



Genistein induces apoptosis and topoisomerase II-mediated DNA breakage in colon cancer cells

G.I. Salti, S. Grewal, R.R. Mehta, T.K. Das Gupta, A.W. Boddie Jr, A.I. Constantinou *

University of Illinois at Chicago, College of Medicine, Department of Surgical Oncology (M/C 820), 840 South Wood Street, Chicago, IL 60612, USA

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Abstract

The present study was undertaken to determine if (a) genistein induces topo II-mediated DNA damage in HT-29 colon cancer cells; and (b) if this damage is required to induce apoptosis. DNA damage was evaluated using the comet assay. Apoptosis was determined by the ethidium bromide/acridine orange staining technique. DNA breakage was noted within 1 h of treatment. Apoptosis was only induced with high concentrations ($\geq 60 \mu\text{M}$) of genistein. Marked inhibition of HT-29 cell growth was evident at concentrations ranging from 60 to 150 μM . This was associated with a cell cycle arrest at G₂/M. Similar findings were obtained in SW-620 and SW-1116 colon cancer cell lines. Aclarubicin, a topo II antagonist, reduced genistein-induced DNA breaks but did not reduce apoptosis. These data suggest that, in colon cancer cells, topo II serves as the enzymatic target of genistein. Furthermore, topo II-mediated DNA cleavage is not required for the induction of apoptosis. © 2000 Published by Elsevier Science Ltd. All rights reserved.

Keywords: Genistein; Colon cancer; Comet assay; DNA breakage; Apoptosis

1. Introduction

The isoflavone genistein, a major component of soy, was reported to be of chemotherapeutic value when conjugated to tumour-specific antibodies. Genistein conjugated to CD19 and EGF was reported to constitute an effective biotherapy in the treatment of lymphoma, leukaemia and breast carcinoma [1,2]. Genistein is known to induce apoptosis in a variety of tumour cell lines. Several possible mechanisms for the anticancer effects of genistein have been proposed [3–8]. These include topo II inhibition, induction of differentiation, inhibition of protein tyrosine kinase activity and inhibition of angiogenesis. Its role as a chemopreventive agent, although intensely investigated, remains controversial. Genistein treatment during the prepubertal period could suppress the development of chemically induced mammary cancer [9]. In human breast cancer cell lines, genistein acts as a growth stimulator at low concentrations and as a growth inhibitor at high concentrations [10]. Genistein's oestrogenic effect was shown to cause proliferation of cultured human breast

cancer cells (MCF-7) and the induction of oestrogen-specific (pS2) gene expression in ovariectomised athymic mice [11]. Dietary genistein significantly increased azoxymethane-induced non-invasive and total adenocarcinoma multiplicity in male F344 rats [12]. The present study was undertaken to evaluate the effect of genistein on colon cancer cells *in vitro*. We sought to determine if genistein induces topo II-mediated DNA damage in these cells, and if this damage is required to induce apoptosis.

2. Materials and methods

2.1. Chemicals

Genistein was obtained as a generous gift from the National Cancer Institute (NCI) (R.K. Varma). VP-16 and aclarubicin were obtained from Sigma Chemicals (St Louis, MO, USA).

2.2. Cell lines and cell culture conditions

HT-29, SW-620 and SW-1116 colon cancer cell lines and the mitoxantrone-resistant human leukaemia HL-60/MX2 cell line were obtained from ATCC (Manassas, VA, USA). The merbarone-resistant leukaemia cell line CEM/

* Corresponding author. Tel.: +1-312-413-1155; fax: +1-312-996-9365.

E-mail address: andreasc@uic.edu (A.I. Constantinou).

M70-B1 was kindly offered by W.T. Beck (University of Illinois at Chicago, USA). The breast cancer cell line ZR75-1 was obtained from R.G. Mehta (University of Illinois at Chicago, USA). Cells were maintained in RPMI-1640 medium (MEM-E was used for ZR75-1 cells) supplemented with 15% fetal bovine serum (FBS), 2 mM L-glutamine and 1% antibiotic–antimycotic solution (10 units/ μ l penicillin, 10 μ g/ μ l streptomycin and 25 μ g/ml amphotericin B). They were grown at 37°C in a humidified atmosphere of 5% CO₂.

2.3. Comet assay

DNA strand breaks were evaluated using single-cell gel electrophoresis (comet) assay. We modified the technique described by Singh and colleagues [13] and Duthie and associates [14]. Briefly, cells ($2\text{--}4 \times 10^5$) were suspended in 1 ml phosphate buffered saline (PBS) following treatment are transferred onto fully frosted slides precoated with 1% high-melt agarose. Thirty microlitres of this cell suspension were added to 80 μ l of 1% low-melt agarose. A coverslip was placed on top, and the slides were kept in the dark at 4°C for 5 min. The coverslips were then removed, and the slides were immersed in a lysis solution (100 mM Na₂EDTA, 2.5 M NaCl, 10 mM Tris, pH 10.0 and 1% TritonX100) for 1 h, then into the electrophoresis buffer (300 mM NaOH, 1 mM Na₂EDTA) for 40 min at 4°C. Electrophoresis was then performed in fresh solution for 30 min (0.5 V/cm). Slides were immersed into a neutralising solution (0.4 M Tris, pH 7.5). After staining with ethidium bromide solution (1 μ g/ml) for 20 min and destaining with 10 mM MgSO₄ for 20 min to decrease background staining, the slides were examined using a fluorescent microscope. A weak electric field draws negatively charged DNA towards the anode; the amount of DNA able to migrate depends on the size of DNA molecule and the number of broken ends. This allows for the evaluation of individual cells as ‘comets’. A semi-quantitative visual scoring system was devised to quantify the degree of DNA damage. Comets are graded 0, 1, 2, 3 and 4, ranging from the absence of detected comet tails (0) to the presence of a large tail with no comet head [4]. One hundred cells are evaluated per treatment. The DNA damage score (arbitrary units, au) is based on the equation ($\Sigma n_1 + 2n_2 + 3n_3 + 4n_4$), where n_1 is the number of comets with small tails, n_2 is the number of comets with equal-sized heads and tails, n_3 is the number of comets with predominant tails and n_4 is the number of comets with no heads. Therefore, our scoring system ranges from 0 to 400 [15].

2.4. Assessment of apoptosis

A technique reported by Duke and Cohen [16] was followed. It is based on the differential staining of

viable/apoptotic cells in a mixture of acridine orange/ethidium bromide. Cultured cells are centrifuged and suspended in PBS and the mixture of dyes is added. Fluorescent microscopy is used to identify non-viable cells whose nuclei stain bright orange. Viable cells exclude ethidium bromide and stain bright green. Quantitative assessments can be made by determining the percentage of apoptotic cells, whose nuclei are highly condensed or fragmented.

2.5. Immunocytochemistry

Anti-Ki-67 antibody (MIB-1; Immunotech, Westbrook, ME, USA) was diluted 1:50 in PBS. Dako LSAB2 System/Peroxidase Kit (Dako Corporation, Carpinteria, CA, USA) was used for immunostaining. Cells were washed in PBS, then fixed, in subsequent order, with 10% buffered formalin (4 min), cold methanol (4 min) and cold acetone (2 min). They were then incubated with the antibody for 2 h. Antibody link (10 min), streptavidin (10 min) and AEC (Biogenex, 5 min) were added. Cells were stained with haematoxylin and counterstained with Scott’s tap water substitute. After supermounting and permounting, 200 cells were evaluated, and the percentage of cells with positive staining was calculated and recorded.

2.6. Effect of pre-incubation with aclarubicin on genistein- and VP-16-induced DNA breakage and apoptosis

Cells were pre-incubated with aclarubicin (0.4 μ M) then treated with either genistein (100 μ M) or VP-16 (20 μ M) for 1 h. In one set of experiments, the comet assay was performed as described above. Similar experiments were performed in HL-60/MX2 and CEM/M70-B1 cells. In another set of experiments, the medium was removed. Cells were washed twice with PBS and fresh media was added free of drugs. HT-29 cells were evaluated for apoptosis 4 days later. In these experiments, we used a concentration of 50 nM aclarubicin because this concentration did not induce apoptosis in HT-29 cells.

2.7. Effect of pre-incubation with aclarubicin on the uptake of [¹⁴C] genistein in HT-29 cells

Cells were subcultured into wells (10⁵ cells/ml) and allowed to assimilate for 1 h. Cells were pre-incubated with aclarubicin (0.4 μ M) for 20 min, then treated with [¹⁴C]genistein (100 μ M) for 1 h. The medium was then removed, and cells were washed twice with ice-cold PBS. Cells were spun down, washed twice with PBS, and solubilised with 0.2 M NaOH. They were then analysed for [¹⁴C]genistein uptake using a Beckman liquid scintillation spectrometer.

Cell count was performed using a Coulter counter (Coulter Corporation, Miami, FL, USA). Cell cycle analysis was performed using flow cytometry and propidium iodide staining as described by Vindeløv and colleagues [17].

3. Results

3.1. DNA breakage

DNA strand breakage was assessed at 1 and 48 h following treatment with genistein (2–200 μM). Fluorescent comets with varying sizes of 'tails' were evident after 1 h when HT-29 cells were treated with $\geq 10 \mu\text{M}$ genistein. Fig. 1 shows an example DNA strand breakage with 100 μM genistein. Treatments with 2 and 5 μM did not show a significant difference from untreated controls, which, for the most part, appeared spherical. After 48 h of treatment in the presence of 10–30 μM genistein, there was less DNA breakage than after 1 h treatment with the same concentrations, indicating that

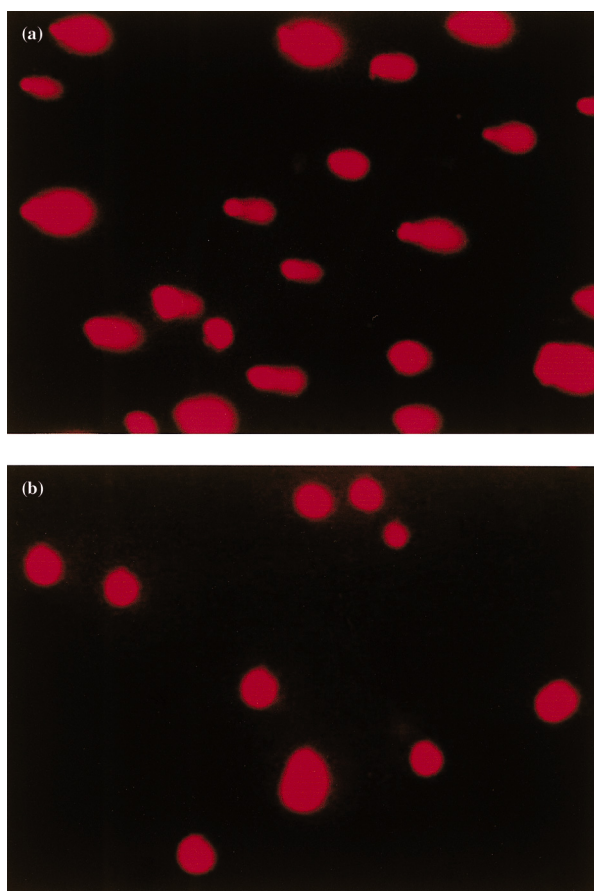


Fig. 1. DNA strand breakage using the comet assay and evaluated by fluorescent microscopy (100 \times). (a) HT-29 cells treated with 100 μM genistein for 1 h. The greater the number of DNA breaks, the larger the comets tails. (b) Untreated HT-29 cells.

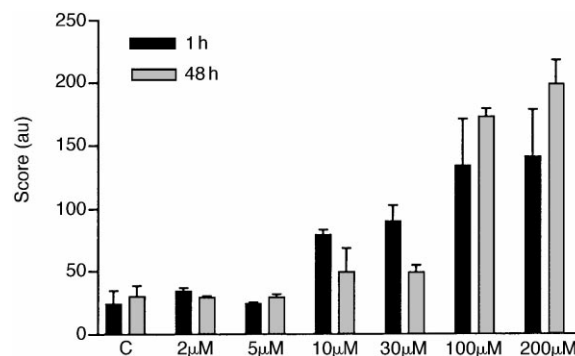


Fig. 2. DNA breakage induced by genistein in HT-29 cells at 1 and 48 h. Dose-dependent ($\geq 10 \mu\text{M}$) DNA breaks were detected 1 h after treatment. Cells treated with 10 and 30 μM genistein showed reduced levels of DNA breakage at 48 h.

HT-29 cells can repair the initial DNA breaks induced by genistein at these concentrations. At higher drug concentrations, DNA breakage was more extensive after 48 h when compared with 1 h of treatment. These results are shown in Fig. 2. DNA breakage by high concentrations of genistein was also evident in SW-620 and SW-1116 colon cancer cell lines, HL-60 human leukaemia cell line and ZR75-1 human breast cancer cell line (not shown).

3.2. Apoptosis

The percentage of apoptotic cells detected by the acridine orange/ethidium bromide staining technique after treatment with genistein (60 and 150 μM) for 4 days was evaluated, and the results are shown in Figs. 3 and 4. Apoptosis was not demonstrated in cells treated with $\leq 30 \mu\text{M}$ genistein. Treatment of HT-29 with 60 μM genistein resulted in $54\% \pm 3.5$ apoptotic cells, compared with $94\% \pm 1.4$ apoptotic cells with 150 μM genistein treatment. In addition, genistein caused apoptosis in SW-620 and SW-1116 cell lines (data not shown).

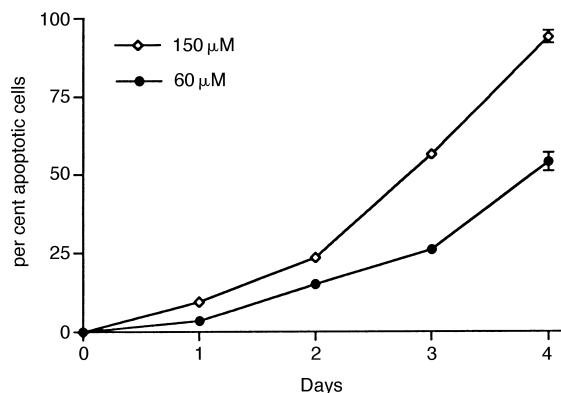


Fig. 3. Apoptosis in HT-29 cells induced by genistein (60 and 150 μM) and evaluated by the acridine orange/ethidium bromide staining technique over 4 days.

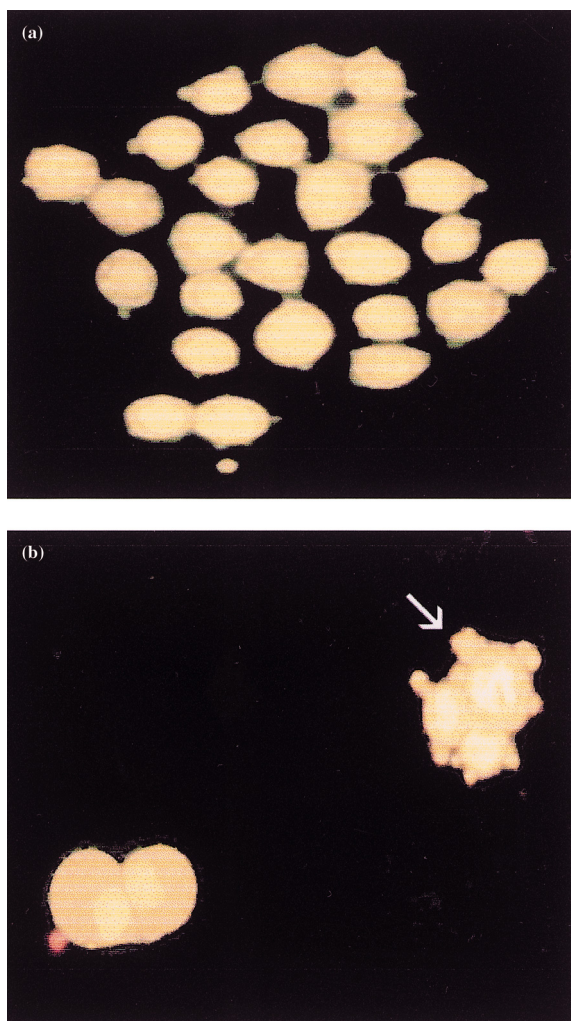


Fig. 4. Apoptosis in HT-29 cells using acridine orange/ethidium bromide staining technique and evaluated by fluorescent microscopy (400 \times). (a) Control HT-29 cells. (b) Cells treated with 100 μ M genistein for 3 days. Apoptotic nuclei appear highly condensed or fragmented (arrow).

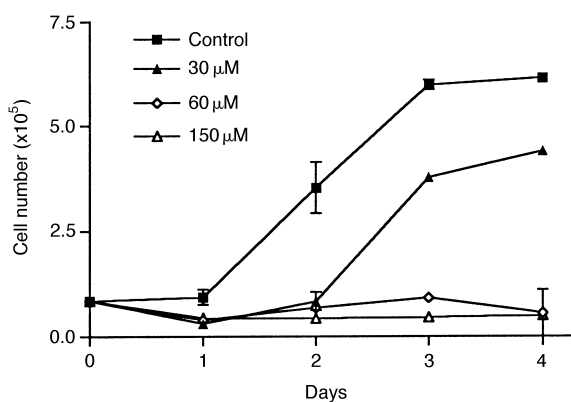


Fig. 5. The effect of genistein (30–150 μ M) on the growth of HT-29 cells.

3.3. Cell growth

The effect of genistein on the growth of HT-29 cells *in vitro* was evaluated at different concentrations. Fig. 5 demonstrates the tumoricidal effects of 60 and 150 μ M genistein. Interestingly, when cells were grown in the presence of 30 μ M genistein, there was an initial growth inhibition over the first 2 days. However, cell growth promotion was evident after 2 days. This growth promotion may be related to the ability to repair DNA breaks as evidenced by the comet assay (above). Similar findings were obtained in SW-620 and SW-1116 colon cancer cell lines (data not shown).

Cell proliferation was noted at lower (1–2 μ M) genistein treatments. This correlated with an increase in Ki-67 proliferation antigen immunostaining. Fig. 6 shows the expression of Ki-67 antigen in HT-29 cells treated with 9 μ M genistein and in untreated cells.

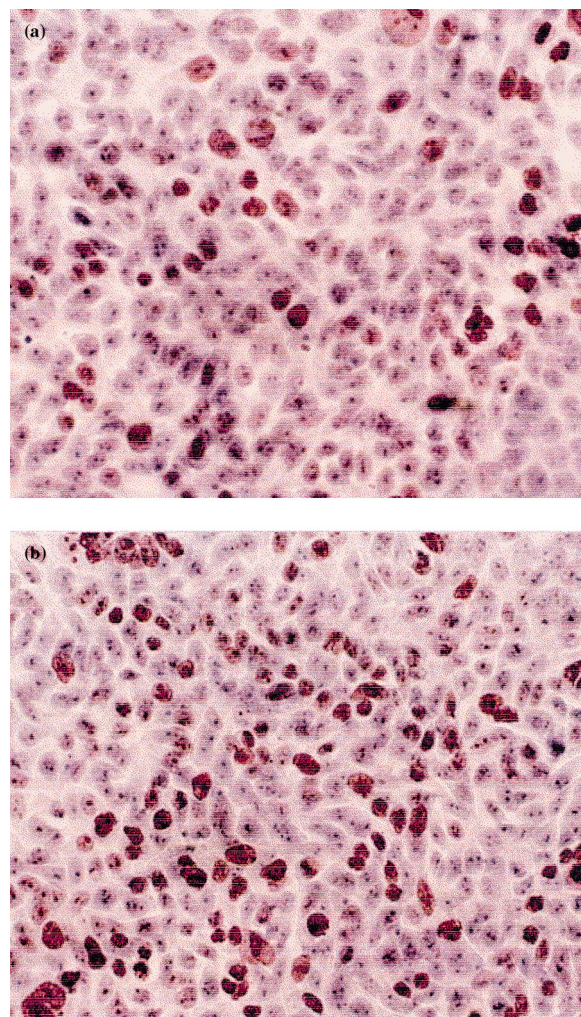


Fig. 6. The expression of Ki-67 proliferation antigen by immunocytochemistry in HT-29 cells. (a) Untreated cells. (b) Cells treated with 2 μ M genistein for 3 days. Treated cells display a higher percentage of staining (brown).

3.4. Cell cycle analysis

A delay in G₂/M became evident in HT-29 cells 3 days after treatment with 100 μ M genistein (Fig. 7). This was also associated with a sub-G₁ apoptotic peak. Also evaluated were treatments with 2 and 30 μ M genistein, which did not differ from untreated controls (data not shown).

3.5. Effect of pre-incubation with aclarubicin on genistein- and VP-16-induced DNA breakage and apoptosis in HT-29 cells

Pre-incubation with aclarubicin (0.4 μ M) reduced the level of DNA breakage induced by both genistein and VP-16 (Fig. 8a). This reduction was 66.6% for genistein-induced DNA breaks and 58.6% for VP-16-induced DNA breaks. Pre-incubation with aclarubicin did not alter the uptake of [¹⁴C]genistein into HT-29 cells (data not shown). In the mitoxantrone-resistant HL-60/MX2 cells, genistein induced a lower level of DNA breakage than in all other cell lines. Pre-incubation of these cells with aclarubicin did not alter DNA cleavage (Fig. 8b). Similar results were obtained with VP-16. In CEM/M70-B1 cells, genistein induced a lower level of DNA damage than VP-16. Aclarubicin did not alter DNA cleavage induced by either drug. Pre-incubation of HT-29 cells with aclarubicin (50 nM) did not reduce the percentage of apoptotic cells induced by either genistein or VP-16 evaluated 96 h following incubation in drug-free culture media (Fig. 8c).

This concentration of aclarubicin did not induce apoptosis in HT-29 cells. However, apoptosis was evi-

dent when cells were treated with 0.4 μ M of aclarubicin. Both concentrations caused an immediate (within 1 h) reduction in genistein and VP-16-induced DNA breakage in HT-29 cells. This reduction was greater when aclarubicin was used at 0.4 μ M. Even at the higher concentration, aclarubicin did not cause an immediate increase in DNA breaks.

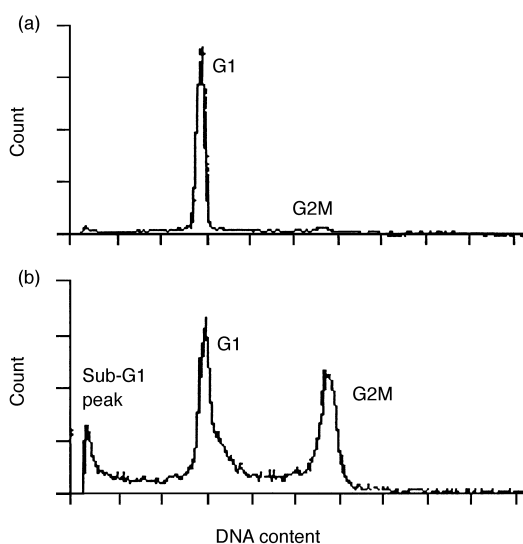


Fig. 7. Cell cycle analysis by flow cytometry in HT-29 cells. (a) Untreated cells. (b) Cells treated with 100 μ M genistein for 3 days undergo a G₂/M arrest.

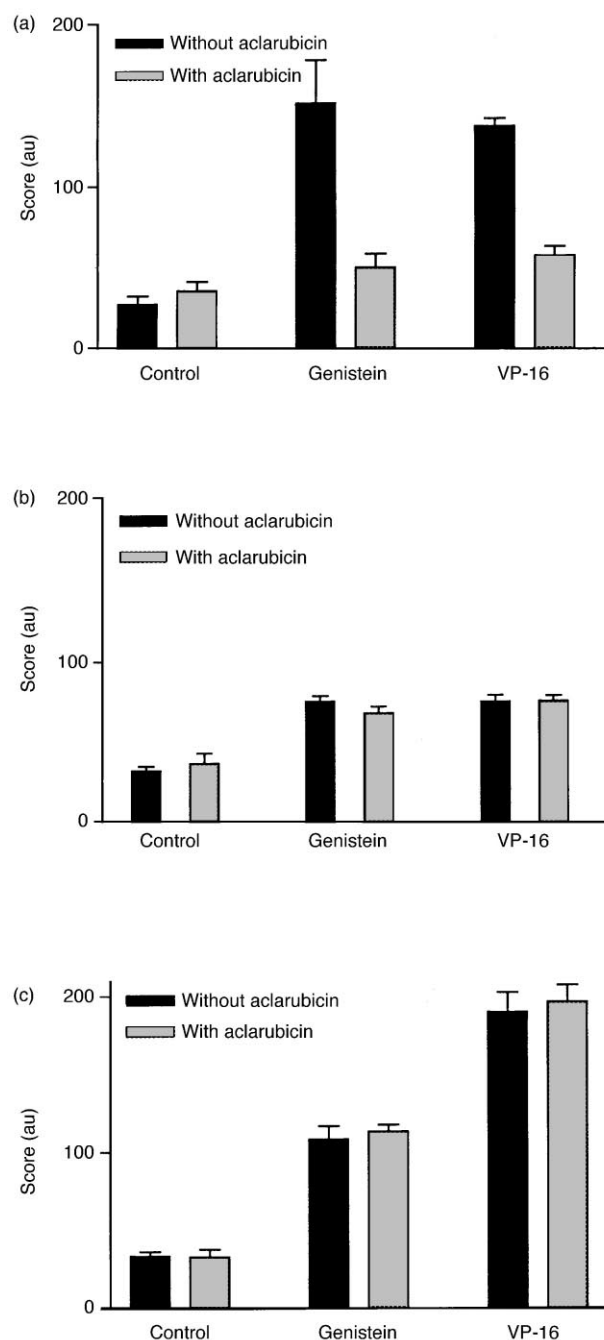


Fig. 8. The effect of genistein (100 μ M) and VP-16 (20 μ M) on HT-29 cells with and without pre-incubation with aclarubicin (0.4 μ M), evaluated using the comet assay on HT-29 cells (a), HL-60/MX2 cells (b) and CEM/M70-B1 cells (c).

4. Discussion

We have shown that genistein elicits a concentration-dependent effect on HT-29 colon cancer cells. At low physiologically relevant concentrations (1–2 μM), genistein caused an increase in cell proliferation as shown by the immunohistochemical staining for the proliferation antigen Ki-67. No detectable changes in the level of DNA cleavage were noted at these concentrations. At high concentrations ($\geq 60 \mu\text{M}$), genistein caused G_2/M arrest and induced apoptosis. In addition, there was a significant level of DNA damage caused at high concentrations, which increased when evaluated after 48 h. This is probably the result of internucleosomal DNA damage and nuclear fragmentation, two hallmarks of apoptosis. The dual nature of genistein is well documented in the literature, but has not been previously evaluated in HT-29 cells. Our results also offer insight into the effects of genistein (10–30 μM). We have shown an ability of HT-29 cells to repair genistein-induced DNA breakage 48 h after treatment. This ability to repair DNA may explain the cell proliferation that occurred 72 h following treatment with 30 μM genistein. Under such conditions, the percentage of cells that stained for Ki-67 was comparable with untreated controls. The ability of cells to repair DNA damage induced by various topoisomerase II inhibitors has previously been described and is not unique to HT-29 cells. It has been previously reported in human leukaemia (HL-60) cells [18], human lung adenocarcinoma (A549) [19] and in Chinese hamster lung fibroblasts (DC3F) [20].

Several mechanisms of action of genistein have been proposed [3–8]. These include topo II inhibition, inhibition of tyrosine kinase activity, induction of differentiation and inhibition of angiogenesis. In the current study, we investigated whether genistein induces topo II-mediated DNA damage in HT-29 cells, and if this damage was required to induce apoptosis. Inhibitors of eukaryotic topo II can be categorised into two groups, depending on the stage of the catalytic cycle they inhibit [21]. In the first group belong the topo II poisons that stabilise the covalent enzyme–DNA complex known as the cleavage complex. VP-16 is a representative topo II poison and is capable of inducing DNA cleavage. The second group of agents prevent the formation of covalent enzyme–DNA complexes and enzymatic turnover. They are referred to as topo II antagonists or catalytic inhibitors. Aclarubicin belongs to this group. Unlike topo II poisons, these agents do not induce DNA cleavage.

Using *in vitro* studies, we and others have shown that genistein inhibited the activity of DNA topo II and stabilised the cleavable complex [9,22]. In the current study, we sought to make use of the comet assay to further delineate the mechanism of action of genistein. In assays with purified topo II, aclarubicin has been shown to antagonise topo II-mediated DNA cleavage

induced by VP-16 [23]. In our study, aclarubicin antagonised DNA damage induced by both genistein and VP-16 in HT-29 cells in a similar manner, as determined by the comet assay. In HL-60/MX2 cells, the level of DNA cleavage induced by both drugs was lower than in HL-60 cells (data not shown) and HT-29 cells, and aclarubicin did not antagonise the effect of either drug. These cells have reduced topo II α levels and no topo II β [24]. Although CEM/M70-B1 cells have a markedly reduced level of topo II α , they have normal topo II β expression [25]. Genistein induced a lower level of DNA damage than VP-16 in this cell line, and aclarubicin did not antagonise the DNA cleavage induced by either drug. These observations suggest that topo II α may be preferentially targeted by genistein.

Markovits and colleagues [26], based on their observation that genistein-resistant CEM cell lines contained normal levels of topo II α and markedly reduced topo II β levels, came to a different conclusion. They suggested that the resistance to genistein in these cells results from the decreased topo II β expression that is also responsible for cross-resistance to topo II inhibitors.

Topo II-mediated DNA breaks in CEM/M70-B1 were markedly reduced in cells treated with genistein than in those treated with VP-16, indicating that these two inhibitors target different topo II isoforms. Perrin and colleagues [27] tested the ability of genistein to differentially inhibit the catalytic activity of either topo II α or topo II β using a DNA decatenation assay. Genistein showed equivalent effects on both enzymes. Our data, deduced from cell culture systems, strongly suggest that topo II α is the preferred enzymatic target of genistein, whereas topo II β is the preferred enzymatic target of VP-16.

Our results also suggest that genistein-induced topo II-mediated DNA breaks are not required for the induction of apoptosis. Furthermore, pre-incubation with aclarubicin did not alter the degree of apoptosis induced by either genistein or VP-16. The process of apoptosis most likely involves initial drug–target interactions and late nucleosomal fragmentation. The absence of a temporal relationship between early topo II–drug interaction and nucleosomal fragmentation is consistent with previous observations [28,29].

The exact mechanism of action of low concentrations of genistein in HT-29 cells remains unknown. The presence of low levels of oestrogen receptors (ER) in colon cells has been reported [30]. Using two different antibodies against ER, we were unable to detect ER in HT-29 cells by immunocytochemistry (data not shown). At these low concentrations, promotion of HT-29 cell growth has occurred. In addition, there was an increased percentage of cells that stained positive for the proliferation antigen Ki-67. These concentrations can be attainable in the serum of individuals or animals ingesting diets rich in genistein. Caution should,

therefore, be exercised when genistein is being considered as a chemopreventive agent against colon carcinogenesis. It is important to evaluate genistein's activity on different organs or sites.

Our results thus demonstrate the possible use of genistein as a chemotherapeutic agent when introduced at high concentrations in patients with colon cancer. Numerous topoisomerase inhibitors are currently being used or evaluated for the treatment of various malignancies. Genistein's ability to induce apoptosis and inhibit the growth of colon cancer cells renders it a possible chemotherapeutic drug for the treatment of colon cancer, which continues to be a major public health problem in the United States.

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