Genistein at Maximal Physiologic Serum Levels Induces G0/G1 Arrest in MCF-7 and HB4a Cells, But Not Apoptosis

Marcela S. Tsuboy,¹ Juliana C. Marcarini,¹ Alecsandra O. de Souza,² Natália A. de Paula,³ Daniel J. Dorta,² Mário S. Mantovani,⁴ and Lucia R. Ribeiro¹

¹Biosciences Institute, São Paulo State University (UNESP), Rio Claro, São Paulo, Brazil.

²Department of Chemistry, Faculty of Philosophy, Sciences, and Letters; ³Department of Clinical Medicine, Hospital of the Faculty of Medicine; University of São Paulo (USP), Ribeirão Preto, São Paulo, Brazil.

⁴Department of Biology, State University of Londrina (UEL), Londrina, Paraná, Brazil.

ABSTRACT Several studies have demonstrated that a balanced diet can contribute to better human health. For this reason, soy-based food and pure isoflavones (pills) are one of the most consumed. The association of this consumption and lower risks of chronic diseases and cancer is well established for the Asian population and has been attracting the attention of people worldwide, especially women at menopause who seek to alleviate the symptoms associated with the lack of estrogen. Despite positive epidemiological data, concerns still exist because of conflicting results found in scientific literature with relation to the role of isoflavones in breast and hormone-related cancers. The aim of our study was to investigate the cytotoxicity, induction of apoptosis, and changes in apoptosis-related genes of maximal physiological serum levels of the isoflavone genistein (Gen) in MCF-7 tumoral cells and in HB4a non-tumoral cells. In addition, induction of cell cycle arrest was also investigated. Only supraphysiological levels of Gen (50 and 100 μ M) were cytotoxic to these cell lines. Concentrations of 10 and 25 μ M did not induce apoptosis and significant changes in expression of the studied genes. Positive results were found only in cell cycle analysis: G0/G1 delay of MCF-7 cells in both concentrations of Gen and at 25 μ M in HB4a cells. It is the first study investigating effects of Gen in the HB4a cell line. Thus, despite the lack of apoptosis induction (generally found with high concentrations), Gen at physiologically relevant serum levels still exerts chemopreventive effects through the modulation of cell cycle.

KEY WORDS: • breast cell lines • cancer chemoprevention • cell cycle • cell death • physiological concentration • soy isoflavone

INTRODUCTION

GENISTEIN (Gen) is one of the most studied soy isoflavones, but it still arouses the interest of researchers worldwide. Many beneficial health effects have been attributed to the consumption of Gen, including reduction of cancer risk, osteoporosis, and cardiovascular diseases.^{1,2} In addition, Gen and soy-based food and supplements have gained special attention from women at menopause because of the association of their ingestion with the amelioration of symptoms caused by climacteric.³ These findings are supported by epidemiological observations from Asian populations that have lower rates of cancer incidence and other chronic diseases. Nevertheless, the role of soy foods in an overall healthy diet has become a confusing and contentious issue in recent years because of the conflicting

results found in scientific literature.⁴ One unresolved issue relates to the effect of isoflavones on breast tissue and the reproductive system. There have been studies indicating that there might be promotion or inhibition of breast cancer by the isoflavones.⁵ Moreover, depending on the study, Gen can inhibit or increase the action of tamoxifen, selective estrogen receptor modulator generally prescribed for patients with breast cancer. So, understanding and interpreting the enormous amount of soy research conducted is challenging, because it requires understanding the strengths and weaknesses of a wide variety of experimental models and designs.⁴

In relation to cancer, Gen chemopreventive effects includes modulation of various signaling cascades, but induction of apoptosis and cell cycle arrest are among the most studied ones. However, in most of these studies, non-physiological concentrations of Gen (> 30 μ M) are tested. Such data may produce results that are physiologically irrelevant, thus hindering predictions of efficacy.⁶ According to Steiner *et al.*,⁷ another limitation to investigate the

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Address correspondence to: Marcela S. Tsuboy, MSc, PhD, Instituto de Biociências, UNESP, Univ Estadual Paulista, Rio Claro, SP 13506-900, Brazil, E-mail: tsuboyma@yahoo.com.br

preventive effects of isoflavones is the lack of data regarding their effects in non-tumoral cell lines.

In front of these observations, the aim of our study was to evaluate the cytotoxicity, induction of apoptosis, changes in the expression of pro- and anti-apoptotic genes focusing on concentrations of Gen considered the maximal physiologically achievable through diet or through pharmacological administration, using two human mammary cell lines: a non-tumoral lineage (HB4a) and a tumoral one (MCF-7). In addition, we also investigated whether these concentrations of Gen are able to cause cell cycle arrest.

MATERIALS AND METHODS

Reagents

Gen (CAS 446-72-0), camptothecin (CAS 7689-03-4), and resazurin (CAS 62758-13-8) were purchased from Acros Organics. Doxorubicin (CAS 29042-30-6; Adri-blastina[®]) is manufactured by Actavis Italy and distributed by Pfizer. Most part of the reagents used to maintain cell cultures was from Gibco (Invitrogen Corporation): Dulbecco's modified Eagle's medium (cat no. 12800-058), fetal bovine serum, antibiotics (cat no. 15240-062), insulin, and trypsin. Hydrocortisone was from Pharma Nostra. Propidium iodide (PI) was from Sigma-Aldrich, and annexin V-fluorescein isothiocyanate (FITC) was from BD Pharmingen[™]. Oligo sequences were purchased from Prodimol Biotecnologia. RNase A, and the reagents used in the analysis of gene expression were from Invitrogen Corporation: Trizol LS, DNase I, dNTP mix, RNase Out[™], M-MLV reverse transcriptase, DEPC water, and Platinum[®] SYBR[®] Green qPCR SuperMix-UDG kit.

Test compound solutions and concentrations

Soy isoflavone Gen (99% purity) was dissolved in dimethyl sulfoxide (DMSO), and it was used in cell culture with a DMSO maximum concentration of 1%. In cytotoxicity assay (see *Resazurin-based assay—cytotoxicity evaluation*), doxorubicin (dissolved in phosphate buffer saline [PBS]) was used at 10 $\mu\text{g/mL}$. In flow cytometry tests (see *Annexin V-FITC/PI analysis of apoptosis induction* and *Cell cycle analysis by flow cytometry*), camptothecin (dissolved in DMSO) was used at 4 $\mu\text{g/mL}$.

Except for resazurin-based assay (see *Resazurin-based assay—cytotoxicity evaluation*), in the other biological tests (see *Quantitative real-time polymerase chain reaction*, *Annexin V-FITC/PI analysis of apoptosis induction*, and *Cell cycle analysis by flow cytometry*), we have focused on concentrations of 10 and 25 μM of Gen, which represents, respectively, maximal dietary and pharmacological concentrations that have been already found in human plasma.^{8–11} It is worth noting that there is no consensus in literature about the isoflavones serum levels in the human body. However, some authors consider that the concentration of 10 μM seems to be the maximum concentration found after consumption of soy in the diet, and because of this we have chosen this concentration to perform our tests.^{8,9,11} A

pharmacological concentration of 25 μM was tested, because it was found in the plasma of men with prostate cancer after the administration of Gen pills.¹⁰ The variability in the results found in literature may be, at least in part, due to the form of soybean processing, the source of isoflavones used (taken as food or pills), the time elapsed between soy intake and blood samples for analysis, and the personal factors such as CYP450 polymorphisms and intestinal microbiota.

Cell lines and culture

MCF-7 cells were kindly provided by Professor João Ernesto de Carvalho (UNICAMP), and HB4a cell line was kindly provided by Professor Silvia R. Rogatto from Hospital do Câncer A.C. Camargo. Both lineages were cultivated in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics at 37°C, 5% CO₂, and 95% relative humidity. Insulin and hydrocortisone (both at final concentration of 5 $\mu\text{g/mL}$) were also added to HB4a culture. The cell density for the experiments was determined by pilot tests, and it aimed at avoiding the detachment of cells resultant from total confluence on the last day of each assay.

Resazurin-based assay—cytotoxicity evaluation

Approximately 4.5×10^4 cells were seeded in each well of a 24-well microplate. After 24 h of stabilization, cells were treated with Gen for 24 or 48 h. After this period, the medium was removed, wells were washed with PBS, and cells were incubated with resazurin 60 μM for 3 h. Fluorescence was measured with VICTOR 3 (Perkin Elmer) at 530–560 nm of excitation and 580–600 nm of emission ranges.

The final concentrations of Gen used were 0.1, 1, 10, 50, and 100 μM . The medium with 1% DMSO constituted control treatment, and doxorubicin 10 $\mu\text{g/mL}$ was the control of induction of cytotoxicity. Experiments were performed in biological and technical triplicate (with three wells/treatment; $n = 9$). The tests were standardized based on the protocols described by Nakayama *et al.*,¹² O'Brien *et al.*,¹³ and McMillian *et al.*¹⁴

Quantitative real-time polymerase chain reaction

Gene expression was analyzed after 12 h treatment of cells ($10^6/25 \text{ cm}^2$ flasks) with Gen 10 or 25 μM . Experiments were performed in biological duplicate and technical triplicate ($n = 6$).

According to the manufacturer's instructions, total RNA was extracted using Trizol LS reagent, followed by DNase I treatment. Verification of RNA quality was done in agarose gel (28S and 18S rRNA pattern of bands) and by A₂₆₀/A₂₈₀ ratio (Biophotometer; Eppendorf). Complementary DNA (cDNA) was synthesized using dNTP 2.5 mM mix; OligodT 10 pmol/ μL ; RNase Out; M-MLV reverse transcriptase; RNA 500 ng/mL; and DEPC water. Real-time polymerase chain reactions (RT-PCR, triplicate) were performed in Engine Opticon detection system (Bio-Rad) using Platinum

SYBR Green qPCR SuperMix-UDG kit in a total of 20 μ L of reaction mix (2 μ L of 1:5 cDNA; 10 μ L SYBR Green; 0.5 μ L of 10 μ M of each specific gene primer; and 7 μ L of H₂O). Target cDNAs were amplified in separate tubes: 3 min at 95°C, then 35 cycles of denaturation (95°C for 20 s), annealing (at 60°C for 30 s), and extension (72°C for 20 s) per cycle. Melting curves (50–95°C ready every 0.5°C) for each PCR reaction were generated to ensure the purity of the amplification product. Primers sequences were designed with IDT tool available at <http://idtdna.com/Scitools/Applications/Primerquest>. In order to assess the induction of apoptosis in these mammary cell lines, primers of Caspase-3 (*CASP-3*), Caspase-7 (*CASP-7*), BCL2-associated X protein (*BAX*), and BCL2-like 1 (*BCL-xL*) were used, as well as glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as a reference gene,¹⁵ besides cyclooxygenase-2 (*COX-2*).¹⁶ Primers sequences used and amplicon size (bp) was as follows: *CASP-3* (NM_032991.2) 5'-GTG CTA CAA TGC CCC TGG AT-3' and 5'-GCC CAT TCA TTT ATT GCT TTC C-3' (199 bp); *CASP-7* (NM_001227.3) 5'-TCA CCA TGC GAT CCA TCA AGA CCA-3' and 5'-TTT GTC TGT TCC GTT TCG AAC GCC-3' (149 bp); *BAX* (NM_004324.3) 5'-TTT CTG ACG GCA ACT TCA ACT GGG-3' and 5'-TGT CCA GCC CAT GAT GGT TCT GAT-3' (122 bp); *BCL-xL* (NM_138578.1) 5'-TGG GCT CAC TCT TCA GTC GGA AAT-3' and 5'-ATG TAG TGG TTC TCC TGG TGG CAA-3' (121 bp).

Annexin V-FITC/PI analysis of apoptosis induction

Approximately 10⁵ cells of HB4a or MCF-7 were seeded in each well of a six-well microplate. After 24 h of stabilization, cells were treated with camptothecin (4 μ g/mL) and Gen 10 or 25 μ M for another 24 h. At the end of the treatment, the medium was removed from the wells and washed with PBS before the addition of trypsin (0.01%) to the cells. The same medium and PBS (that were reserved in Falcon tubes) were added to the cells, and the cellular suspension was centrifuged (Hitachi Himac CR21E, 1000 rpm, 5 min, 4°C). Supernatant was discarded and the cell pellet was resuspended in cold PBS followed by another centrifugation (Hitachi Himac CR21E, 1000 rpm, 10 min, 4°C). Cells were then labeled with annexin V-FITC (1:100) and PI (5 μ g/mL) in Falcon tubes protected from the light. Ten thousand events were analyzed in a BD FACS CANTO flow cytometer. It was performed in biological duplicate with three wells of each treatment in each biological repetition ($n=6$).

Cell cycle analysis by flow cytometry

MCF-7 and HB4a cells (10⁵/well) were seeded in a six-well microplate and after 24 h of stabilization, treated with camptothecin (4 μ g/mL) and Gen 10 or 25 μ M for another 24 h. After detachment of the cells (0.01% trypsin) and centrifugation (Hitachi Himac CR21E, 1000 rpm, 5 min, 4°C), PBS and 70% ice-cold ethanol were added to the pellet. Then, the pellet was resuspended and left at –20°C for 24 h. After this period, cells were centrifuged (Hitachi Himac CR21E, 1000 rpm, 5 min, 4°C), supernatant was

discarded, and cold PBS and RNase A were added to each tube. Samples were incubated for 30 min at 37°C, followed by staining with hypochthonic fluorochrome solution (PI 5 μ g/mL; sodium citrate 0.1%; and Triton X-100 0.1%) for 1 h before flow cytometric analysis (10,000 events; BD FACS CANTO). Experiments were performed in biological duplicate with three wells of each treatment in each biological repetition ($n=6$).

Statistical analysis

Results obtained in cytotoxicity and in flow cytometry tests were submitted to analysis of variance followed by Dunnett's test compared with control group ($\alpha=0.05$), using GraphPad Prism5 software. Gene expression was analyzed with relative quantification method using *GAPDH* as a reference gene according to Pfaffl with REST[®] software (*Relative Expression Software Tool*).^{17,18} The software is available online at: <http://gene-quantification.de>

RESULTS

Resazurin-based assay—cytotoxicity evaluation

After 24 h of treatment with Gen, HB4a cells were not affected by any of the investigated concentrations, but Gen 100 μ M was cytotoxic to MCF-7 cells after the same period (Figs. 1 and 2). Treatment with Gen 50 and 100 μ M for 48 h induced cytotoxicity in HB4a and MCF-7 (Figs. 1 and 2). Gen 10 μ M was not cytotoxic for both cells in these conditions.

Quantitative RT-PCR

The values of *R* (ratio obtained in REST software for gene expression) are presented in Tables 1 and 2. As can be seen,

FIG. 1. Fluorescence intensity (mean value $\pm$ standard deviation [SD]) measured after treatment of HB4a (24, 48 h) with different concentrations of genistein (Gen) in resazurin-based assay ($n=9$). *Indicates cytotoxic effect ($P < .05$; analysis of variance [ANOVA] followed by Dunnett's test compared with control group). Gen [x], cells treated with Gen x μ M; Doxorubicin, cell treated with doxorubicin in 10 μ g/mL.

FIG. 2. Fluorescence intensity (mean value $\pm$ SD) measured after treatment of MCF-7 (24, 48 h) with different concentrations Gen in resazurin-based assay ($n=9$). *Indicates cytotoxic effect ($P<.05$; ANOVA followed by Dunnett's test compared with control group).

COX-2 expression in mammary tumoral MCF-7 cells was null, and in HB4a, it was practically similar to the control group.

We can observe that expression of the investigated genes was practically unchanged in the tested conditions for both cell lineages. Gene expression of caspases 3 and 7 (*CASP3*; *CASP7*; known as execution caspases) was very similar to those found in control HB4a and MCF-7 cells. *BAX* expression slightly changed in HB4a cells with treatment of GEN. 25 μ M and in MCF-7 with treatment of GEN. 10 μ M. *BCL-xL* expression was negatively regulated in HB4a cells in front of Gen 10 μ M treatment, and in MCF-7 cells with Gen 25 μ M treatment.

Annexin V-FITC/PI analysis of apoptosis induction

Analysis of apoptosis induction by flow cytometry in HB4a and MCF-7 is shown in Figures 3 and 4. Apoptotic

TABLE 1. RELATIVE GENE EXPRESSION OF *CASP-3*, *CASP-7*, *BAX*, *BCL-xL*, AND *COX-2* AFTER TREATMENT OF 10 OR 25 μ M OF GENISTEIN IN HB4A CELLS

	Genistein 10 μ M	Genistein 25 μ M
<i>CASP-3</i>	1.212 $\pm$ 0.213 $P=.184$	1.074 $\pm$ 0.207 $P=.647$
<i>CASP-7</i>	1.046 $\pm$ 0.173 $P=.86$	1.305 $\pm$ 0.259 $P=.104$
<i>BAX</i>	1.208 $\pm$ 0.161 $P=.244$	1.295 $\pm$ 0.125 $P=.031$
<i>BCL-xL</i>	-1.85 $\pm$ 0.169 $P=.008$	1.181 $\pm$ 0.237 $P=.168$
<i>COX-2</i>	-1.198 $\pm$ 0.256 $P=.37$	1.002 $\pm$ 0.278 $P=.983$

Data are presented as R value $\pm$ standard deviation (SD) and P -value. Data were submitted to REST[®] software.¹⁸ Negative R value means down-regulation of the gene.

TABLE 2. RELATIVE GENE EXPRESSION OF *CASP-3*, *CASP-7*, *BAX*, AND *BCL-xL* AFTER TREATMENT OF 10 OR 25 μ M OF GENISTEIN IN MCF-7 CELLS

	Genistein 10 μ M	Genistein 25 μ M
<i>CASP-3</i>	1.113 $\pm$ 0.231 $P=.601$	1.556 $\pm$ 0.389 $P=.142$
<i>CASP-7</i>	1.181 $\pm$ 0.261 $P=.457$	1.098 $\pm$ 0.278 $P=.862$
<i>BAX</i>	1.452 $\pm$ 0.224 $P=.040$	-1.172 $\pm$ 0.268 $P=.105$
<i>BCL-xL</i>	1.356 $\pm$ 0.247 $P=.134$	-1.546 $\pm$ 0.18 $P=.027$

Values are presented as R value $\pm$ SD and P -value. Data were submitted to REST[®] software.¹⁸ Negative R value means down-regulation of the gene. *COX-2* expression was null in this cell line and it is according to literature.¹⁹⁻²¹

HB4a and MCF-7 cells were found only after treatment of cells with camptothecin, that is Gen 10 and 25 μ M were not able to induce apoptosis in these lineages.

Cell cycle analysis by flow cytometry

Figures 5 and 6 show the cell cycle distribution of HB4a and MCF-7 cells after treatment with Gen 10 and 25 μ M. Percentage of cells in sub-G0 phase had significantly increased only with treatment of cells with camptothecin (Figs. 5 and 6). Gen 10 μ M slightly decrease the number of HB4a cells at S phase. Gen 25 μ M was able to arrest HB4a cycle at G0/G1 phase and as a consequence of this arrest, the percentage of cells in S and G2/M phases has also decreased (Fig. 5). For MCF-7 cells, Gen 10 and 25 μ M arrested cell cycle at G0/G1 phase (Fig. 6).

DISCUSSION

Recently, different societies and institutes (*e.g.*, National Cancer Institute and North American Menopause Society) have called attention to the possible estrogen-like effects of natural isoflavones (*e.g.*, Gen) and possible promotion or propagation of estrogen-sensitive cancers, such as the breast one. Data supporting this idea are based on *in vitro* and *in vivo* studies showing stimulating tumor cell proliferation and growth.²²⁻²⁵ It is well known that diverse phytoestrogens such as Gen possess a biphasic effect, that is, they can promote or inhibit cell proliferation depending on the concentration. Accordingly, our results of resazurin-based assay clearly showed this effect: cytotoxic effect of Gen only at high concentrations (50 and 100 μ M). Gen 100 μ M was cytotoxic to tumoral MCF-7 cells in both experimental times analyzed (24 and 48 h), but it showed cytotoxicity in non-tumoral HB4a cells only after 48 h. Gen 50 μ M was cytotoxic to both cells lines only after 48 h of treatment. Increase in fluorescence measured was observed in MCF-7 cells treated with Gen 0.1–10 μ M. In resazurin-based assay, the higher the fluorescent signals, the more viable cells are present in the sample, as only viable cells convert resazurin into resofurin.¹²⁻¹⁴ Contradictory results have long been

FIG. 3. Analysis of apoptosis induction by flow cytometry (annexin V-fluorescein isothiocyanate [FITC]/propidium iodide [PI]) in HB4a cells treated with genistein for 24 h. Results are the mean value $\pm$ SD of $n=6$. *Statistically significant ($P<.05$; ANOVA followed by Dunnett's test compared with control group).

Treatment HB4a cells	Viable cells (D)	Apoptotic cells (C)	Late apoptotic cells (B)	Necrotic cells (A)
Control	86.66 $\pm$ 3.21	6.32 $\pm$ 1.93	5.72 $\pm$ 1.54	1.2 $\pm$ 0.4
Camptothecin 4 μ g/mL	36.78 $\pm$ 10.79*	32.26 $\pm$ 7.81*	30.38 $\pm$ 3.90*	0.6 $\pm$ 0.51
Genistein 10 μ M	81.34 $\pm$ 7.12	9.94 $\pm$ 4.73	7.65 $\pm$ 2.37	1.4 $\pm$ 0.4
Genistein 25 μ M	80.05 $\pm$ 7.22	9.00 $\pm$ 2.37	9.68 $\pm$ 5.55	0.9 $\pm$ 0.49

demonstrated in studies of Gen treatment of different cell lines. Depending on the study, inhibitory effects of Gen are observed even at low concentrations that generally were found as non-cytotoxic and which had stimulatory effects on the proliferation of cancer cells. Results from Zava and Duwe,²⁶ for example, revealed that Gen has cell growth-inhibitory actions over a physiologically achievable concentration range (10 nM–20 μ M) in MCF-7. The cytotoxic effect of low Gen (0.01 μ M) was also reported by Chen and Anderson in OVCAR-3 (ovarian cancer cell).²⁷ Despite such observations, cytotoxic effects are generally found with high isoflavones concentrations,⁷ and in the majority of studies

using MCF-7 as an experimental model, low concentrations of soy extracts or pure isoflavones stimulated cell growth.²⁸ As mentioned earlier, cytotoxicity and cell death by apoptosis of high Gen concentrations (above 30 μ M) has been found in different cell lines: MCF-7,^{29–32} MDA-MB-231,³² HeLa,³⁰ SK-OV-3,³³ HO-8910,³⁴ V79,³⁵ JURKART-T,²⁹ WM451,³⁶ PC-3,^{37,38} nontumorigenic CRL-2221 human prostate epithelial,³⁸ NIH 3T3,³⁹ and so on.

As reviewed by many authors^{40–42} and others, Gen modulates expression or the activity of various molecules involved in apoptosis signaling, cell cycle regulation, antioxidant defense, xenobiotic metabolism, cell survival, inflammation,

FIG. 4. Analysis of apoptosis induction by flow cytometry (annexin V-FITC/PI) in MCF-7 cells treated with genistein for 24 h. Results are the mean value $\pm$ SD of $n=6$. *Statistically significant ($P<.05$; ANOVA followed by Dunnett's test compared with control group).

Treatment MCF-7 cells	Viable cells (D)	Apoptotic cells (C)	Late apoptotic cells (B)	Necrotic cells (A)
Control	81.71 $\pm$ 5.33	7.56 $\pm$ 3.92	5.24 $\pm$ 1.98	3.46 $\pm$ 0.93
Camptothecin 4 μ g/mL	43.44 $\pm$ 9.43*	39.85 $\pm$ 7.44*	20.2 $\pm$ 5.86*	4.13 $\pm$ 0.66
Genistein 10 μ M	81.73 $\pm$ 8.42	12.05 $\pm$ 7.34	5.45 $\pm$ 2.91	2.80 $\pm$ 0.88
Genistein 25 μ M	78.66 $\pm$ 2.80	10.76 $\pm$ 7.93	8.33 $\pm$ 1.85	3.52 $\pm$ 0.49

FIG. 5. Cell cycle distribution of HB4a after genistein treatment for 24 h (flow cytometry). Results are the mean value $\pm$ SD of $n=6$. *Statistically significant ($P < .05$; ANOVA followed by Dunnett's test compared with control group). CPT, cells treated with camptothecin 4 μ g/mL.

metastasis, and angiogenesis. Moreover, the chemopreventive effects of Gen are associated to epigenetic events and microRNA modulation.^{43–46} So, the variability of results found in literature is also related to these mechanisms.

One of the most reported biological activities of Gen is the induction of apoptosis. However, when we deeply analyze and compare the results, we can see that in most of the studies, Gen-induced apoptosis is through supraphysiological concentrations. Our quantitative RT-PCR and annexin V-FITC/PI analysis showed that maximal dietary and pharmacological concentrations investigated (10 and 25 μ M) did not induce apoptosis in either non-tumoral HB4a cells or tumoral MCF-7 cells. Gen-induced apoptosis, when observed, is resultant from a decrease in Bcl-2 and Bcl-xL protein expression, an increase in Bax, Bak, and caspase activation, mainly of caspase-3.^{47,48} These alterations in mitochondrial

and caspases proteins can also be due to the generation of reactive oxygen species by the isoflavones.^{49,50}

Positive results were found in our flow cytometric analysis of cell cycle: Gen delayed MCF-7 cells at G0/G1 phase in both treatments (10 and 25 μ M), and for HB4a cells, G0/G1 phase arrest was seen only after the treatment of cells with 25 μ M of Gen. Cell cycle modulation is another known chemopreventive effect that is attributed to Gen; nevertheless, G2/M arrest is most encountered.^{7,4,48,51–54} Consistent with our results, Kuzumaki *et al.* found G0/G1 arrest induced by Gen (but up to 60 μ M) in BALB/c 3T3 and B16-F1 cells mediated through induction of p21 and suppression of cyclin E, key protein regulators of G1/S transition of cell cycle.^{57,58} In the study by Chen and Donovan,⁵⁹ Caco-2BBE cells were arrested in G0/G1 stage after a low dose of Gen (3.7 μ M for 48 h). In this case, the lower cyclin D level suggested that cells were not entering the cell cycle, as it regulates entry into and progression of the cell cycle at G1.^{59,60} Gen treatment (10, 20, and 40 μ M for 24, 48, and 72 h) of LNCaP also increased the percentage of cells in G0/G1 phase.⁶¹ The authors demonstrated that this antiproliferative effect was mediated by up-regulation of p21^{WAF1} and p27^{KIP1}, two negative cell-cycle regulators that act as cyclin-dependent kinase inhibitors.⁵⁴

Cancer chemoprevention strategies include prospection of phytochemicals and new synthetic drugs that show higher cytotoxicity (and other desirable effects) in cancer cells than in their normal counterparts.⁴⁷ In our study, we observed cell cycle arrest induced by Gen not only in the tumoral cell line MCF-7, but also in the non-tumoral HB4a, although MCF-7 cells were more sensitive to Gen treatment. Results seen in HB4a cells (decreased percentage of cells in S phase with both concentrations; accumulation of cells in G0/G1; and decrease of cells in G2/M with Gen 25 μ M) can also indicate a chemoprevention action of Gen, as cell cycle arrest allows time for damaged cells to be repaired and to not accumulate mutations that are favorable for cancer development at earlier stages. As reviewed by Klein and King,⁴⁹ Gen itself can induce mutagenicity. So, apparently, Gen can induce and protect cells from its own biological damage. The balance between cell survival and death signs will determine cell fate after treatment of Gen.

Despite the absence of apoptosis in our study, Gen at physiologically relevant serum levels still exerts chemopreventive effects, as seen by the induction of G0/G1 arrest of the cells. This is the first study investigating effects of Gen in the non-tumoral cell line HB4a, and one of the few comparing the breast cancerous cell line and its normal lineage. We hope our data will improve understanding of the biological effects of Gen in order to reach a consensus on the controversial issue “isoflavones and breast carcinogenesis.”

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FIG. 6. Cell cycle distribution of MCF-7 after genistein treatment for 24 h (flow cytometry). Results are the mean value $\pm$ SD of $n=6$. *Statistically significant ($P < .05$; ANOVA followed by Dunnett's test compared with control group). CPT, cells treated with camptothecin 4 μ g/mL.

AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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