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# Genistein Inhibits Tumour Cell Growth *in vitro* but Enhances Mitochondrial Reduction of Tetrazolium Salts: A Further Pitfall in the Use of the MTT Assay for Evaluating Cell Growth and Survival

**M.C. Pagliacci, F. Spinozzi, G. Migliorati, G. Fumi, M. Smacchia, F. Grignani, C. Riccardi and I. Nicoletti**

**The natural isoflavone genistein inhibits the growth of a number of tumour cell lines *in vitro*. During investigations on the antiproliferative effects of genistein we observed that, with respect to direct cell counting, a tetrazolium (MTT) colorimetric assay consistently underestimated the growth inhibitory activity of the substance. Cell proliferation was markedly inhibited by genistein in three tumour cell lines (MCF-7, human breast tumour; Jurkat cells, human T-cell leukaemia; L-929, mouse transformed fibroblasts) when cell number was evaluated by direct counting, whereas a 72-h MTT assay failed to reveal any growth-inhibitory effect. Cell cycle analysis by propidium iodide staining and flow-cytometry revealed a G2/M cell cycle arrest after genistein treatment. Genistein-treated cells displayed an increase in cell volume and in mitochondrial number and/or activity, as revealed by enhanced formazan generation and increased uptake of the vital mitochondrial dye rhodamine 123. These results suggest that alterations in cell cycle phase redistribution of tumour cells by genistein may significantly influence mitochondrial number and/or function and, consequently, MTT reduction to formazan. This may constitute an important bias in analysing the effects of genistein, and possibly other drugs that block the G2/M transition, on growth and viability of cancer cells *in vitro* by MTT assay.**
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## INTRODUCTION

COLORIMETRIC ASSAYS are extensively used for evaluating the effect of growth factors, hormones and drugs on growth and survival of both normal and tumour cells in culture [1, 2]. Two reagents, tetrazolium (MTT) and formazan (XTT) [3] are commonly employed as indicators of cell number and viability, since they are converted to a coloured formazan derivative via mitochondrial dehydrogenase activity by viable cells. Although a number of factors, such as pH, medium glucose content and age of cultures, influence MTT and XTT reduction by living cells [4, 5], there is, in general, a good concordance between the number of viable cells in the culture and the production of formazan, which can be easily measured by colorimetric methods after solubilisation in dimethylsulphoxide. Because of its simplicity, precision and low-cost, the MTT assay is currently used in cytotoxic drug screening protocols [3, 5, 6]. It has, also, been proposed as a valid alternative to the [$^3$H]thymidine uptake methods for analysing cell proliferation [7]. Our laboratory has successfully applied the test to investigating the growth-inhibitory effect of drugs and cytokines [8, 9].

During the course of experiments aimed at analysing the

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mechanisms of the growth-inhibitory effects of the isoflavone genistein, we observed that the substance markedly reduced tumour cell growth when cells were directly counted with a haemocytometer, but that the MTT assay constantly underestimated the growth-inhibitory effect of the drug. These observations prompted a series of studies, which are herein described, for investigating the cause of the discrepancy between cell number and MTT reduction after genistein treatment. We demonstrated that tumour cells arrested in G2/M cell cycle phase by genistein have an enhanced formazan producing capacity due to an increase in mitochondrial activity.

## MATERIALS AND METHODS

Genistein, media, sera and antibiotics were purchased from ICN-Flow Biomedicals (Bucks, U.K.), rhodamine 123 from Molecular Probes INC (Eugene, Oregon, U.S.A.); MTT [3-(4,5-dimethyl-thyazol-2-yl)-2,5-diphenyltetrazolium bromide], propidium iodide and other laboratory reagents from Sigma (St Louis, Missouri, U.S.A.).

### *Growth of tumour cell lines*

A panel of tumour cell lines (MCF-7 breast tumour cells, L-929 mouse transformed fibroblasts and Jurkat T-leukaemia cells) was used. All lines were routinely grown in RPMI 1640 (ICN-Flow) containing 7.5% fetal bovine serum. Cultures were maintained in 75 $cm^2$ culture flasks in a humified atmosphere of 95% air and 5% carbon dioxide. Adherent lines were passaged at 70–80% confluency.

### *Analysis of cell growth*

Exponentially growing MCF-7 and L-929 cells from stock cultures were washed twice with sterile Hanks' balanced salt solution (HBSS) and removed from the dish with 2 ml 0.05% trypsin and 0.02% EDTA in HBSS. The cells were then collected by centrifugation at 500 *g* for 3 min, washed three times with RPMI 1640-1% bovine serum albumin (BSA) and dispersed by gentle passages through a Pasteur pipette. Jurkat cells were directly suspended in RPMI 1640-1% BSA and washed. Adherent cells (MCF-7 and L-929) were seeded at 50 000/ml in culture medium [RPMI 1640 plus 7.5% fetal bovine serum (FBS)] either in 35 mm diameter culture dishes for cell counting and flow cytometric analyses or in 96-well culture plates (5000 cells/100 μl) for MTT assay. After a 24 h preculture period to ensure attachment, medium was removed, the cultures were washed twice with HBSS–1% BSA and fresh medium supplemented with appropriate genistein concentrations (from 2 to 200 μmol/l) was added. Jurkat cells (500 000 cells/ml) were grown in 5 ml culture tubes for flow cytometric analysis and in U-bottomed 96-well culture plates (50 000 cells/100 μl) for MTT assay. Pre-established concentrations of genistein were directly added to cells suspended in RPMI 1640 plus 7.5% FBS. At the selected times, cells were counted directly in a haemocytometer after trypsinisation and dispersion or indirectly by a colorimetric assay (MTT assay) that measures the reduction of MTT to a violet-coloured formazan by living cells [10] as previously decribed [8].

### *Cell cycle analysis*

A quantitative measure of cell cycle distribution was obtained by flow cytometric analysis of DNA histograms according to Fried *et al.* [11], as previously described [9]. Briefly, at the pre-established times cells cultured in 35 mm plastic dishes (MCF-7 and L-929) were detached by mechanical scraping, collected in 5 ml plastic tubes, and washed twice with cold phosphate buffered saline (PBS). Thereafter 2 ml fluorochrome solution (propidium iodide 0.05 mg/ml dissolved in 0.1% Na citrate with 0.1% Triton-X 100) was added to the cell pellet. Jurkat cells were washed and stained as above directly in culture tubes. The tubes were placed at 4°C in the dark for 60–90 min, the cells dispersed by repeated pipetting and the stained cells transferred to test tubes for DNA analysis. Cell fluorescence was measured in a FACSCAN flow cytometer (Becton Dickinson, Mountain View, U.S.A.) by an argon ion laser at 488 nm for excitation. Red DNA fluorescence due to propidium iodide staining was read in the band above 620 nm. Data (forward scatter, right-angle scatter and DNA fluorescence of single nuclei) were recorded in a Hewlett-Packard (HP 9000 model 310) computer after an electronic gating of nuclear aggregates by the doublet discriminating module (DDM, Becton Dickinson). The percentage of nuclei in the different phases of cell cycle (G0/G1, S and G2/M) was calculated from the histogram of the DNA fluorescence area with DNA cell cycle analysis software (Cell-Fit, Becton Dickinson). A minimum of $10^4$ cells/sample were analysed.

### *Rhodamine 123 uptake*

Purified rhodamine 123 (Molecular Probes Inc, Eugene, Oregon, U.S.A.) was dissolved at 1 mg/ml in water as stock solution. Further dilutions were made in RPMI 1640. Cells, cultured as described above (see analysis of cell growth) were treated with rhodamine 123 at the selected times. The vital dye was added to cell cultures at the final concentration of 10 μg/ml and incubation continued for a further 15 min. Cultures were then washed in PBS–1% BSA, scraped or collected, the cell pellet resuspended in 1 ml PBS and analysed by a FACSCAN (Becton Dickinson) flow cytometer according to Darzynkiewicz *et al.* [12].

### *Statistical analysis*

All data are the mean ± S.D. Due to the non-normal distribution of the data, non-parametric tests (Wilcoxon's rank sum test and Kruskal Wallis' and Friedman's analysis of variance) were adopted for statistical evaluation of the results.

## RESULTS

### *Effects of genistein on tumour cell growth, cell cycle progression and mitochondrial MTT reduction*

Incubation of tumour cell lines with different genistein concentrations resulted in a marked and dose-dependent reduction in cell number after a 4-day culture period (Fig. 1). Jurkat and

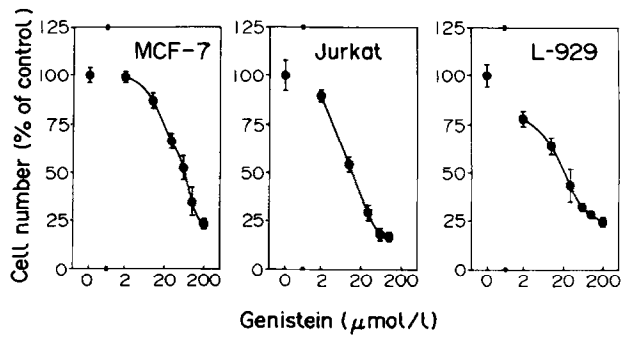


**Fig. 1. The effect of genistein on cell number, expressed as per cent of control cultures, after a 96 h period of incubation. Results are the means ± S.D. of three experiments in quadruplicate.**

L-929 cells were more sensitive to the growth inhibition exerted by the isoflavone than MCF-7 cells. The lowest concentration used (2 μmol/l) produced a significant reduction in cell number in both Jurkat and L-929 tumour cells, while MCF-7 cells were growth-inhibited only by concentrations above 10 μmol/l. Cell cycle analysis revealed a progressive accumulation of cells in the G2/M phase of the cycle, which preceded any significant reduction in cell number and/or increase in the percentage of trypan-blue positive dead cells (Fig. 2). Long-term observation of cultures, however, demonstrated a progressive increase in the percentage of dead cells at later times (after the 48th hour in Jurkat cells, after the 72nd hour in the L-929 and MCF-7 tumour cell lines) after genistein treatment, Genistein, therefore, exerts an early cytostatic effect on the G2/M transition, which is followed by cell death of cycle-arrested cells.

There was a marked discrepancy between the results of direct cell counting and formazan generation when a standard 72-h MTT assay [1] was adopted for evaluating the growth inhibitory effect of genistein (Fig. 3). The number of viable cells, as evaluated by the MTT assay, greatly exceeded the haemocytometer measured cell number in genistein-treated plates. The maximum discrepancy was observed in MCF-7 and L-929 cells at a genistein concentration of 30 μmol/l. Since direct chemical interaction between genistein and the MTT reduction process was ruled out (the same genistein concentrations did not exert any acute effect on formazan generation in cultured cells), the capacity of long-term treated cells to reduce MTT to formazan

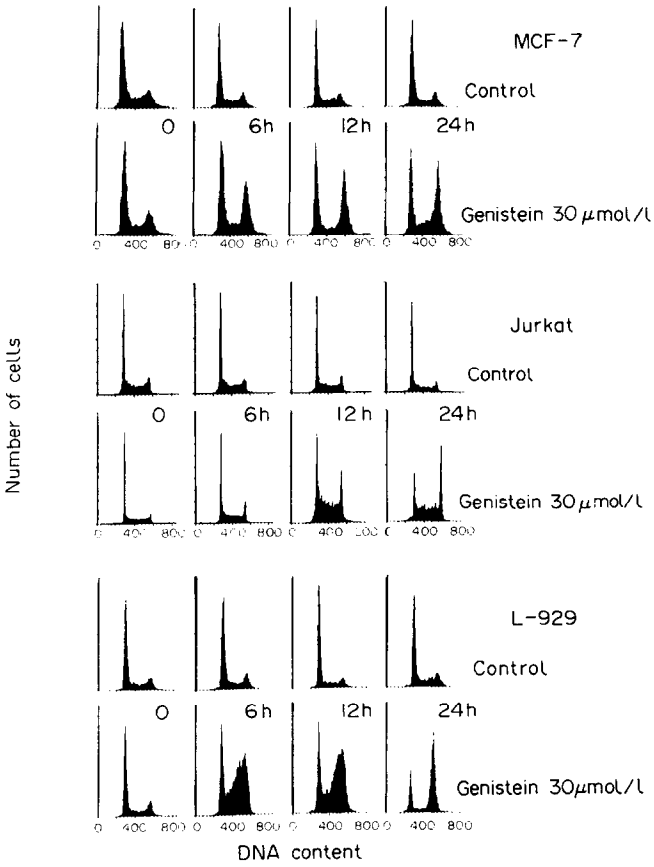


**Fig. 2. Time course examination of genistein effect on cell-cycle distribution of tumour cells, as measured by flow-cytometric analysis of DNA histograms in a representative experiment. Genistein produces a progressive accumulation of cells into the G2/M cell cycle phase.**

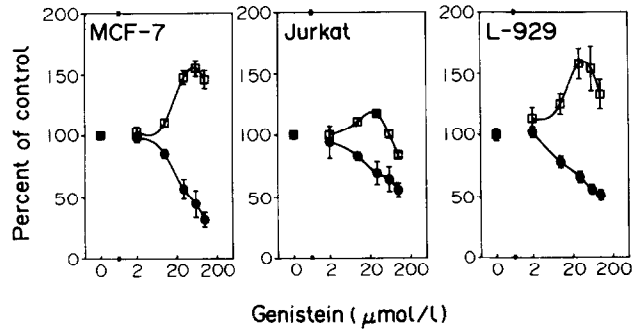


**Fig. 3. The effect of 72 h incubation with genistein on cell number measured with a haemocytometer (●) or calculated by MTT-assay (□). Values (means ± S.D. of three experiments in quadruplicate) are expressed as a per cent of controls.**

was clearly increased. An arbitrary "reduction index", calculated as the ratio between the optical density of the plates after 4 h incubation with MTT and the corresponding number of viable cells (counted with a haemocytometer), revealed a significant increase in the relative formazan production, which was particularly evident in genistein-treated L-929 and MCF-7 cell cultures (Fig. 4). Direct light microscope examination of the cells confirmed that the drug increased the number of mitochondria and/or enhanced their function. Cells in genistein-treated cultures were increased in volume, rich in mitochondria, and had an increased content of formazan crystals after 4 h incubation with MTT (Fig. 5).

*Effect of genistein on mitochondrial uptake of rhodamine* 123

Mitochondria accumulate and retain the fluorescent compound rhodamine 123 through an energy-dependent mechanism linked to their transmembrane potential [13]. The dye is taken up by mitochondria in living cells, without being accumulated in other cell organelles. Changes in mitochondrial number, organisation, distribution and shape, induced either by natural causes (i.e. viral transformation) or drugs, can be visualised after staining with rhodamine 123 [14]. Furthermore, cells with varying mitochondrial number and/or mitochondrial membrane potential can be reproducibly measured by flow cytometry [12, 15]. Flow-cytometric analysis of our tumour cell lines after vital

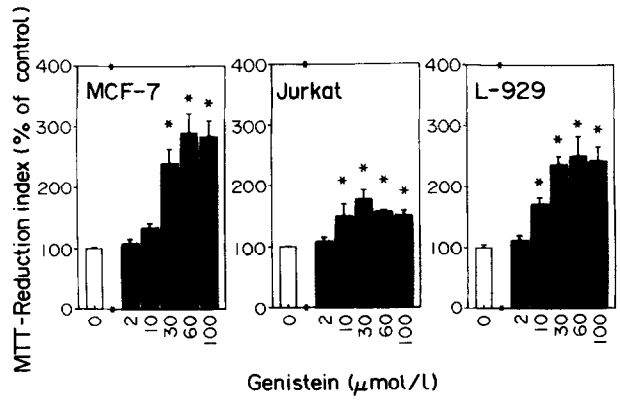


**Fig. 4. The effect of genistein on MTT reduction index (ratio between OD of plates at 570 nm and number of viable cells counted by haemocytometer after 72 h of incubation). Values (means ± S.D. of three experiments in quadruplicate) are expressed as per cent of controls. *$P < 0.01$ by Kruskal–Wallis' analysis of variance.**

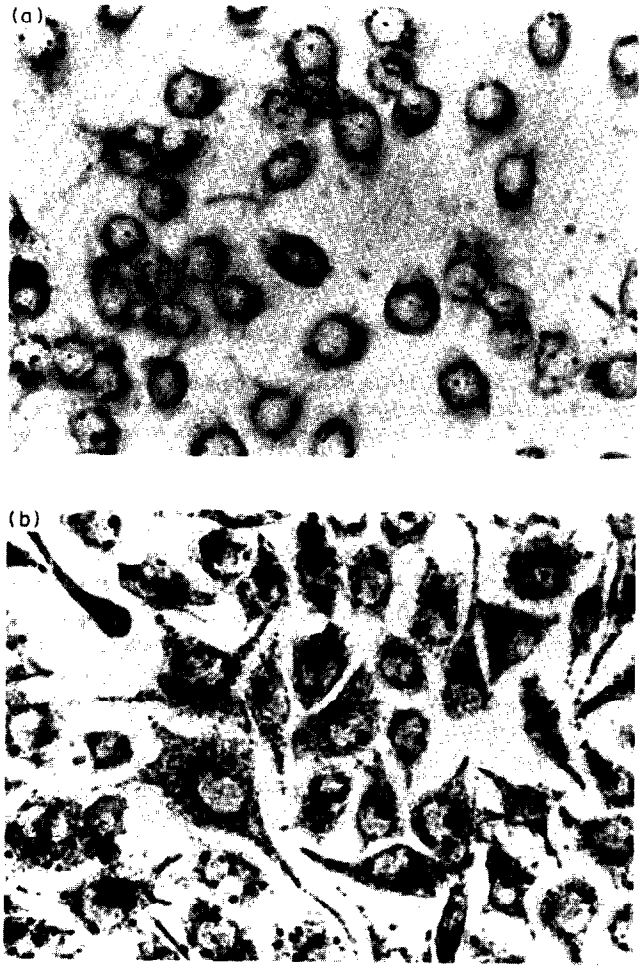


Fig. 5. Light microscope images of control (a) and genistein-treated (60 μmol/l, b) L-929 mouse fibroblasts. Cells, after 48 h of culture, were incubated with MTT for a 4 h period before observation (× 300).

staining with rhodamine 123 revealed a clear increase in the fluorescence intensity of genistein-treated cultures, which paralleled the increase in the percentage of cells in the G2/M phase of the cell cycle (Figs 6 and 7).

Time-course analysis (data not shown) showed that the uptake of rhodamine 123 by genistein treated cells increased progressively over that of control cells starting at about the 6th hour of incubation. Cell fluorescence reached its maximum between 48 and 72 h and fell abruptly after 96 h, when cell cycle blocked cells were almost completely dead. The enhanced fluorescence emission of genistein-treated cells correlated well with the MTT reduction index at 72 h, indicating that mitochondrial number and/or function in tumour cells were clearly increased by genistein at that time.

## DISCUSSION

In 1983 Mosmann demonstrated that, under appropriate conditions, MTT reduction to formazan is proportional to the number of metabolically viable cells in culture [1]. Since then, many laboratories have utilised the MTT assay as a rapid, precise and reproducible method for estimating cell growth. The test has also been extensively used by the U.S.A. National Cancer Institute for large scale screening of potential new antineoplastic drugs [2, 16].

When we evaluated the growth inhibitory potential of the natural isoflavone genistein by MTT assay, we found a marked

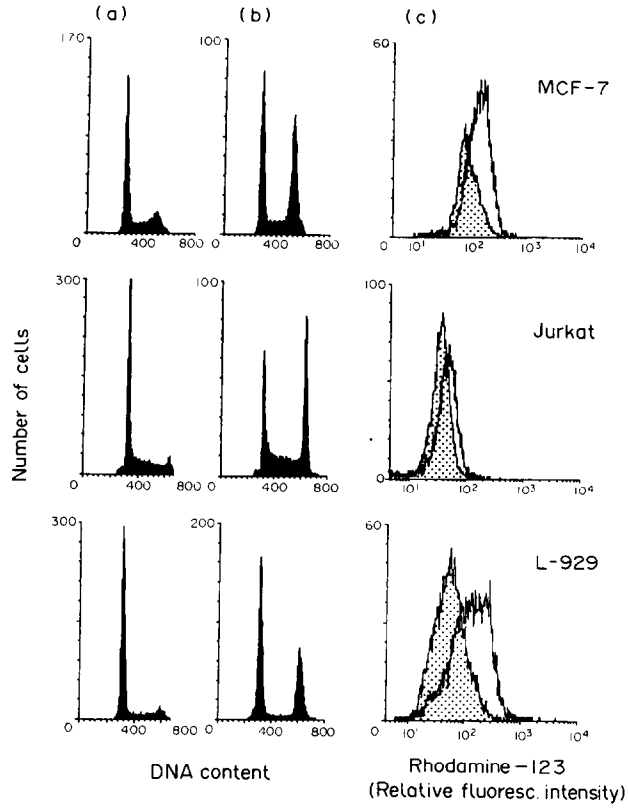


Fig. 6. The effect of genistein on cell cycle progression and rhodamine 123 uptake in a representative experiment. (a) DNA histograms of control tumour cell cultures. (b) DNA histograms of genistein-treated (30 μmol/l for a 48 h period) tumour cell lines. A marked increase in the G2/M cell cycle phase peak is visible. (c) Rhodamine 123 fluorescence emission of parallel cultures (controls: dotted area; genistein-treated: clear area).

discrepancy between the real number of viable cells and that estimated by the colorimetric assay. None of the critical factors known to affect formazan generation (medium glucose concentration, cell culture age, medium pH) [4, 5] was significantly different in genistein-treated cells with respect to controls, with the exception of a slight increase in mean glucose concentration in genistein-treated cultures.

Direct light microscope examination revealed that genistein produced an evident increase in cell volume, changes in cell

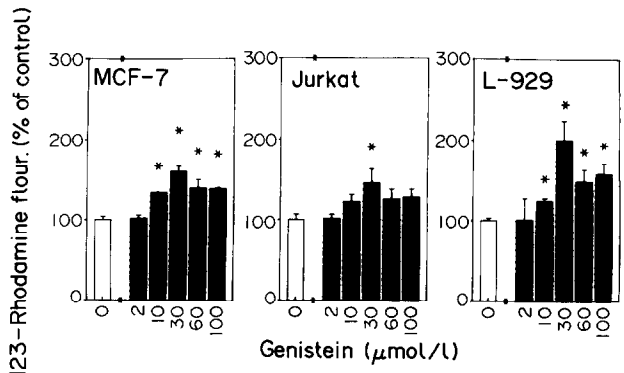


Fig. 7. The effect of genistein on rhodamine 123 uptake (means ± S.D. of four experiments) by tumour cells. Results are expressed as a percent of controls. *$P < 0.01$ by Kruskal–Wallis' analysis of variance.

morphology and an impressive increase in mitochondrial number and/or function, as revealed by the marked increase in tetrazolium salt deposits after 4 h incubation with MTT. This may well explain why the MTT assay underestimates the growth inhibitory effect of genistein under our experimental conditions.

The effects genistein exerts on tumour cell growth are only partially understood. At molecular level it specifically inhibits receptor-associated tyrosine kinases [17] and affects topoisomerase-II activity [18]. The resultant biological effects are differentiation (i.e. of erythroleukaemic cells) [19], and/or growth inhibition [17, 20, 21]. In our tumour cell models, the growth inhibition produced by genistein was exerted through a G2/M cell cycle block which was followed by cell death. Although a direct effect of genistein on mitochondrial functions cannot be excluded, it is more likely that the profound redistribution of the percentage of cells into G1, S and G2/M cell cycle phases after genistein treatment accounts for the enhanced MTT reductive capacity of genistein treated tumour cells. Although information on the relationships between mitochondrial reduction of MTT salts and cell cycle progression is lacking, other mitochondrial activities seem to vary with cell cycle phase. Mitochondrial uptake of rhodamine 123 is a valid tool for investigating the energy state of these organelles [12–14]. The intensity of mitochondrial staining by rhodamine 123 seems to be related to the activity of the oxidoreductive complexes responsible for the maintenance of the electronegativity of the mitochondrial membrane [14]. It has been clearly demonstrated that both the lymphocyte stimulation by phytohaemagglutinin (PHA) [12] and the G2/M block produced by platinum in tumour cells [22], result in increased mitochondrial rhodamine 123 uptake, thus confirming that modifications in the proliferative state of cells influence mitochondrial activity. Mitochondrial stainability with the fluorescent probe was also increased by genistein, in parallel with the accumulation of cells in the G2/M cell cycle phase. Even though our experiments do not define the mechanisms through which the mitochondrial number and/or function increases in tumour cells after genistein treatment, they do suggest that in certain conditions the inhibition of cell-cycle progression and cell division does not result in a parallel block of mitochondrial replication. Increased mitochondrial uptake of rhodamine 123 [22] and enhanced reduction of MTT salts to formazan may, therefore, be the logical consequence of increase in cell volume and mitochondrial mass after exposition of tumour cells to drugs which block the G2/M transition and cell division without exerting any direct influence on cell metabolism.

The fact that G2/M-arrested, but still viable, cells have an enhanced MTT reduction potential compared to logarithmically-growing control cultures, introduces a new and important bias in the use of the MTT assay for evaluating the effects of antineoplastic drugs. The enhanced formazan production by G2/M arrested cells may lead to considerable underestimation of the growth-inhibiting activity of genistein and possibly of other molecules able to produce a cell cycle specific block without any direct and early antimetabolic effect. In these cases, a preliminary control that formazan generation is linear with respect to cell number at different doses and times of treatment is necessary before the use of MTT assay as a rapid method for analysing the number of viable cells.


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