efficacy of genistein to induce differentiation in HL60 and NB4
45 cells, as determined by the increased cell surface expression of CD11b antigen. Genistein
46 was initially assayed at 10 to 50 μ M, which is in the range of concentrations currently used
47 to study apoptosis in leukaemia cells and other tumour cell models [16, and references
48 therein]. Some of the obtained results are indicated in Fig. 1A. Increased CD11b
49 expression was already detected at 24 h of treatment with 25-50 μ M genistein, and at 96 h
50 with 10 μ M genistein. Differentiation induction by 25 μ M genistein was corroborated by
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measuring NBT reduction (see Fig. 2B). As expected, the isoflavone caused cell
accumulation at G₂/M in HL60 cell cultures, and this effect was already observed at 24
and 96 h of treatment with 25 μ M and 10 μ M genistein, respectively (Fig. 1B), thus
paralleling the initiation of differentiation marker expression. On the other hand, within the
assayed time periods 10 μ M genistein did not cause significant apoptosis, while at the
concentration of 25 μ M apoptosis was detected at hour 72, as revealed by accumulation of
cells with sub-G₁ 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 μ M genistein, respectively). On the ground of the
obtained results, the concentration of 25 μ M 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 μ M ATRA induced CD11b expression (Fig.
2A); caused cell accumulation at G₁ (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 G₂/M blockade caused by 25 μ M 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-
G₁ 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 μ M and 25 μ M, respectively, which according to preliminary
4 determinations caused a moderate toxicity at 72 h of treatment, as in the case of 25 μ M
5 genistein. By contrast to genistein, these agents did not cause G₂/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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18 3.2. *Protein kinase modulation and effects of protein kinase inhibitors*

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23 Earlier studies indicated that ERK activation is involved in the regulation of myeloid cell
24 differentiation [26]. However, genistein was reported to inhibit the MEK/ERK pathway in
25 some cell systems [4,7-8]. For these reasons, we examined the capacity of the isoflavone
26 to modulate the phosphorylation/activation of ERK and upstream kinases (MEK, Raf-1) in
27 HL60 cells. It was observed that 25 μ M genistein caused a rapid and stable increase in the
28 phosphorylation state of these kinases, which was already detected at 6 h of treatment
29 (Fig. 3A). The increase in ERK phosphorylation was corroborated at 24 h in NB4 cells, and
30 still persisted at 48 h in both cell lines (results not shown). Treatment for 24 h with ATRA
31 alone slightly increased Raf-1, MEK and ERK phosphorylation, but the effect of genistein
32 plus ATRA was not higher than that caused by genistein alone (Fig. 3B). The cause-effect
33 relationship between ERK activation and differentiation induction was then examined using
34 the MEK/ERK inhibitors PD98059 (20 μ M) and U0126 (2 μ M), as well as specific siRNAs
35 directed against ERK1 and ERK2. Under the used conditions both the pharmacologic
36 inhibitors and siRNAs efficaciously attenuated ERK phosphorylation (see Fig. 4A (inset)
37 and C). It was observed that co-treatment with PD98059 and U0126 attenuated CD11b
38 surface expression (Fig. 4A, left bar chart) and NBT reduction (Fig. 4B, left bar chart) in
39 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 G₁ and reduced the accumulation at G₂/M in genistein-treated HL60 (Fig. 5A) and NB4 (result not shown) cell cultures. The analysis of proteins responsible for the regulation of G₂ 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 μ 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 G₂/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 μ M

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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 (hour 24) of G₂/M arrest and differentiation (see Fig. 1A,B). On the other hand, co-treatment with ATRA, which as indicated above prevented the late (hour 72) 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 μ M LY294002, which efficaciously decreased the basal Akt phosphorylation level (Fig. 6C), prevented the isoflavone-provoked stimulation of CD11b expression (Fig. 6A) and G₂/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 G₂/M arrest, the kinetic assays indicate 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 G₂ check-point by inhibiting ATM phosphorylation, although other studies indicate that caffeine also delays G₁ to S phase transition [28]. In agreement with this, treatment of HL60 cells with 5 mM caffeine caused cell accumulation at G₁, suppressed the G₂/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 G₂/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 cells [17,18] as well as ERK activation and ERK-regulated G₂/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 μ M genistein caused a moderate (approximately three- to four-fold) increase in intracellular ROS accumulation in HL60 cells, as measured by the increase in H₂DCFDA-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 G₂/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 μ 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 G₂/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 G₂/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 G₂/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 G₂/M arrest is regulated by ATM and Chk2 activation, Cdc2
20 inactivation, and increased p21^{waf1/cif1} 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 G₂ into mitosis. In
6 addition, we and others demonstrated that topoisomerase poisons and other DNA-
7 damaging agents which cause S or G₂/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 G₂/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 G₂/M cycle blockade [16]. As a complement, the present work
37 demonstrates that ROS accumulation does not mediate genistein-provoked ERK
38 activation or G₂/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 G₂/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- κ 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-G₁ 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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(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

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 μ M, and genistein at 25 μ 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 α -tubulin
13 (α -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 μ M, and genistein at 25 μ 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 μ M) and U0126 (U, 2.5 μ M) or the p38-MAPK inhibitor
41 SB203580 (SB, 10 μ 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 μ M and genistein at 25
3 μ M. The asterisks indicate significant differences in relation to the control. All other
4 μ 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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11 Fig. 5 - Effects of MEK/ERK and p38-MAPK inhibitor on genistein-provoked cell cycle
12 arrest and cell cycle-related protein alterations. (A) Changes in cell cycle distribution in
13 HL60 cell cultures treated for 48 h with 25 μ M genistein and 20 μ M PD98059 or 10 μ M
14 SB203580, alone and in combination. (B) Relative levels of total cyclin B1 and p21^{waf1/cif1}
15 and of phosphorylated (p-) ATM, Chk2 and Cdc2 (Tyr15) proteins in cells subjected for 24
16 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 μ M) and 25 μ 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 μ M genistein and 20 μ 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 μ 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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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 H₂DCFDA. 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 μ M, NAC at 10 mM, and ATO at 2 μ 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 G₂ 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.

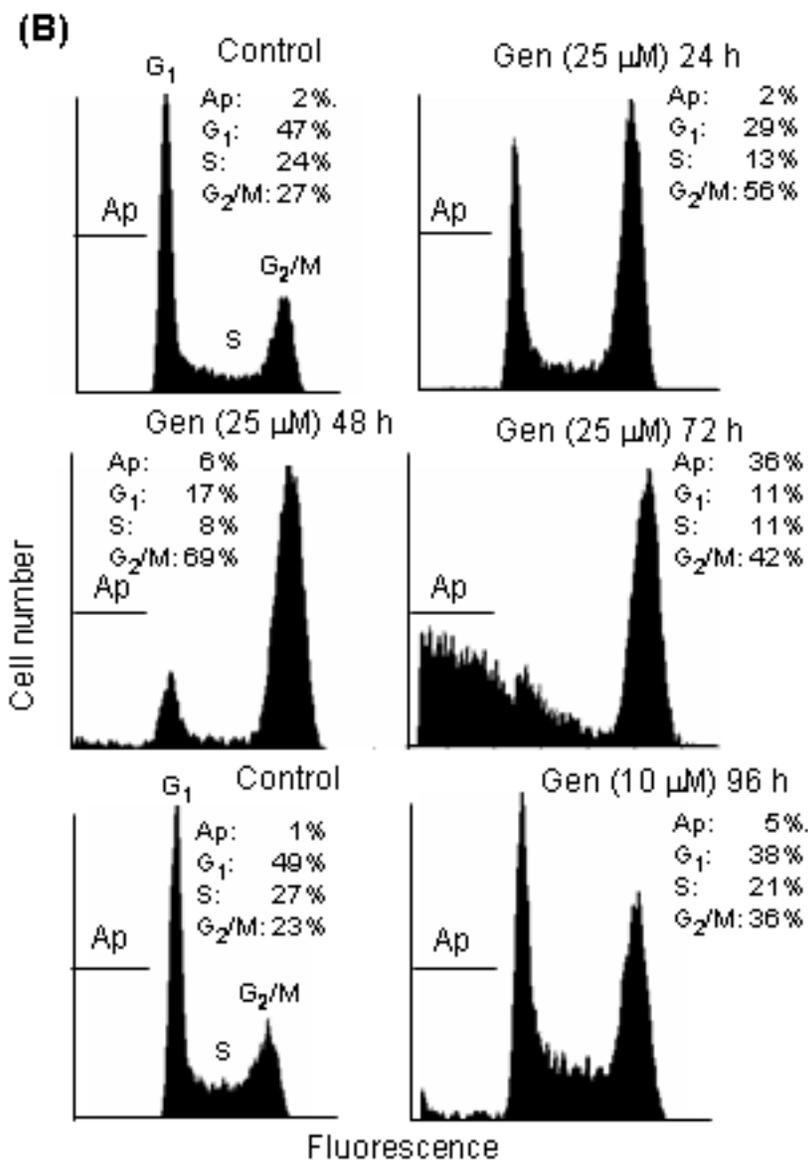
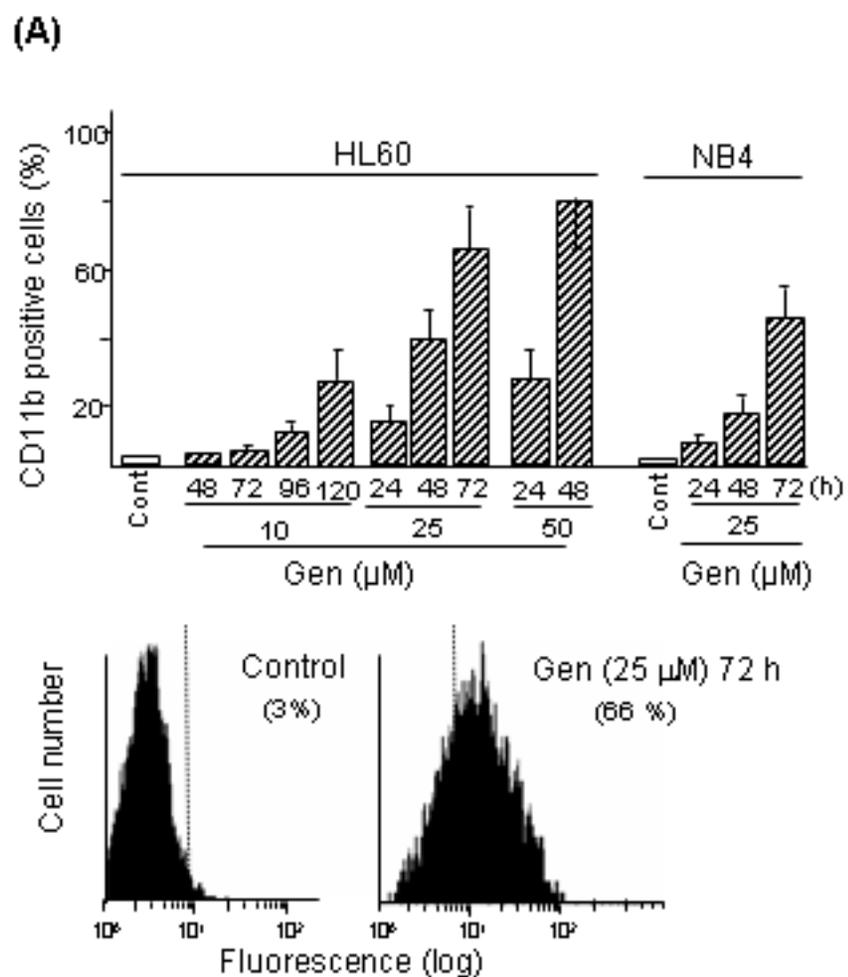


Fig 1

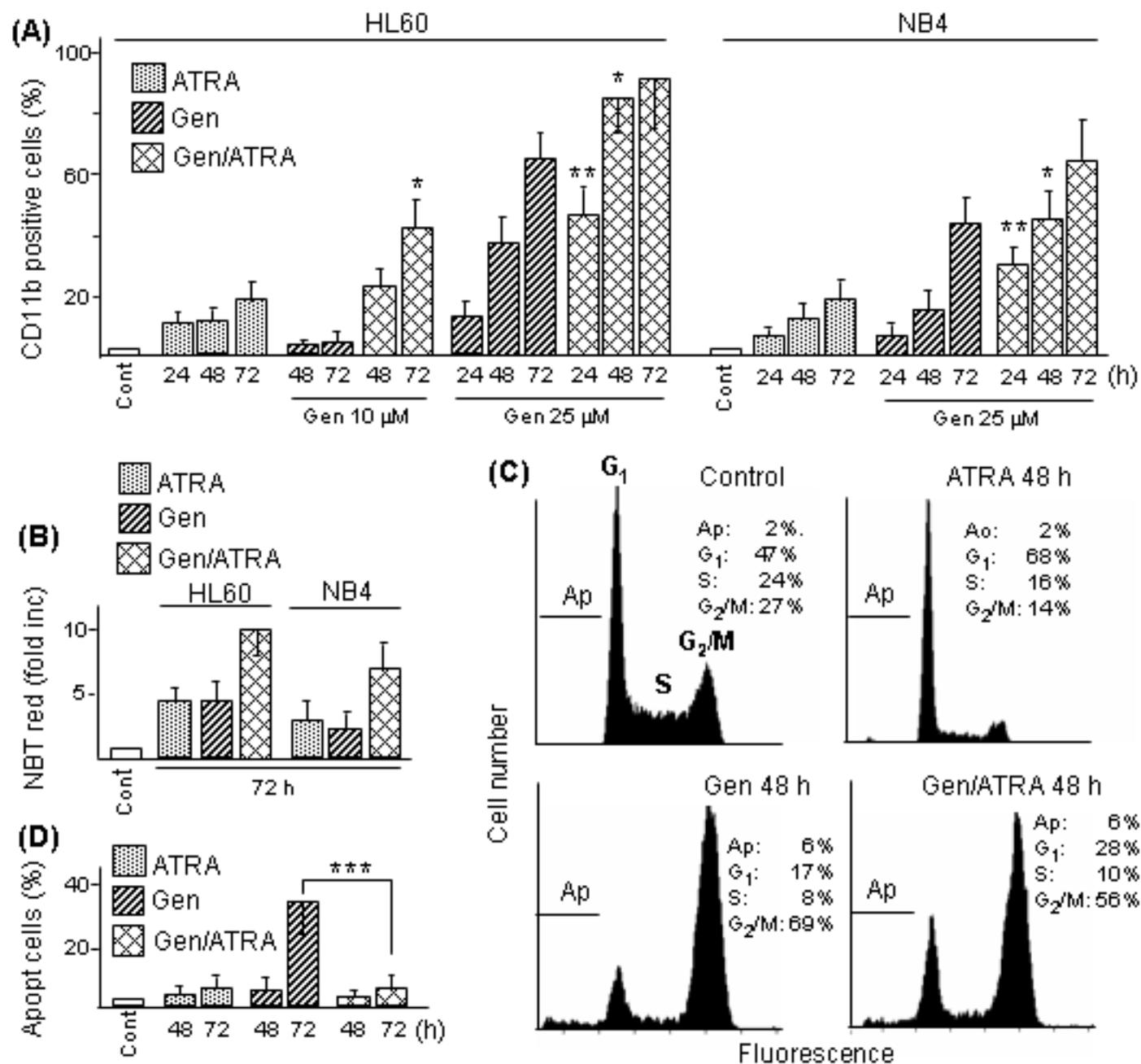


Fig 2

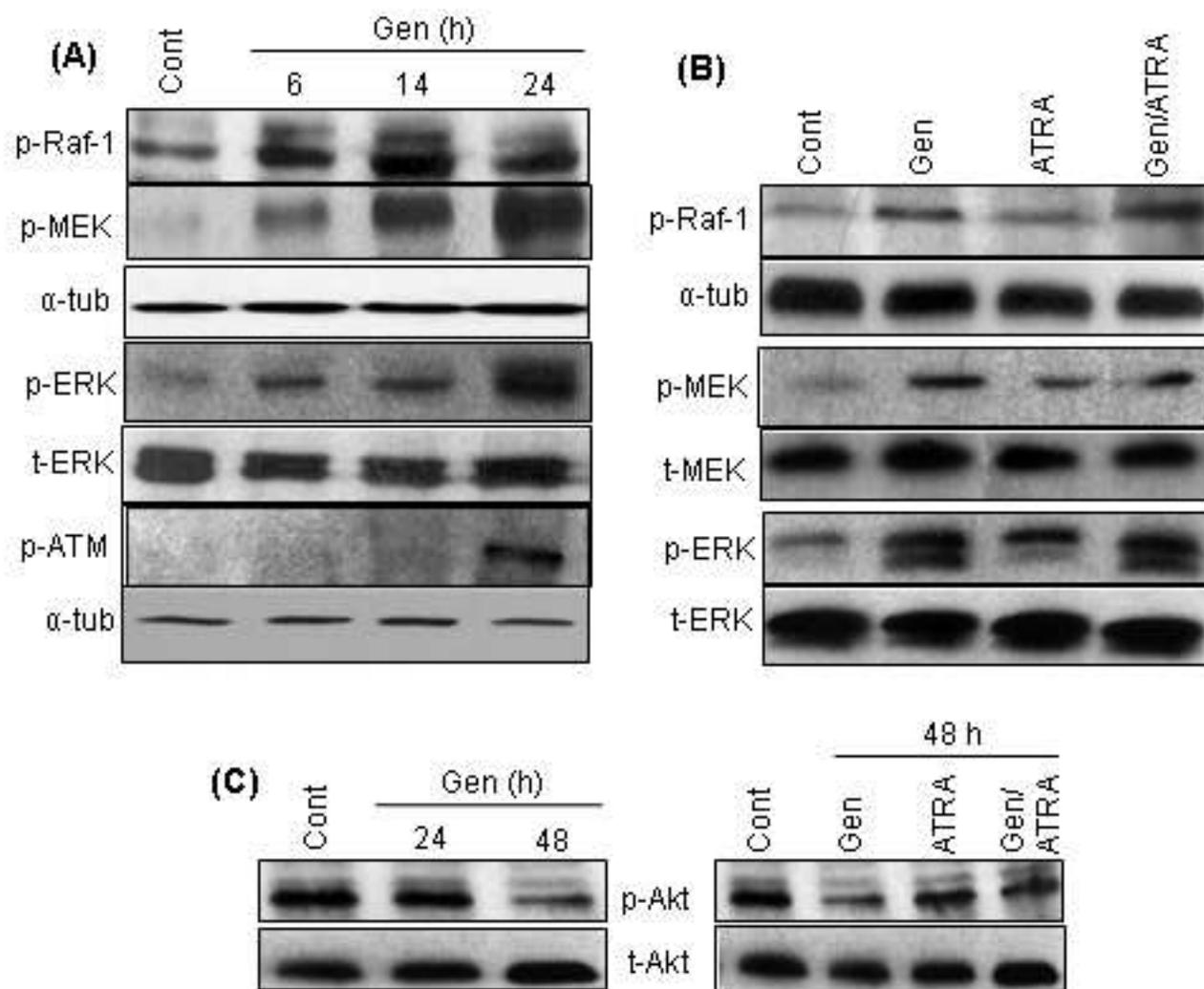


Fig 3

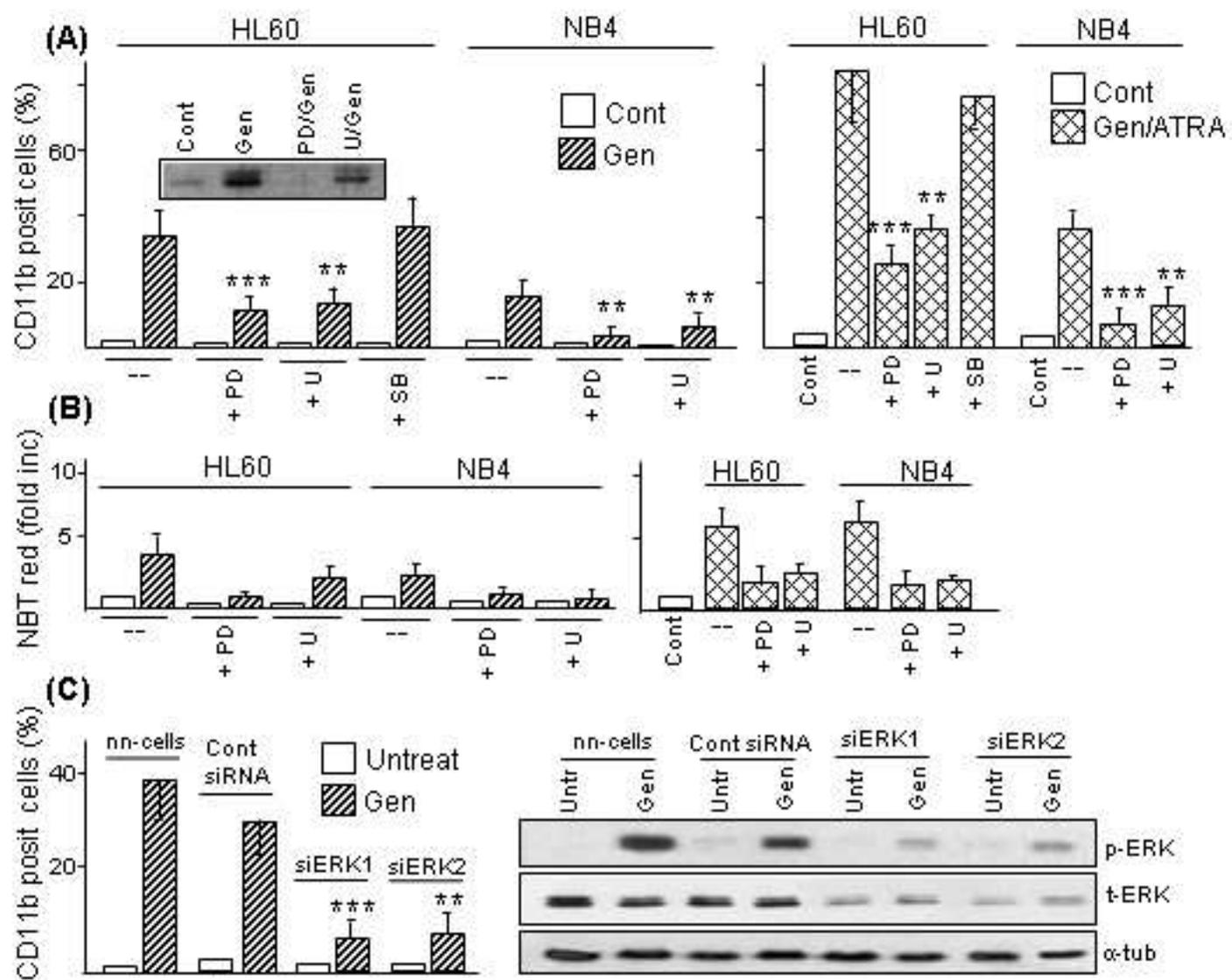


Fig 4

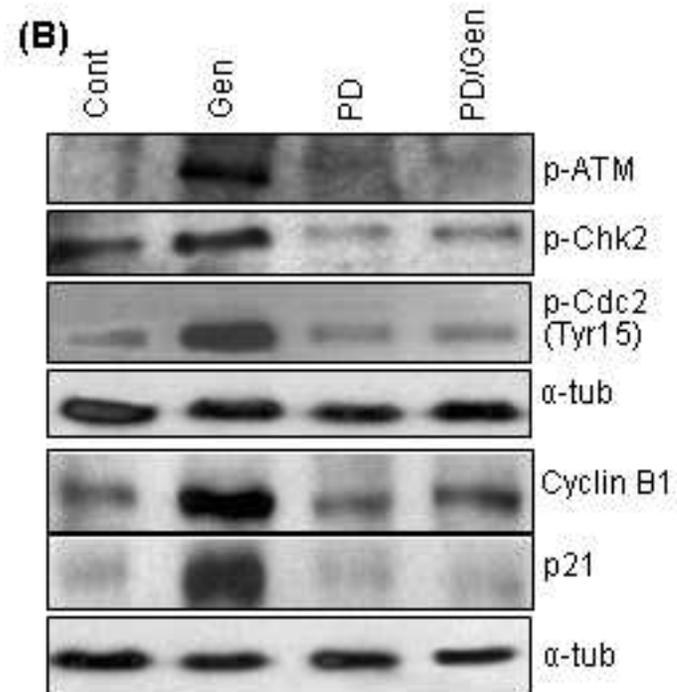
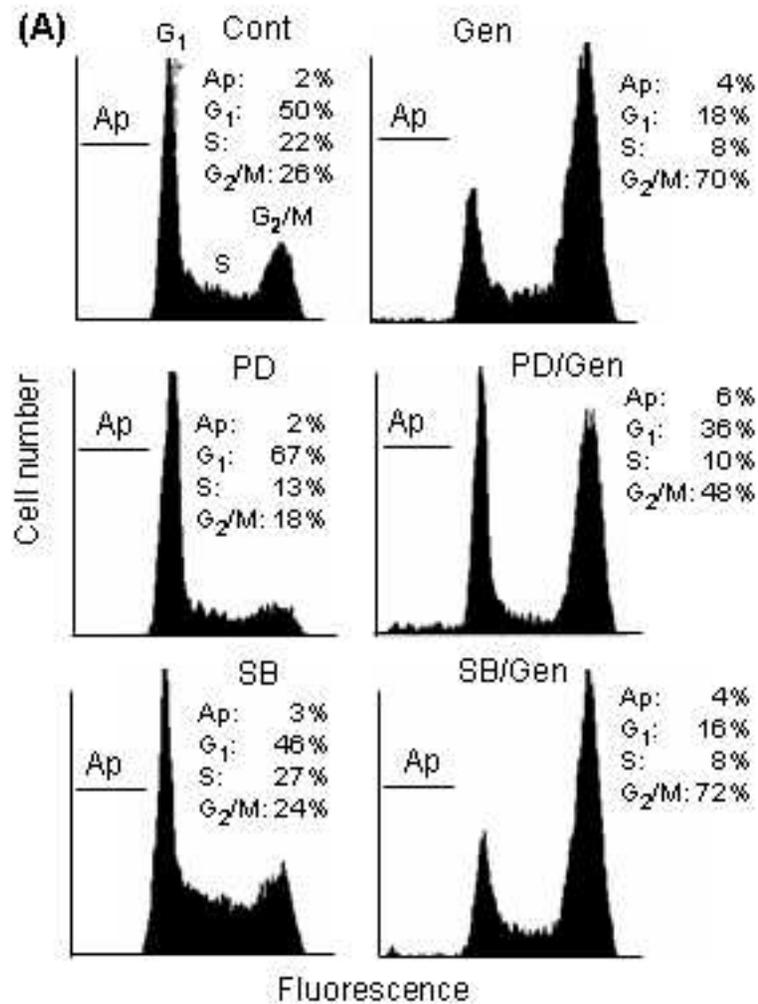


Fig 5

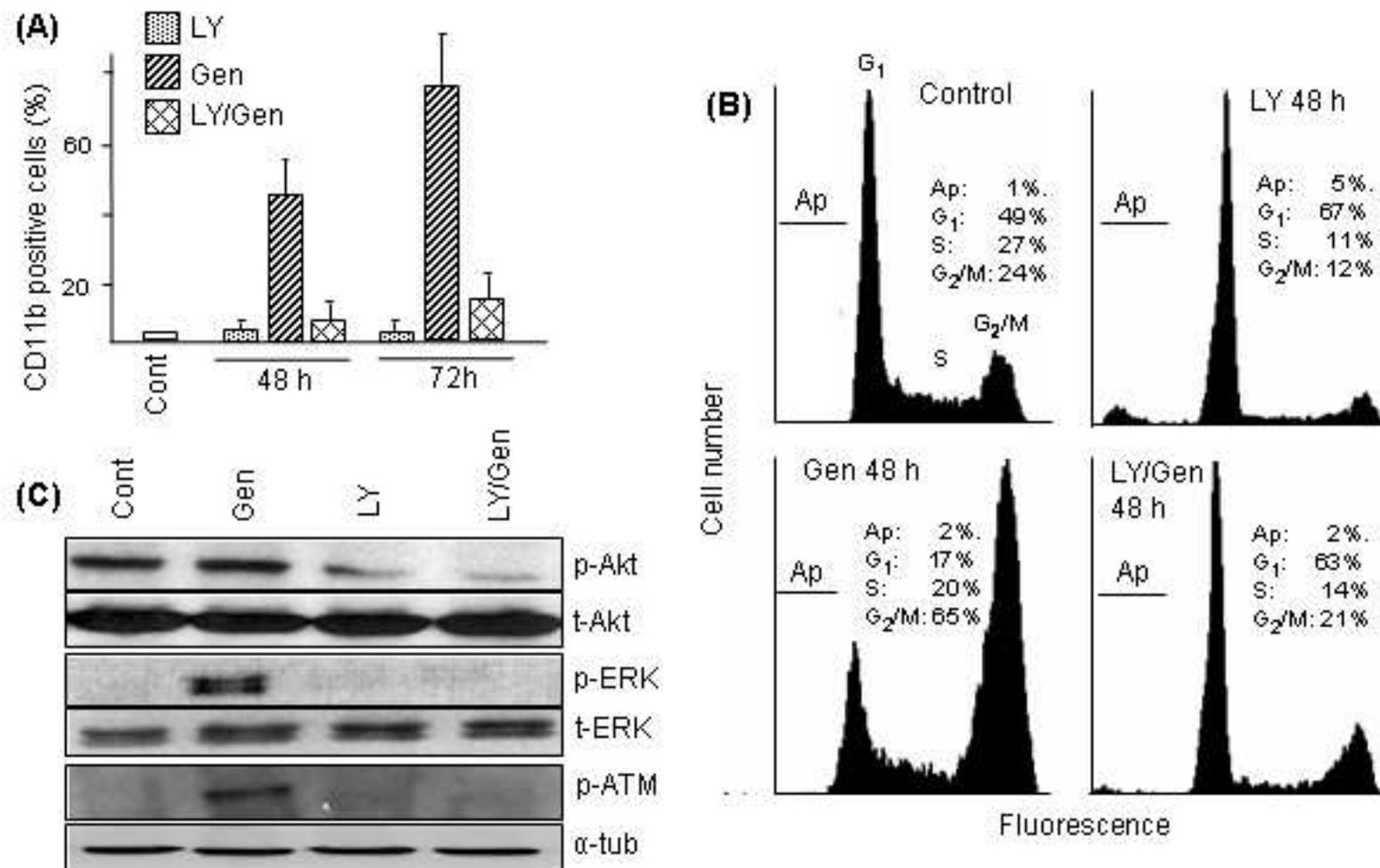


Fig 6

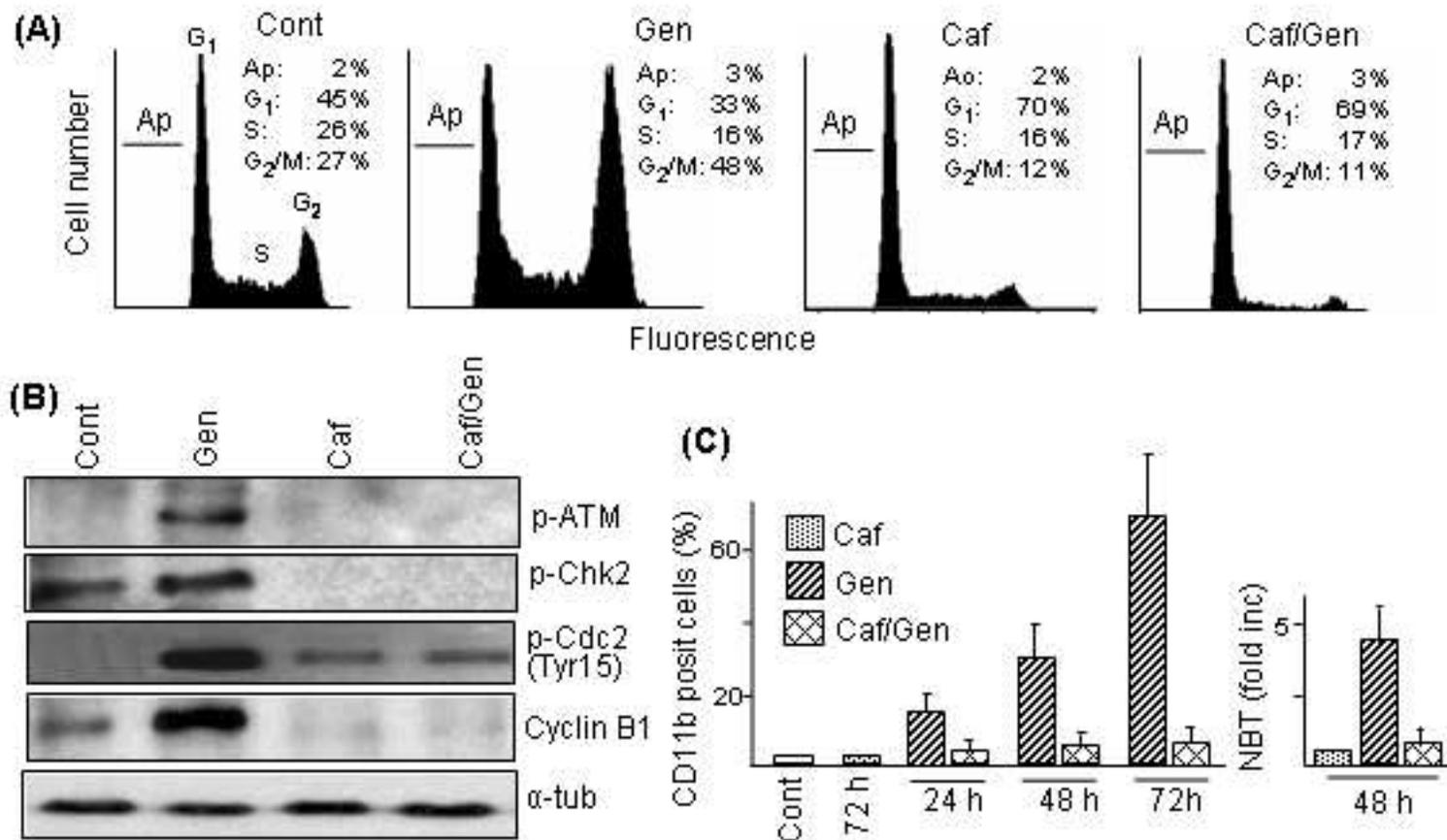


Fig 7

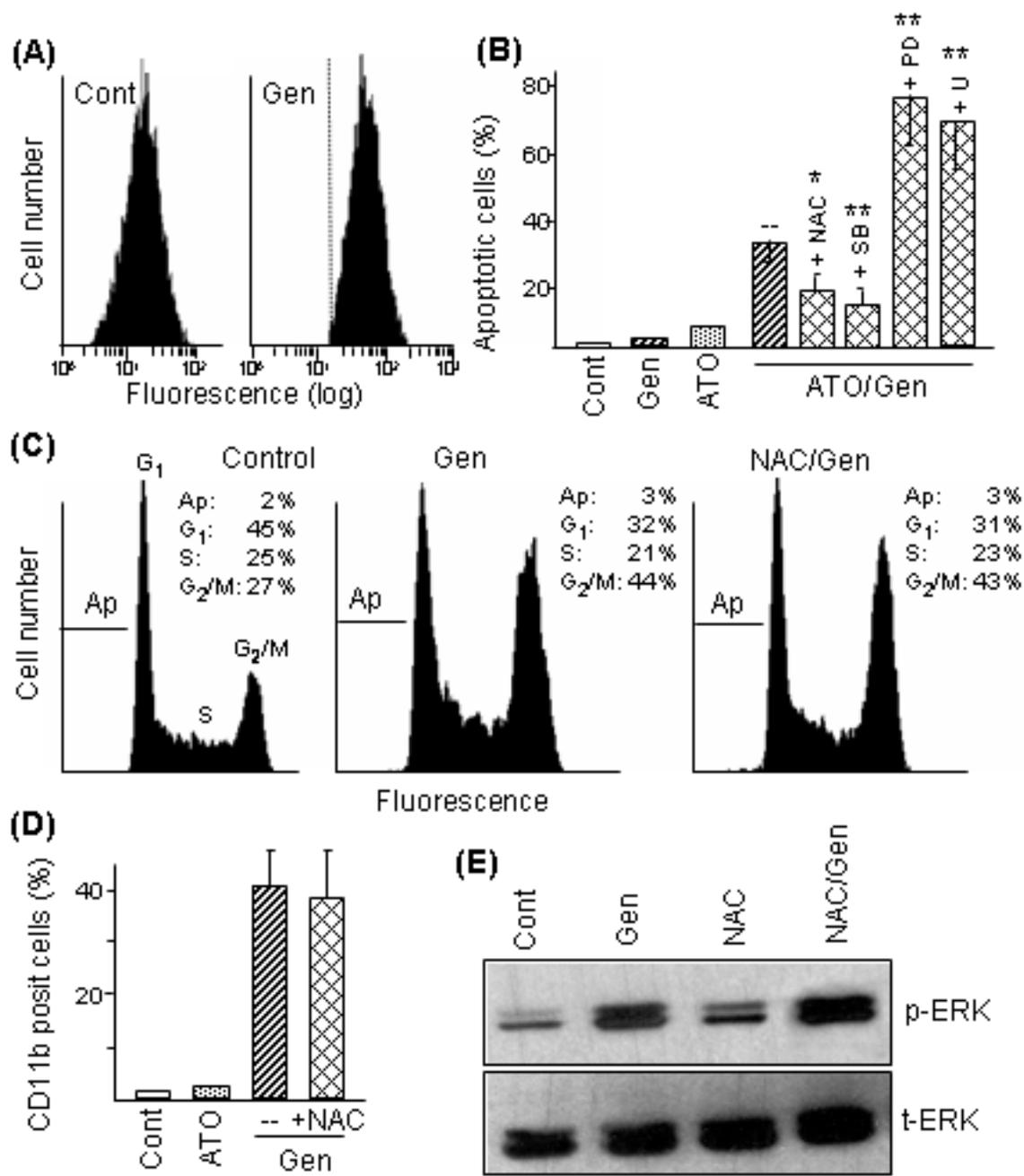


Fig 8

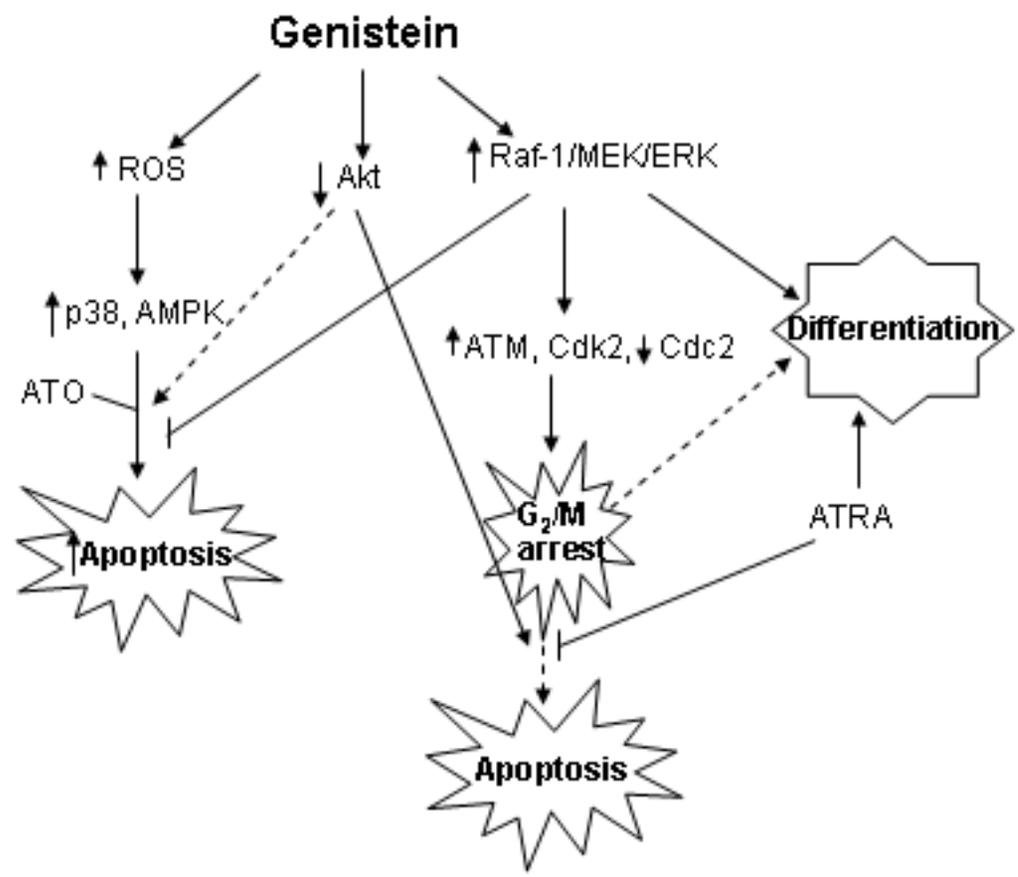


Fig 9