

Laboratory Investigation

Synergistic effect of genistein and BCNU on growth inhibition and cytotoxicity of glioblastoma cells

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Key words: synergism, chemotherapy, genistein, isoflavones, BCNU, glioblastoma multiforme

Summary

Objective: Recent experiments have shown that dietary soy isoflavones such as genistein can significantly suppress invasiveness and growth of a number of human malignancies. This study examined whether genistein, at a concentration typical of plasma levels following soy diet intake, in combination with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU, carmustine) exhibited an additive or synergistic inhibitory effect on the growth of glioma cells.

Methods: The human glioblastoma multiforme (GBM) cell line U87 and the rodent C6 glioma were treated with genistein at 4 μM , combined with BCNU (0–50 μM). Monolayer cell growth and cytotoxicity, as measured by colonogenic survival in soft agarose, were then compared in control and drug-treated cultures. Presence of apoptosis, using the DNA ladder assay and laser scanning cytometry (LSC), was investigated in all cell lines at those concentrations where an enhancement of antiproliferative effect of BCNU in presence of genistein was observed.

Results: A 32–41% increase in monolayer growth inhibition and a 28–42% increase in colony cytotoxicity in the U87 cell line were observed when genistein (4 μM) was added to BCNU in the 0–10 μM dose range. In the C6 cell line, a 30–36% increase in monolayer growth inhibition and a 39–54% increase in colony cytotoxicity were observed with the BCNU dose range of 0–50 μM . All experiments showed a significant increase in growth inhibition and a decrease in colonogenic survival ($P < 0.05$). We were unable to detect apoptosis in any of the lines when genistein was combined with BCNU.

Conclusion: These results indicate that genistein at typical adult dietary plasma levels can significantly enhance the antiproliferative and cytotoxic action of BCNU. The implication for treatment of GBM may be a reduction in the chemotherapeutic dose recommendations of these agents and subsequently a decrease in the risk of treatment sequelae for these patients.

Introduction

The adjuvant use of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU, carmustine) in the treatment of both pediatric and adult malignant gliomas is limited by significant and possibly life-threatening toxicity. Dose-limiting myelosuppression as well as pulmonary and hepatic toxicity are major limitations [1]. Research on the use of novel agents that reduce toxicity, increase effectiveness, and reduce the required dosages of the BCNU and other chemotherapeutics is justified.

One group of such agents is the isoflavones, in the family of receptor tyrosine kinase (RTK) inhibitors [2]. As the most anticarcinogenic and antiproliferative members of this family, the isoflavones genistein and

daidzein occur naturally at high concentrations in soybeans and soy-protein foods [3,4]. Genistein is a potent and relatively specific inhibitor of epidermal growth factor receptor tyrosine kinase (EGFR-TK) activity with a wide range of Inhibitory Concentration (IC_{50}) values (2.6–79 μM) for proliferation of many human tumor lines *in vitro* [2,3]. Genistein inhibits *in vitro* endothelial cord formation (IC_{50} : 150 μM), endothelial cell proliferation stimulated by basic fibroblast growth factor (bFGF), as well as plasminogen activator (PA) and PA-inhibitor-1 in vascular endothelium, all of which play an important role in tumor angiogenesis [5]. Genistein can also inhibit the activity of topoisomerase II *in vitro* with IC_{50} values of 110 μM [6–8]. Reports of the experimental *in vitro* and *in vivo*

use of isoflavones have shown encouraging results in blocking malignant transformation, growth and invasiveness of a number of human tumors such as breast cancer, colorectal carcinoma, glioblastoma multiforme and leukemia [3,9–17]. In the majority of these studies, however, the effective genistein concentrations are much higher than plasma levels reached following consumption of soy-based diets or formula [3].

Although the bioavailability of soy-based foods is still under investigation, a series of human trials has shown isoflavone (genistein and daidzein) plasma levels of 3–6 μM following oral intake of soy milk formulations in adults and infants [3,4,18,19]. These low plasma concentrations may not justify the use of dietary isoflavones in their current form as sole chemotherapeutic agents for brain tumors such as glioblastoma multiforme. Nevertheless, when combined with a primary chemotherapeutic agent, genistein may exhibit a significant synergistic effect on growth inhibition and cytotoxicity. Such an effect has been previously shown by combining genistein with cisplatin on human medulloblastoma cell lines assayed for growth inhibition *in vitro* [20]. In the present study, we show that genistein may also exhibit a significant synergistic effect on growth inhibition and cytotoxicity of malignant gliomas. The implication of this may be a reduction in the current standard chemotherapy dose recommendations and subsequently a decrease in the risk of their potentially devastating treatment sequelae.

Materials and methods

Two glioblastoma multiforme cell lines were used. The lines U87 (human) and C6 (rodent) were acquired from American Type Culture Collection (Manassas, VA).

Monolayer growth inhibition assay

Exponentially growing glioblastoma multiforme cells were seeded at a density of 1×10^5 in each well of a 6-well culture dish (Falcon, Lincoln Park, NJ) containing 3 ml complete growth media consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, four times the prescribed concentration of nonessential amino acids, 1% L-glutamine, 100 IU/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin (Life Technologies, Inc., Bethesda, MD). Tumor cells were cultured under standard tissue culture conditions (100% humidity, 95% air and 5% CO_2) for

24 h. All lines exhibited anchorage dependent growth. The growth media were then removed and replaced with 3 ml fresh complete media containing varying concentrations of BCNU in the absence or presence of genistein at 4 μM (the highest reported plasma level in adults). BCNU and genistein (Sigma, St. Louis, MO) concentrations in growth media were established from stock solutions made with ethyl alcohol and dimethyl sulfoxide (Sigma), respectively.

BCNU concentrations used for inhibition of cell line U87 monolayer growth were 0, 1, 2, 5 and 10 μM . Concentrations used for inhibition of cell line C6 monolayer growth were 0, 5, 10, 20 and 50 μM . For control cultures, drug vehicle ethyl alcohol and DMSO were used (0.02% solution in Dulbecco's phosphate-buffered saline, DPBS). The effect of 4 μM genistein alone on monolayer growth was studied separately in all lines. All wells were replenished with media containing drug or vehicle every other day for 4–5 days. Cells were then harvested by trypsinization and counted using a Coulter counter. Viable cell counts were performed using hemocytometer and trypan blue exclusion. Cell counts at each drug concentration were performed in triplicate and each experiment was repeated three times.

Cytotoxicity assay

As a measure of cytotoxicity, tumor cell colony formation and colonogenic survival in soft agarose was used as previously described [21,22]. Briefly, tumor cells were treated with BCNU in the presence or absence of genistein for 24 h. The cells were then seeded at a density of $1-2 \times 10^3$ in 3 ml of 0.5% agarose in serum-enriched complete media (supplemented with 20% fetal calf serum) in 60 mm culture dishes (Falcon, Lincoln Park, NJ) base-coated with 0.25% agarose in complete media. The selected drug concentrations for inhibition of colonogenic survival were based on maximum observed additive or synergistic effects from monolayer growth inhibition assay. These concentrations were 1, 2 and 5 μM for U87 cells and 5, 10 and 20 μM for C6 cells. The cultures were maintained under standard tissue culture conditions for at least 10 days. The number of colonies containing more than 50 cells in diameter was then counted at each drug concentration in each dish. Colonies were fixed using methyl alcohol and stained with methylene blue, and counted under an inverted microscope at a magnification of 40 \times . The percent inhibition of colony formation (as

the measure of inhibition of colonigenic survival) in drug-treated dishes was calculated based on the number of colonies (>50 cells in diameter) in control dishes [21,22].

DNA ladder assay

The DNA ladder assay for apoptosis was performed using the Apoptotic DNA Ladder Kit (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's specifications. Briefly, both tumor cell lines (U87 and C6) were treated with genistein alone (4 μ M), BCNU alone, or combinations of genistein with BCNU. Controls consisted of positive controls provided by the manufacturer treated with 300 μ M of H₂O₂ for 24 h, and glioblastoma multiforme cells that were treated with drug vehicle DMSO (0.02% solution in DPBS). Drug incubation interval was 48 h for all cell lines. Cells were then trypsinized, spun down and resuspended in 200 μ l of DPBS (Life Technologies Inc., Bethesda, MD). Subsequently, cells were lysed and the DNA was eluted and treated with 2 μ g/ml RNase A (Qiagen, Valencia, CA) for 20 min at room temperature. Drug-treated samples and controls were then electrophoresed on a 1% agarose gel at 2 V/cm until the loading buffer reached the end of the platform. The DNA was then visualized using a UV light source and photographed. Concentrations chosen for BCNU for this apoptosis assay were those that caused maximum additive or synergistic effects in the presence of genistein in monolayer and colonigenic assays (see above).

Laser scanning cytometry

Laser scanning cytometry (LSC) and detection of apoptotic cells were performed using a Compucyte Laser Scanning Cytometer with an Argon laser and Wincyte software (Cambridge, MA) as previously described [23]. As mentioned above, all tumor cell lines were treated with genistein alone (4 μ M), BCNU alone, or combinations of genistein with BCNU. Controls consisted of cells treated with 300 μ M of H₂O₂ for 24 h, and glioblastoma multiforme cells that were treated with drug vehicle DMSO (0.02% solution in DPBS) only. Drug incubation interval was 48 h for all cell lines. For this assay, cells were grown and treated in Lab-Tek 4-chamber slides (Nalge Nunc International, Naperville, IL). Following the incubation period the cells were fixed in 70% ethanol at

−20°C for 15 min and were subsequently incubated with a mixture of 0.1% Triton X-100 (Sigma), 20 μ g/ml of Propidium Iodide (Sigma, excitation and emission wave lengths of 488 and 620 nm respectively) and 100 μ g/ml RNase A (Life Technologies Inc.) in DPBS for 25 min at room temperature. The slides were then rinsed with DPBS, covered with mounting medium (50% glycerin in DPBS) and a glass cover slide (Nalge Nunc International), and stored at −20°C until further use. The Compucyte LSC was used to detect and count the cells with a fractional DNA content or 'sub-G₁ peak,' which would be typical for apoptosis [23].

Statistical methods

A regression model was designed to estimate cell or colony count based on concentrations of BCNU and the presence or absence of genistein. Regression analysis allowed for a simple way of calculating point estimates of percent inhibition as well as confidence intervals for these point estimates. The point estimate of percent inhibition for each level of BCNU is calculated as follows:

$$\frac{\{(\text{predicted cell count with no genistein}) - (\text{predicted cell count with genistein})\}}{\text{predicted cell count with no genistein}}$$

Data display marked heteroscedasticity (variance among the variances) (Figures 1–4). Log-transformations of both the cell count and colony count were required to stabilize the variance. Data for each experiment were then examined using a two-way ANOVA

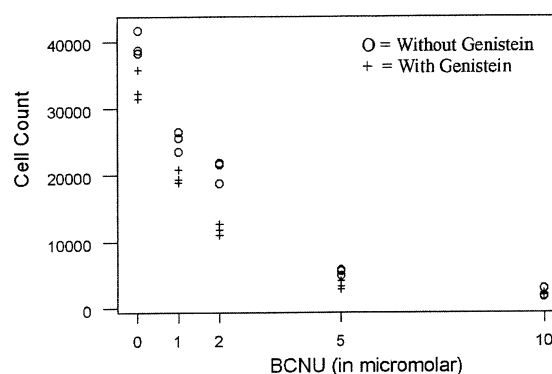


Figure 1. Plot of cell counts obtained with U87 cell line at each BCNU concentration, both in the presence and absence of genistein.

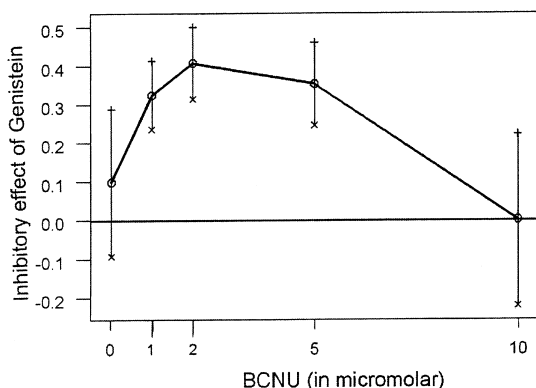


Figure 2. Point estimates and confidence intervals for the inhibitory effect of genistein on U87 monolayer cell growth at a range of BCNU levels (0–10 μM).

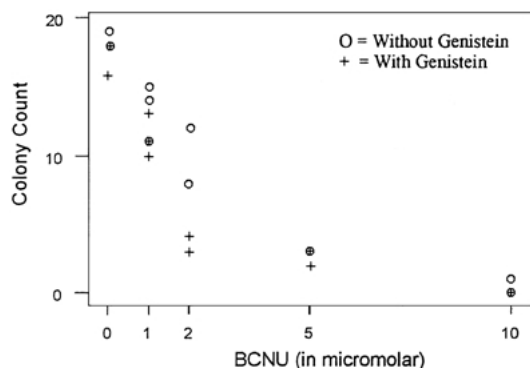


Figure 3. Plot of colony survival obtained with U87 cell line at each BCNU concentration, both in the presence and absence of genistein.

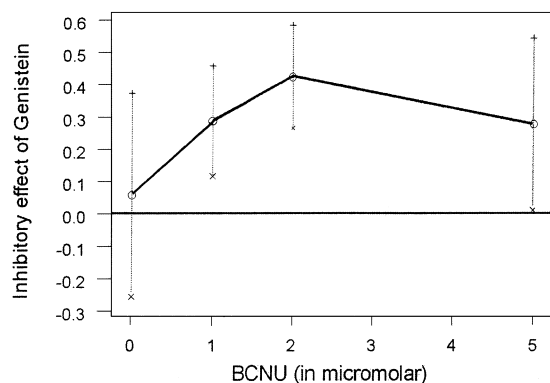


Figure 4. Point estimates and confidence intervals for the inhibitory effect of genistein on U87 colonogenic survival at a range of BCNU levels (0–10 μM).

model that allowed for non-linear interaction between BCNU and genistein by employing both a linear and a quadratic term. (Models were fit in Proc GLM, SAS Version 8.0, SAS Institute Inc., Cary, NC). The resulting models fit well, with an r^2 of approximately 0.98.

Results

Effect of genistein and BCNU on monolayer cell growth and cytotoxicity in U87 cell line

Figure 1 is a plot of cell counts at each of the indicated BCNU concentrations both in the presence and absence of genistein. (The six points represent the three repetitions with and without genistein.) Figure 2 illustrates the point estimates and confidence intervals for the inhibitory effect of genistein on U87 monolayer cell growth at the different BCNU levels. Only the confidence levels for BCNU at a concentration of 0 and 10 μM include the zero value, indicating that the effect of genistein at 1, 2 and 5 μM is to significantly increase the percent inhibition of monolayer cell growth ($P < 0.01$, 95% confidence interval). Genistein caused a 32%, 41% and 35% inhibition in monolayer growth at BCNU concentrations of 1, 2 and 5 μM respectively.

Figure 3 is a plot of colony survival at each of the indicated BCNU concentrations both in the presence and absence of genistein. Figure 4 illustrates the point estimates and confidence intervals for the inhibitory effect of genistein on U87 colony formation at the BCNU levels used. No colonies formed at a BCNU levels of 10 μM . Therefore, the percent inhibition at this level of BCNU is undefined and must be excluded from the subsequent analysis. Genistein caused a 29%, 42% and 28% inhibition in U87 colony formation at BCNU concentrations of 1, 2 and 5 μM respectively.

Effect of genistein and BCNU on monolayer cell growth and cytotoxicity in C6 cell line

Figure 5 is a plot of cell count at each of the indicated BCNU concentrations both in the presence and absence of genistein. Figure 6 illustrates the point estimates and confidence intervals for the inhibitory effect of genistein on C6 monolayer cell growth at the different BCNU levels. Only the confidence level for BCNU at a concentration of 50 μM includes the zero point, indicating that the effect of genistein at 0, 5, 10, 20 μM is to

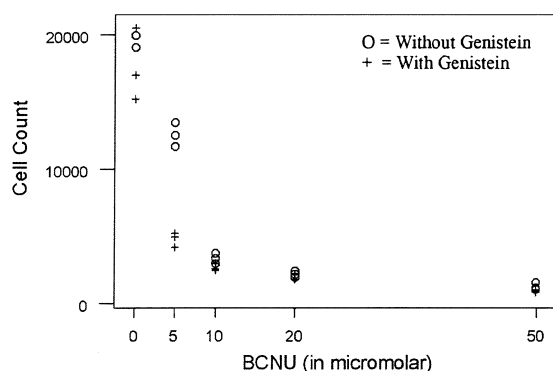


Figure 5. Plot of cell counts obtained with C6 cell line at each BCNU concentration, both in the presence and absence of genistein.

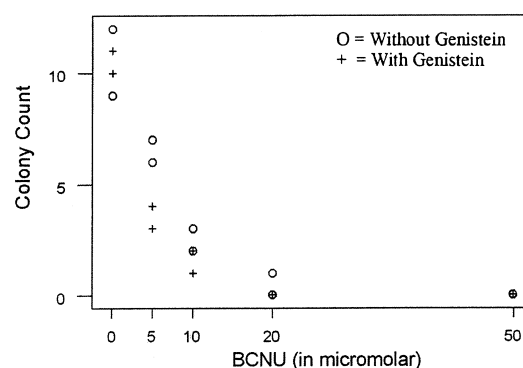


Figure 7. Plot of colony survival obtained with U87 cell line at each BCNU concentration, both in the presence and absence of genistein.

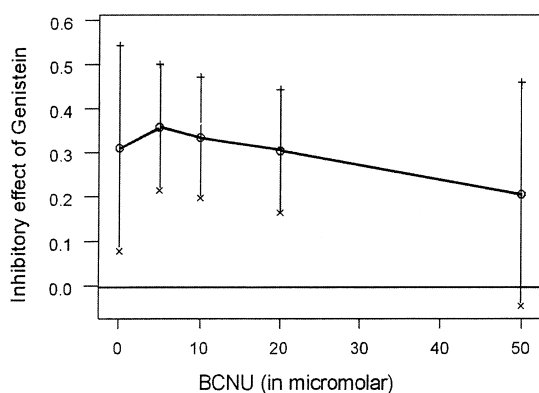


Figure 6. Point estimates and confidence intervals for the inhibitory effect of genistein on C6 monolayer cell growth at different BCNU levels (0–50 μ M).

significantly increase the percent inhibition of monolayer cell growth ($P < 0.01$, 95% confidence interval). Genistein caused a 31%, 36%, 33% and 30% inhibition in monolayer growth at BCNU concentrations of 0, 5, 10 and 20 μ M respectively.

Figure 7 is a plot of cell count at each of the indicated BCNU concentrations both in the presence and absence of genistein. Figure 8 illustrates the point estimates and confidence intervals for the inhibitory effect of genistein on C6 colony formation at the different BCNU levels. No colonies formed at a BCNU level of 20 and 50 μ M. Again, the percent inhibition by genistein at these levels of BCNU remained undefined because of the effect of BCNU and was therefore excluded from the analysis. Genistein caused a 39% and 54% inhibition in C6 colony formation at BCNU concentrations of 5 and 10 μ M respectively.

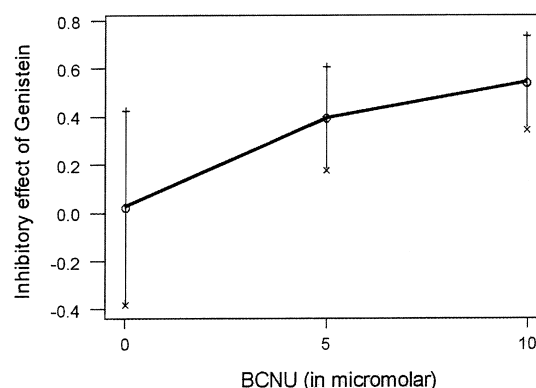


Figure 8. Point estimates and confidence intervals for the inhibitory effect of genistein on C6 colonogenic survival at different BCNU levels (0–50 μ M).

Effect of genistein and BCNU on induction of apoptosis

As assessed by a DNA ladder assay and LSC, no nuclear fragmentation typical of apoptotic cells in the U87 and C6 lines studied was detected. This was true using both methodologies in the presence or absence of genistein (4 μ M) at BCNU concentrations of 1, 2 and 5 μ M in U87 cells and 5 and 10 μ M in C6 cells where maximum additive and synergistic effects were observed.

Discussion

Soy-derived isoflavones have recently been the subject of considerable medical interest, since a number

of epidemiological studies have shown a lower incidence of some human cancers in Asian populations consuming a diet high in soy [9,24–27]. The primary isoflavone in a soybean-based diet and food proteins is genistein [2–4]. Genistein blocks RTKs involved in signal transduction and has been shown to inhibit topoisomerase II activity and angiogenesis [2,3,5–7,28]. In addition, genistein has been reported to induce differentiation, and to have weak estrogenic and antiestrogenic properties [29]. These actions are believed to underlie its anticarcinogenic effects [7]. The experimental *in vitro* and *in vivo* use of genistein has shown encouraging results in blocking growth, invasiveness and angiogenesis of a number of human tumors such as breast cancer, colorectal carcinoma, glioblastoma multiforme and leukemia [3,9–17]. However, the majority of these effects are observed at concentrations that are much higher than levels following consumption of soy-based diets or formula [3,7]. A series of human trials has shown isoflavone (genistein and daidzein) plasma levels of up to 4 μM following oral doses of 2 mg/kg body weight with concentrated soy milk formulations in adults [4,7,30]. Plasma isoflavone concentrations in 4-month-old infants with cow milk allergy who were fed soy-based formula have been reported to be between 3 and 6 μM [18,19]. Although such low plasma concentrations may not justify the use of dietary genistein in its current form as a sole chemotherapeutic agent, there emerging evidence that even at these plasma levels, a significant synergistic antiproliferative effect with standard chemotherapy for some tumors may exist [16,20,21,31–33].

In the current study, we explored this potential for a human glioblastoma multiforme cell line. We proposed that while genistein alone at typical physiologic dietary plasma levels may not have a significant effect on the growth of human glioblastoma multiforme cells, it may enhance antiproliferative effects of other chemotherapeutic agents. The implication, particularly for malignant gliomas, would be that one could use soy-based formula as a source of genistein to potentially reduce the recommended dose of chemotherapeutic agents such as BCNU. We examined the antiproliferative synergistic effect of genistein at 4 μM (the maximum reported plasma level in adults) with BCNU (0–50 μM) on two glioma cell lines. Our experiments showed a significant increased effect on monolayer cell growth inhibition and cytotoxicity when BCNU was supplemented with genistein.

We were able to detect a 32–41% increase in monolayer growth inhibition and a 28–42% increase

in colony cytotoxicity in the U87 cell line when genistein (4 μM) was added to BCNU in 0–50 μM dose range. When the C6 cell line was used, a 30–36% increase in monolayer growth inhibition and a 39–54% increase in colony cytotoxicity were observed in the 0–10 μM concentrations. All experiments showed a significant increase in growth inhibition and decrease in colonogenic survival (all $P < 0.05$).

Genistein alone (without BCNU) caused a 31% inhibition of C6 monolayer cell growth. This would seem to support the contention that genistein has growth inhibitory properties for tumor cells (see below). However, the percentage inhibition of genistein alone could be as low as 8% given the confidence level. In the other three groups, the percent inhibition for genistein alone was not significant (the confidence intervals included zero), suggesting that genistein alone may not have tumoricidal effects but does enhance the effects of BCNU when used in combination. We propose that further experiments examining the effect of genistein alone at different concentrations would be useful in elucidating this discrepancy.

The significance of these findings may be that low-dose genistein can enhance the therapeutic effect of BCNU and therefore possibly decrease the risk of myelosuppression and pulmonary toxicity that is the limiting factor with current therapeutic dose recommendation and peak plasma levels [1].

Relatively little is known about the effect of isoflavones such as genistein on the growth of primary brain tumors. Recent studies on neuroblastoma and pediatric kidney tumors have shown that genistein induces apoptosis and can inhibit proliferation and colonogenic growth *in vitro*. The reported IC_{50} values for these actions range from 5 to 25 μM [34,35]. RTK inhibitors such as genistein can also induce neural differentiation in medulloblastoma cells [36]. In the current study, we were not able to detect apoptosis by DNA ladder assay and LSC detection of sub- G_1 peak in any of the glioblastoma cell lines treated with combinations of genistein and BCNU at the tested concentrations. Therefore, apoptosis does not appear to be an underlying mechanism for the effects seen in this study.

Combinations of genistein in the 0–10 μM dose range in combination with tamoxifen or adriamycin have been reported to exhibit marked synergistic antiproliferative effect in human breast cancer lines [21,33]. At higher genistein doses, a synergism with tiazofurin in leukemic and ovarian cancer cells has been shown [31,32]. We have previously shown by similar methods that genistein at typical dietary plasma levels

can significantly enhance the antiproliferative and cytotoxic action of cisplatin and vincristine on human medulloblastoma cell lines [20]. The current study further supports the possibility of administering genistein as an adjuvant therapy for malignant gliomas. The use of commercially available soy-based formulas is particularly attractive since genistein accounts for 65% of the isoflavones in the formula [18,19]. Soy-based formulas have been widely used to feed infants with cow milk allergy since early 1920s without short- or long-term sequelae [37,38]. Although a number of studies have shown that soy-based diets are well tolerated and have no effects on weight gain and development, there is a paucity of data on adverse effects of even low-dose dietary genistein alone or in combination with other chemotherapeutic agents, which necessitates caution and further research [18,39,40]. In addition, the pharmacokinetics and pharmacodynamics of genistein in normal and neoplastic brain tissue as well as its penetration of blood–brain barrier is largely unknown. Still, the prospect of using a safe dietary supplement such as soy to reduce the dose of chemotherapy and its adverse effects is exciting and deserves further investigation.

Acknowledgements

This work was supported by The Andy Fund, an organization dedicated to brain tumor research at the University of Vermont, in memory of Andrea Nelson.

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