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Original Paper

Genistein Inactivates bcl-2, Delays the G2/M Phase of the Cell Cycle, and Induces Apoptosis of Human Breast Adenocarcinoma MCF-7 Cells

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The aim of this study was to identify the molecular mechanism of action of the isoflavone, genistein. Genistein at 0.15 mM caused MCF-7 apoptotic cell death, which was accompanied by cell cycle delay in the G2/M phase. Twenty-four hours post-treatment, 47.3% of the MCF-7 cells accumulated at G2/M, compared with 19.9% in the untreated controls. At 0.15 mM, genistein caused an increase in the steady-state levels of the wild-type tumour suppressor p53, which was attributed to stabilising the tumour suppressor protein, since p53 mRNA levels did not increase. Prior to the upregulation of p53, which became evident within 6 h of genistein treatment, there was increased bcl-2 phosphorylation at 30 min post-treatment. Although early changes (30–120 min) in the phosphotyrosine peptide patterns were not detected, after 24 h, genistein inhibited phosphorylation of several peptides. These results suggest that genistein's dual roles of protein tyrosine kinase inhibitor and topoisomerase II inhibitor are essential for the initiation of apoptosis. © 1998 Elsevier Science Ltd. All rights reserved.

Key words: apoptosis, genistein, p53, bcl-2, topoisomerase

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INTRODUCTION

A NATURAL COMPONENT of soy, the isoflavone genistein, has recently been found to be of chemotherapeutic value when combined with tumour-specific antibodies in the treatment of B-cell precursor leukaemia [1]. This same soy ingredient has been intensively investigated in recent years as a chemopreventive agent, mainly against hormonally regulated breast and prostate cancers in animal models [2]. Epidemiological studies also provide evidence for the possibility of preventing breast cancer through the consumption of soybeans and specifically soy isoflavones [3].

The isoflavone genistein was identified initially as a phyto-oestrogen [4] shown to compete with oestradiol in MCF-7 cells for the oestrogen receptor (ER) and to elucidate other typical oestrogen-induced responses [5]. Subsequently, genistein was identified as a specific protein tyrosine kinase inhibitor [6, 7] and a topoisomerase (topo) II inhibitor [8, 9].

Genistein, when introduced at relatively high concentrations in cultures of T-cell leukaemia and other tumour cell

lines, delayed cell cycle progression and induced apoptosis [10–13]. Using genistein as a tool, we examined key components of the biochemical cascade of apoptosis in MCF-7 cells.

MATERIALS AND METHODS

Cells and culture conditions

MCF-7 cells were cultured in RPMI-1640 medium supplemented with 15% fetal bovine serum, penicillin (100 units/ml), and glycine. MDA-MB-468 and MCF-7 cells were obtained from the American Type Culture Collection (Rockville, Maryland, U.S.A.). MDA-MB-468 cells were cultured in MEM medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and glycine. All cells were plated at 5×10^4 in 1 ml medium and cultured at 37°C in a humidified atmosphere of 8% CO₂ in air. Genistein (4', 5,7-trihydroxyisoflavone) was purchased from Indofine Chemical Company (Somerville, New Jersey, U.S.A.) and VP-16 was a gift from Bristol-Myers (Wallingford, Connecticut, U.S.A.). Cell cultures were treated 24 h after plating with the specified doses. Genistein arrests growth and induces differentiation of MCF-7 cells at concentrations ranging from

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10 to 45 μM [14]. After 4 days of treatment with 31 μM genistein, MCF-7 cell growth is inhibited by 50% when compared with untreated controls [15]. Cell viability at this concentration was nearly 100%. The present studies were performed at cytotoxic concentrations. Cell numbers were determined by counting in haemocytometer chambers, and viability was monitored by trypan blue exclusion.

Assessment of apoptosis

The main method used to evaluate apoptosis was that of Duke and Cohen, which is based on the differential staining of viable/apoptotic cells in a mixture of two dyes [16]. Briefly, MCF-7 cells were centrifuged and suspended in 50 μl of phosphate buffered saline (PBS) containing acridine orange and ethidium bromide at 100 $\mu\text{g/ml}$ each. Microscopic examination of 10 μl aliquots, using a fluorescein filter, identified the non-viable cells whose nuclei stain bright orange and are highly condensed or fragmented. These cells can be distinguished from viable cells with normal nuclei that exclude ethidium bromide and stain bright green. Necrotic cells stain uniformly with ethidium bromide. Using these methods, quantitative assessments were made by determining the percentage of apoptotic cells in five independent fields totalling at least 200 cells.

An *in situ* apoptosis method (ApopTag, purchased from Oncor, Gaithersburg, Maryland, U.S.A.) was used to detect directly digoxigenin-labelled genomic 3'-OH DNA ends generated during apoptosis. A third method of assessing apoptosis is based on gel electrophoresis and the characteristic pattern of DNA laddering generated in apoptosing cells. Briefly, after 48 h of treatment with dimethylsulphoxide (DMSO) or the test agent (dissolved in DMSO), 3×10^6 cells were suspended in PBS containing 0.25% Nonident P-40 and 0.1 mg/ml RNase A (Sigma Chemical Co., St Louis, Missouri, U.S.A.). Following 30 min incubation at 37°C, the cells were treated with proteinase K (Sigma) at a final concentration of 1 mg/ml and incubated for an additional 30 min. After the addition of a loading buffer, 25 μl samples were loaded on a 1.5% agarose gel and subjected to electrophoresis at 35 V for 4 h. DNA was stained with ethidium bromide for 20–30 min, and gels were photographed over UV light.

Cell cycle analysis

This was evaluated using flow cytometry exactly as reported previously [17].

Northern blot analysis

RNA isolation, fractionation, and hybridisation were essentially as described previously [18]. The probes used for the analysis included *p53*, a 1.3 kb full-length cDNA (Oncogene Research Products, Cambridge, Massachusetts, U.S.A.), and *GAPDH* (a generous gift from F. Collart), a 0.78 kb *Pst1/Xba1* digest of pHcGAP clone, ATCC#57090 (American Type Culture Collection). The probes were labelled with [^{32}P]dCTP using the random hexamer method. When re-probing was necessary, filters were stripped by washing in 0.2% sodium dodecyl sulphate (SDS), 10 mM Tris-HCl, pH 7.4, at 70°C for 1.5 h.

Western blot analysis

Cells were lysed in boiling sample loading buffer (10 mM Tris-HCl, pH 7.4, 1% SDS). The protein concentration was measured spectrophotometrically using the BioRad DC kit

(Biorad, Hercules, California, U.S.A.). Samples containing 50 μg of protein were electrophoresed over 10% SDS-polyacrylamide gels, transferred to a nitrocellulose membrane (Millipore, Bedford, Massachusetts, U.S.A.), and probed with the appropriate antibody. The monoclonal antibodies *p53* (Ab-1) and *Bcl-2* were obtained from Oncogene Research Products; *cdc-2* and *PY-20* were obtained from Transduction Laboratories. As the secondary antibody, horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma) was used. Antibody binding was detected with enhanced chemiluminescence (ECL; Amersham, Arlington Heights, Illinois, U.S.A.). Densitometric scanning of the bands was performed using a GS-700 imaging densitometer coupled to molecular analyst Vs 1.5 software—both from BioRad.

Bcl-2 phosphorylation and immunoprecipitation

MCF-7 cells were treated with genistein or daidzein at 25 μM or 150 μM for 30 min. The ^{32}P -labelling and immunoprecipitation procedures were identical to those described for topo II [19], except that the primary antibody was an anti-bcl-2-agarose monoclonal antibody conjugate (Santa Cruz Biotechnology, Santa Cruz, California, U.S.A.). Proteins were separated in an SDS-polyacrylamide gel, and after autoradiography the phosphorylation of bcl-2 bands was quantified densitometrically. The same gel was rehydrated, proteins were transferred to membranes, and bcl-2 protein levels of Western blots were detected using an anti-bcl-2 antibody and visualised with ECL (Amersham).

RESULTS

Induction of apoptosis

MCF-7 cells were treated with 150 μM genistein and 2 days later were evaluated for apoptosis. Apoptotic cells exhibited volume reduction, blebbing, chromatin condensation, fragmented nuclei, apoptotic bodies, and stained yellow/orange (Figure 1b). Apoptosis in MCF-7 cells was confirmed with two other assays: the DNA laddering and the *in situ* terminal deoxynucleotidyl transferase assay (data not shown). Genistein treatment for 48 h resulted in 57.5% of the MCF-7 cells undergoing apoptosis (Table 1). Daidzein, which differs from genistein by only a hydroxyl group and does not inhibit topo II [20], was considerably less effective in inducing apoptosis in these cells.

Table 1. Quantification of apoptosis induced by genistein or daidzein in tumour cells

Cell type	Agent	Apoptotic cells* (mean \pm S.D.)
MCF-7	Genistein	57.5 \pm 4.6
	Daidzein	18.6 \pm 3.2
MDA-MB-468	Genistein	76.2 \pm 6.3
	Daidzein	13.6 \pm 2.7
HL-60	Genistein	57.6 \pm 3.2
	Daidzein	9.9 \pm 1.8

*Data represent the percentage of apoptotic cells after 48 h of treatment as determined by the acridine orange/ethidium bromide assay (see Materials and Methods). The number of apoptotic cells among 200 nuclei was counted under fluorescent microscopy. Data represent the mean of at least three independent experiments. S.D., standard deviation.

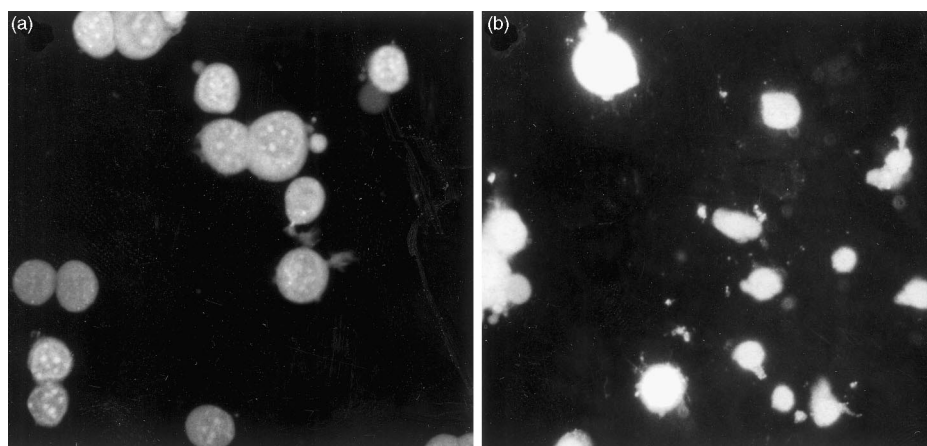


Figure 1. Evaluation of apoptosis of MCF-7 cells treated with genistein as determined by the acridine orange/ethidium bromide assay followed by fluorescent microscopy. (a) Control MCF-7 cells treated for 48 h with dimethylsulphoxide (DMSO; solvent); (b) MCF-7 cells treated for 48 h with 150 μ M genistein. Magnification $\times 400$.

Since genistein is known to have oestrogenic/anti-oestrogenic action, the human breast adenocarcinoma MDA-MB-468 cell line which is ER-negative was used. It has been previously reported that MDA-MB-468 cells also express a transcriptionally active mutant *p53* [21]. Western blot analysis showed that the mutant *p53* protein levels in MDA-MB-468 cells were nine times higher than the levels of wild-type *p53* produced by MCF-7 cells (Figure 2). The latter contain approximately seven times the levels of *bcl-2* found in MDA-MB-468 cells (Figure 2). *Bax* levels were undetectable with Western blots in both cell lines (data not shown), confirming a previous report [22]. Despite these molecular differences, MDA-MB-468 cells underwent apoptosis just like MCF-7 cells in response to genistein treatment (Table 1).

To identify the initiating and subsequent events of genistein-induced apoptosis, MCF-7 cells were treated with 150 μ M of genistein and the protein levels of *p53*, *bcl-2* and *cdc-2* kinase were measured, and the tyrosine phosphopeptide patterns and cell cycle progression compared.

Upregulation of p53

The tumour suppressor *p53* protein levels increased shortly after treatment of MCF-7 cells with a concentration of 150 μ M of genistein (Figure 3). This was evaluated with Western blot analysis of whole cell extracts, using the anti-*p53* antibody (Ab-1). The increase in *p53* protein levels became evident 6 h after treatment, at which time a 25% increase over the control levels was detected. The *p53* protein

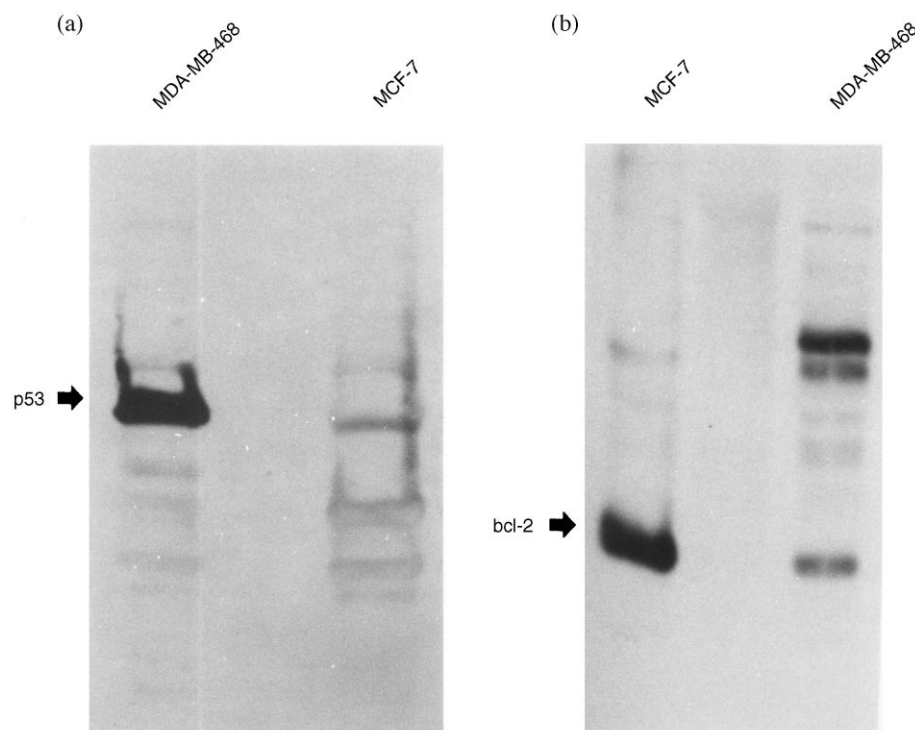


Figure 2. Endogenous protein levels of (a) *p53* and (b) *bcl-2* in MCF-7 and MDA-MB-468 cells. Untreated, logarithmically grown cells were lysed, and the protein levels for *p53* and *bcl-2* determined using Western blot methodology as described in Materials and Methods.

continued accumulating, reaching a 67% increase over the control levels 24 h post-treatment, and then declined. No significant changes in the p53 protein levels were evident when MCF-7 cells were treated for shorter periods. This increase in protein levels was not due to increased transcription, because the p53 mRNA levels did not increase during this period (Figure 3b). In fact, a decline occurred in the p53 transcript, indicating that stabilisation of the protein is more extensive than that measured by just monitoring the corresponding protein levels. Genistein at a dose of 30 μ M did not affect p53 protein levels (data not shown). Similarly, daidzein at up to 150 μ M had no effect on p53 protein levels (data not shown).

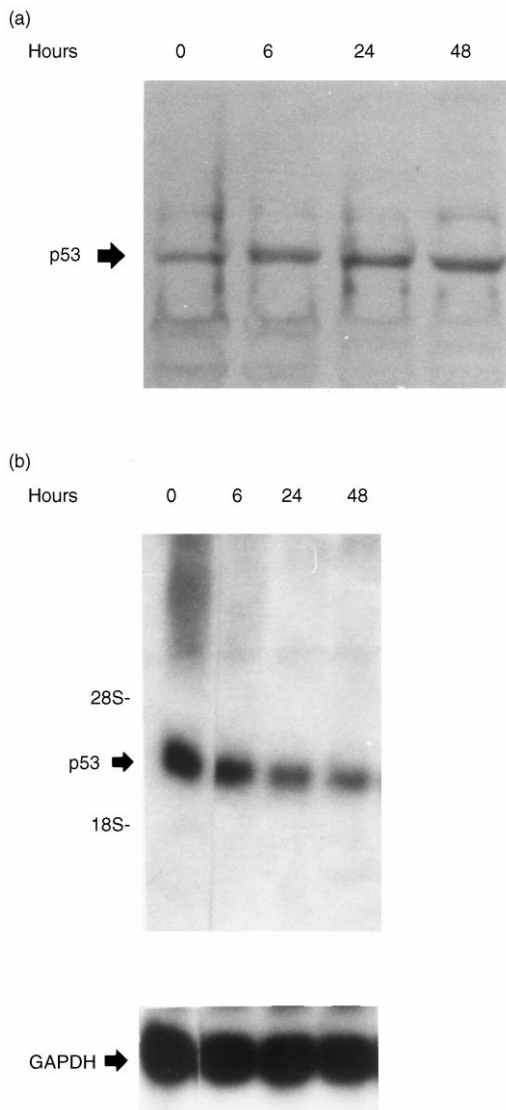


Figure 3. Effect of genistein on p53 protein and RNA levels in MCF-7 cells. (a) Immunoblot analysis of p53 levels in MCF-7 cells treated for 6–24 h with genistein, compared with control cells (0 h of treatment, which were cultured in the presence of dimethylsulphoxide (DMSO)). (b) RNA levels evaluated with Northern blot analysis as described in Materials and Methods using p53 cDNA as a probe. Membranes were stripped and rehybridised with a GAPDH probe to ensure even loading (lower panel).

Downregulation of bcl-2

Experiments were conducted to examine whether, at these high genistein concentrations, there were non-specific reductions in protein levels. The bcl-2 protein levels in MCF-7 remained relatively unchanged during the first 6 h of treatment, but after 24 h a small decrease (17%) became evident, and after 48 h a 46% decrease over the control levels was detectable. The decrease at 48 h was not due to a universal inhibition of protein synthesis, because the levels of cdc-2 kinase remained unchanged for the entire period (Figure 4).

Cell cycle analysis

The effects of genistein on cell cycle progression are shown in Figure 5. Prior to treatment, cells were approximately equally distributed among the three phases of the cell cycle (Figure 5b). A delay in G2/M became evident 12 h post-treatment, and it reached maximum after 24 h, when 47.3% of the cells were found in this phase of the cell cycle. In contrast, only 19.9% of the control cells were found in the G2/M phase. However, in the genistein treated samples, the percentage of cells traversing G2/M declined to 41% after 48 h. A gradual increase in G1 was evident in both the genistein and control cells (treated with the solvent DMSO). The build-up in G2/M and G1 phases was at the expense of the S phase, which declined from 34.7% in untreated cells to 9.7% in cells treated with genistein for 48 h. Cell cycle distribution

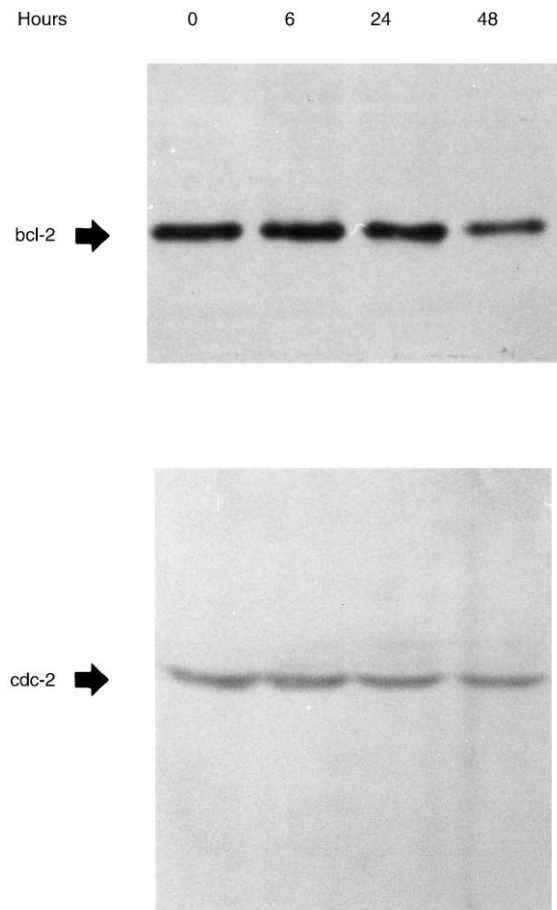


Figure 4. Immunoblot analysis of bcl-2 and cdc-2 levels in MCF-7 cells treated for 0–48 h with genistein at 150 μ M. Time 0 is taken from extracts of cells that have been treated with dimethylsulphoxide (DMSO), which was used as the solvent.

in daidzein treated cells during 48 h of monitoring did not differ from that of the control cells (data not shown).

Protein tyrosine kinase-mediated phosphorylation

Since genistein is known to inhibit protein tyrosine kinases, the patterns of tyrosine phosphopeptides at various times were examined after treatment using a specific antibody (PY-20) in Western blots. No changes were detectable after 30 and 120 min of treatment (Figure 6). After 24 h, however, the phosphorylation of several peptides (shown by arrows), was dramatically diminished.

Bcl-2 phosphorylation

The phosphorylation of bcl-2 has been shown previously to inhibit its ability to interfere with apoptosis. The extent of bcl-2 phosphorylation in intact cells was evaluated, and the

effect of low and high concentrations of genistein and daidzein determined. Increased bcl-2 phosphorylation was evident 30 min after treatment of MCF-7 cells with genistein, while daidzein at the same concentration was ineffective (Figure 7 and Table 2). Lower concentrations of either isoflavone were also ineffective in altering bcl-2 phosphorylation.

Table 2. Effect of genistein or daidzein treatments on the phosphorylation of bcl-2 in MCF-7 cells*

Treatment	ECL†	Autoradiography‡	Ratio§
Control	4422	4872	1.10
Genistein (25 µM)	8872	8717	0.98
Genistein (150 µM)	7740	15 029	1.94
Daidzein (25 µM)	13 810	13 992	1.01
Daidzein (150 µM)	18 435	14 433	0.78

*Treatments were for 30 min after ^{32}P labelling, as described in Materials and Methods and the legend of Figure 7. †Enhanced chemiluminescence of immunoblot shown in Figure 7(b). It represents the immunoprecipitated bcl-2 protein and is used to normalise the phosphorylation data. ‡Determined by scanning densitometrically the ^{32}P -labelled bcl-2 immunoprecipitated bands of the autoradiogram shown in Figure 7(a). §Ratio of phosphorylation to protein levels.

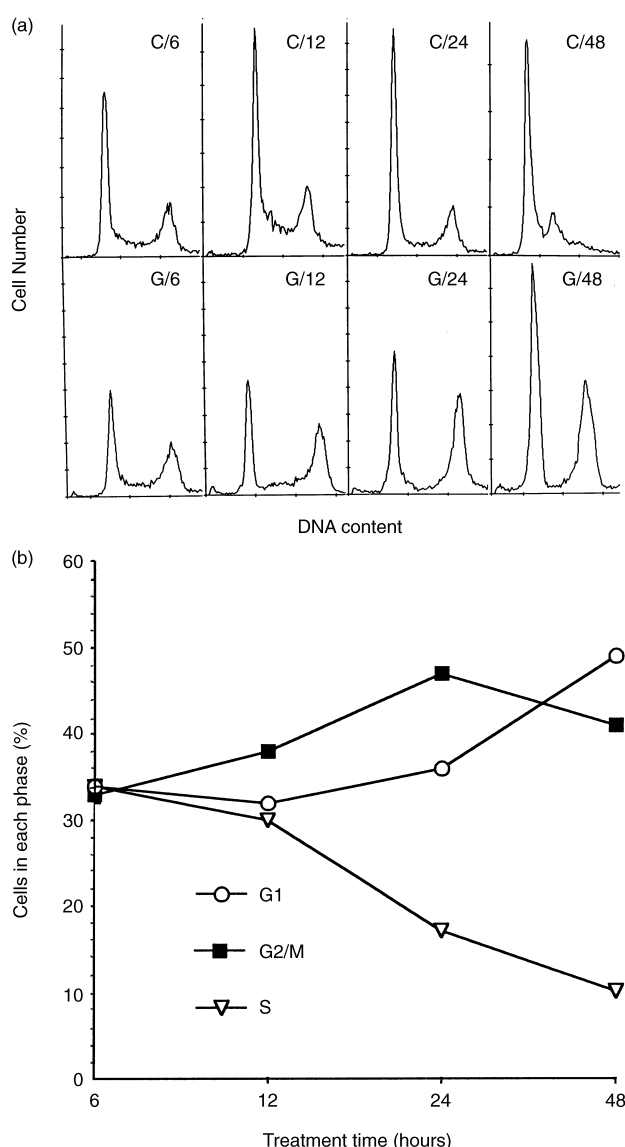


Figure 5. Cell cycle distribution of MCF-7 cells treated for the time shown (h) with 150 µM of genistein (G) or dimethylsulphoxide (DMSO; control cells) (C). (a) The x axis shows propidium iodide fluorescence intensity used to assess the DNA content of the cells. (b) Percentage of cells in each phase of the cell cycle versus genistein treatment time was determined by processing the profiles shown in (a).

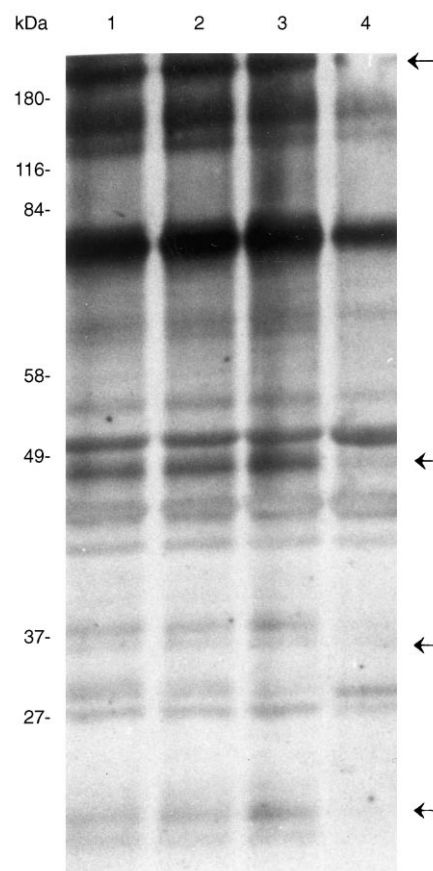


Figure 6. Effect of genistein on tyrosine phosphorylation after treatment of MCF-7 cells with genistein for 30 min (lane 2), 120 min (lane 3), and 24 h (lane 4). Control cells were treated with dimethylsulphoxide (DMSO) for 24 h (lane 1). Phosphotyrosine-containing peptides were detected with the PY-20 antibody followed by ECL. Arrows point to the peptides whose phosphorylation levels were reduced the most. The molecular mass of protein standards is shown in the left column.

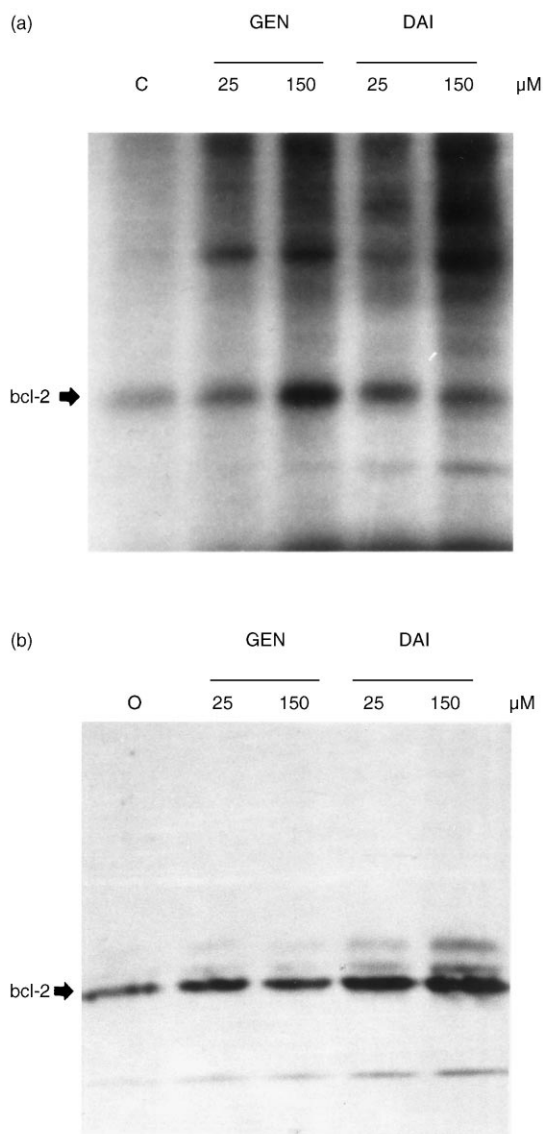


Figure 7. Effects of genistein and daidzein on immunoprecipitated bcl-2 phosphorylation (a) compared with immunoprecipitated protein levels. MCF-7 cells were treated with genistein (GEN) or daidzein (DAI) for 30 min at the concentrations shown. After ^{32}P -labelling (see Materials and Methods), bcl-2 was immunoprecipitated using anti-bcl-2-agarose monoclonal antibody conjugate and phosphorylation detected with autoradiography (a). Following rehydration of the gel, proteins were transferred to membranes, and bcl-2 protein levels were detected with enhanced chemiluminescence (ECL) (b).

DISCUSSION

Two distinctive types of biological response have been reported in genistein treated tumour cells. The first type are antiproliferative, cytostatic, and cell differentiating effects, which have been reported at relatively low genistein concentrations ranging from 10 to 45 μM . At these physiologically relevant concentrations, genistein and other soy isoflavones have been shown to cause alterations in reproductive hormones [23–25] and induce cellular [15, 26, 27] and mammary gland [3] differentiation. The molecular events that may be mediating this response were the subject of a recent review [28].

The second type of biological response are the cytotoxic effects, reported to take place generally in the range of 100–300 μM genistein. Based on its ability to induce apoptotic death of HL-60 cells, B-cell precursor leukaemia, and Jurkat T-leukaemia cells [10–13], genistein was evaluated as an apoptotic agent in human breast adenocarcinoma MCF-7 cells. Although the mechanism of its action has been studied extensively, the sequence of molecular events involved in the apoptotic response is still subject to speculation.

In previous studies, when genistein was introduced at equimolar concentrations to those that induced apoptosis, it inhibited protein tyrosine kinases and topoisomerases, and induced DNA breakage [6, 8, 26, 27]. Although we were unable to identify early genistein-induced alterations in the patterns of tyrosine phosphopeptides in the present study, we detected substantial changes in bcl-2 phosphorylation following their treatment with genistein. It has been proposed that bcl-2 protects cells from programmed cell death by forming heterodimers with bax [29] and reducing the number of bax homodimers. Halder and colleagues have shown that bcl-2 readily undergoes phosphorylation on serine residues [30]. Phosphorylated bcl-2 is incapable of forming heterodimers with bax, and it loses its anti-apoptosis potential [31]. Cells can be driven towards apoptosis when the bcl-2/bax heterodimer complexes are reduced by 50% [32].

It is well accepted that DNA damage leads to upregulation of p53, which, in response to DNA damage, arrests the cell cycle in G1 in a p21-dependent mechanism. By doing so, the time available for DNA repair before entering the critical S and M phases of the cell cycle is extended. The accumulation of p53 in response to DNA damage is due to a post-translational modification that stabilises the protein [33]. Because of its role in the G1 cell cycle checkpoint, p53 has been named the 'guardian of the genome' [34]. If the DNA damage is determined to be severe and beyond repair, the apoptotic pathway is launched in a mechanism that does not depend on p21 or G1 arrest. p53 can induce apoptosis by directly activating death genes such as *bax* or downregulating survival genes such as *bcl-2* [35]. Our data support the latter in genistein treated MCF-7 cells, because bcl-2 expression was reduced 24–48 h post-treatment and bax expression was not increased during this time. The downregulation of bcl-2 protein that became evident 48 h post-treatment (Figure 4) may be due to p53-induced transcriptional controls. Furthermore, our results strongly suggest that an integral and independent early component of the apoptotic signalling is bcl-2 inactivation through phosphorylation. The observed G2/M delay provides additional evidence that genistein's enzymatic target in MCF-7 cells is topo II. Chromosomal segregation requires active topo II whose activity peaks during mitosis; because of that, topo II targeting agents are known to arrest the cell cycle in G2/M [36, 37]. MDA-MB-468 cells, which express a mutant form of p53 [21], were also delayed in G2/M in response to genistein treatment, and these cells also underwent apoptotic death in response to genistein treatment (data not shown). Delay of the cell cycle in G2/M and apoptosis in response to treatment with quercetin, another flavonoid, have also been reported in tumour cells lacking p53 [38] and in normal cells containing the wild-type p53 [39]. Quercetin has been previously reported to inhibit topo II [20]. These data strongly suggest that the G2/M delay is independent of p53 accumulation.

The early increase in the phosphorylation of bcl-2 in response to genistein treatment (Figure 7) is probably due to increased serine phosphorylation, because this is the only type of phosphorylation known to occur on this oncogene product [30]. This observation supports the possibility that genistein (through its action as a protein tyrosine kinase inhibitor) may inactivate a serine phosphatase that normally dephosphorylates bcl-2. Additional support comes from the finding that okadaic acid, a phosphatase inhibitor, induces phosphorylation (and apoptosis) of bcl-2 in lymphoid cells [30]. Bcl-2, when phosphorylated on serine, loses its antiapoptotic function because it can no longer bind to bax and it cannot prevent lipid peroxidation [30].

In conclusion, our results suggest that DNA damage and altered bcl-2 phosphorylation are two early molecular components of the apoptotic pathway initiated by genistein due to its dual action as a protein tyrosine kinase and topo II inhibitor. G2/M delay may also be required for launching apoptotic cell death, but the accumulation of p53 can be functionally dissociated. It will be of interest to determine if the proposed scheme applies to other inhibitors having dual action both against protein tyrosine kinases and topoisomerases, such as tyrophostins. These findings may lead to a more rational and effective approach in the treatment of breast cancer. The combination of DNA targeting agents with protein tyrosine kinase inhibitors may prove especially beneficial in the treatment of breast cancer.

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