Rapid inhibition of ongoing DNA synthesis in human glioma tissue by genistein

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Abstract. The effect of genistein, a protein tyrosine kinase and topoisomerase II inhibitor, on the DNA synthesis rate was studied in 21 human glioma specimens obtained at routine craniotomies for tumor resection. Ongoing DNA synthesis rate was determined by using a method based on the generation of tissue mini-units immediately after tumor resection and short incubation time (0-120 min) with [methyl-^{3}H]-thymidine. A 9-77% inhibition of DNA synthesis rate by 100 μM genistein was observed in 18/21 of the glioma specimens. In these cases, the average percentage of inhibition was 55±20% (mean ± SD, P<0.0001, Student's t-test) and the inhibitory effect was >50% in 12/18 of the cases. In 3 cases genistein increased the DNA synthesis rate. The inhibitory effect of genistein had a short-time onset and was concentration-dependent. Additional experiments in 4 cases showed that herbimycin A had no effect on DNA synthesis rate while etoposide inhibited similarly to that of genistein. Our results suggest that the effect of genistein on DNA synthesis rate in gliomas is independent of protein kinase inhibition and probably mediated by topoisomerase II inhibition. In the RG2 model, 50 μM genistein inhibited ongoing DNA synthesis in glioma cells with little or no effect in normal tissue. The data also encourage further investigations on the therapeutic potential of genistein for gliomas.

Introduction

The uncontrolled growth of gliomas may result in part from excessive activation of protein tyrosine kinases. Consistent with this hypothesis, protein tyrosine kinase inhibitors reduce the proliferation of human glioma cell lines with IC_{50} proliferation values approximating those for inhibition of tyrosine kinase activity in cell free protein extracts (1) and block infiltration of glioblastoma spheroid cells into normal tissue *in vitro* by a mechanism involving inhibition of epidermal growth factor receptor (EGF-R) associated tyrosine kinase (2). The c-erbB gene encoding EGF-R is amplified and over-expressed in up to 50% of malignant gliomas (3). Tyrosine kinase activation of the EGF-R leads to phosphorylation of intracellular proteins, thus initiating an intracellular cascade that leads to cell division.

The isoflavone genistein is found in high amount in soy and has been shown to inhibit the growth of several normal and neoplastic cell lines (4) including human glioma cell lines (1,5). The inhibitory effect on cell proliferation (DNA synthesis) rate is generally attributed to inhibition of tyrosine kinases, especially the EGF-R associated tyrosine kinase activity, topoisomerase II (6) and histidine kinase activity (7) as well as other effects (8). Compared to etoposide (another topoisomerase II poison), the cytotoxic and genotoxic effects of genistein differ significantly (9). Genistein is a small molecule (M.W. 270.2 Da). When injected intraperitoneally to rats, it rapidly appears in brain tissue and in microdialysate fluid from the corpus striatum indicating that genistein is capable of crossing the blood-brain barrier (10).

These data suggest that genistein and other protein tyrosine kinase inhibitors might have a therapeutic potential for tumors expressing high levels of tyrosine kinase activities. In a previous study, we found a strong inhibitory effect of genistein on ongoing DNA synthesis rate in the developing rat cerebral cortex and a much weaker inhibitory effect in adult rat cerebral cortex (11). Although ongoing DNA synthesis is one of the less studied targets for antineoplastic therapy, it might constitute a useful target to treat highly proliferative cancers, specially those arising in low proliferating tissues. In theory, the main advantage of ongoing DNA synthesis inhibitors is their ability to inhibit DNA replication within short period of time. Thus, higher concentrations of drugs might be able to be delivered locally into fast growing tumors with tolerable toxicity to the surrounding normal low proliferative normal tissue.

We studied the effect of relatively high concentrations of genistein on ongoing DNA synthesis rate in mini-units prepared from human gliomas as well as in mini-units prepared from the rat RG2 glioma model.

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Table I. Clinical data of the 25 patients included in the study as well as protein content per microwell, spontaneous DNA synthesis rate and effect of 100 μM genistein.

Patient no. age/gender	Histopathology[a]	Previous treatment[a]	Spontaneous DNA synthesis rate (cpm/mg of protein)[d]	Change (%) of DNA synthesis rate by 100 μM genistein[d,f]
1 40/M	GB	None	140 (4)	ND[e]
2 55/F	ODG	None	153 (3)	ND[e]
3 65/M	GB	None	104 (2)	ND[e]
4 65/M	GB	Surgery + Rx + CCNU[c]	742 (4)	ND[e]
5 65/F	GB	None	204 (3)	-74 (3)
6 57/F	GB	None	176 (3)	-74 (3)
7 63/M	GB	None	776 (2)	-68 (3)
8 58/M	AA	None	196 (2)	-66 (2)
9 44/F	AA	Rx+ CCNU; CCNU[c]	15 (1)	-57 (1)
10 53/M	AA	Surgery + Rx + CCNU	55 (2)	-44 (1)
11 45/M	GB	Surgery + Rx + CCNU[c]	137 (2)	-59 (2)
12 63/M	GB	None	22 (2)	-54 (2)
13 64/M	GB[b]	None	5 (2)	+64 (2)
14 66/F	A ODG	None	79 (2)	-34 (3)
15 43/M	GB	None	124 (4)	-73 (4)
16 48/M	A ODG	Surgery + CCNU[c]	284 (3)	-44 (3)
17 52/F	GB	Surgery + Rx + CCNU	234 (3)	-70 (4)
18 23/F	PA	None	21 (3)	-27 (2)
19 35/M	AA	None	57 (4)	-77 (4)
20 42/M	OA	None	10 (4)	-25 (4)
21 40/F	GB	Surgery + Rx + CCNU[c]	9 (2)	-64 (2)
22 31/M	GB[d]	Rx[c]	10 (4)	+18 (3)
23 55/M	GB	None	221 (4)	-64 (4)
24 65/F	GB	None	3 (4)	+79 (4)
25 62/F	GB	None	5 (3)	-9 (4)

[a]GB, glioblastoma; ODG, oligodendroglioma; AA, anaplastic astrocytoma; A ODG, anaplastic oligodendroglioma; PA, pilocytic astrocytoma; Rx, radiotherapy; CCNU, lomustine. [b]Extensive necrosis areas. [c]Ongoing. [d]The values in brackets indicate the number of determinations. [e]ND, not determined (specimens used only for studying the temporal incorporation of the radioactive precursor); [f]-, inhibition; +, stimulation.

Materials and methods

All studies were approved by the Ethics Committee at Huddinge University Hospital. Tumoral tissue specimens were obtained at routine craniotomies for tumor resection. Genistein (Costa Mesa, CA, USA) was prepared as stock solution (100 mM) in dimethylsufoxid (DMSO, Sigma, Sweden) and stored at -20°C (12). The final dilutions (10-100 μM) were done in Dulbecco's modified Eagle's medium with glutamine and 4,500 mg/l D-glucose (DMEM; Gibco/Life Technologies, Sweden) keeping DMSO concentration below 0.1% (v/v). DMSO alone at the same concentration was used as control.

Generation of tissue mini-units, determination of [methyl-³H]-thymidine incorporation into DNA and ongoing DNA synthesis rate, protein quantitation and determination of effect of genistein were performed as previously reported (11,13). Briefly, mini-units of glioma tissue were generated immediately after tumor resection. These mini-units were then incubated in microwell plates (Nunc, Denmark) with DMEM containing 2 μCi/ml [methyl-³H]-thymidine (Amershan, UK) and genistein or DMSO. Each experimental point was in all but 2 cases determined by at least duplicate. The DNA synthesis rate was calculated as cpm/mg of protein/min. The effect of genistein was determined as change (%) of DNA synthesis rate compared to the corresponding control (Table I) or percentage of control DNA synthesis rate (Fig. 2-4).

Glioma model. The RG2 rat glioma model was performed as previously described (14).

Results

Effect of genistein on ongoing DNA synthesis in tissue mini-units prepared from human gliomas. As shown in Table I,
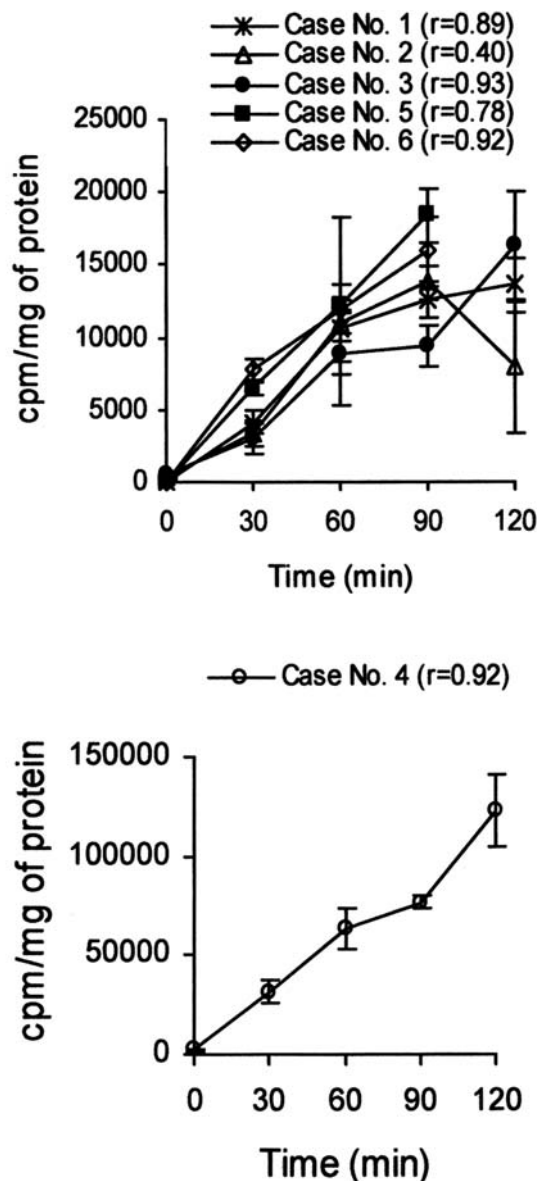
Figure 1. Temporal incorporation of [methyl-³H]-thymidine into DNA in mini-units from 6 different tumor specimens. Each point is the mean ± SEM of triplicates except for 0, 30 and 60 min of case no. 5 and 6 (duplicates). r = correlation coefficient.
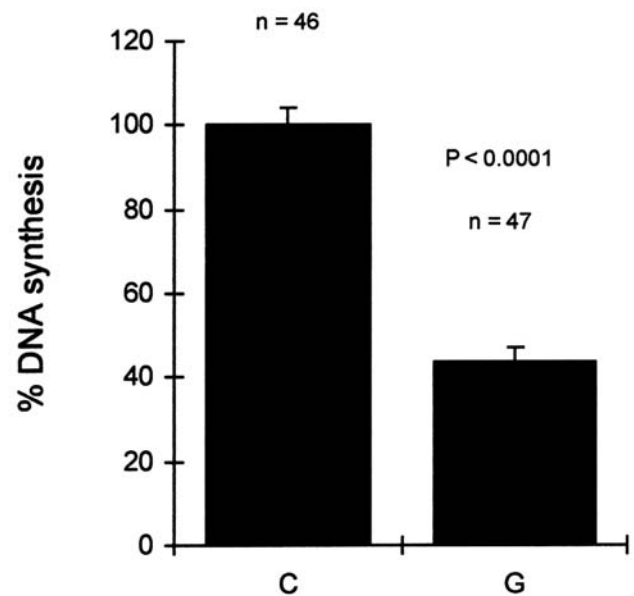

Figure 2. Effect of 100 μM genistein (G) on ongoing DNA synthesis in mini-units prepared from human gliomas. DMSO at equivalent concentration was used as control (C). Data are the mean ± SE of 46/47 (n) determinations from mini-units prepared from cases 5-16 (see Table I for details).

tissue specimens were obtained from 15 males and 10 females with an age range of 23-66 years. The histopathological diagnoses were: glioblastoma (16 cases), anaplastic astrocytoma (4 cases), anaplastic oligodendroglioma (2 cases) and one case each of oligodendroglioma, oligoastrocytoma, and pilocytic astrocytoma. The interval between symptomatic onset and present surgery ranged from <1 month to >5 years. Previous or ongoing treatment was present in 8 patients.

Tissue mini-units generated from 6 different tumor specimens were incubated in DMEM containing 2 μCi/ml [methyl-³H]-thymidine during 0, 30, 60, 90 and 120 min (cases no. 1-4) and during 0, 30, 60 and 90 min (case no. 5 and 6). A quite linear incorporation of the radioactive precursor into DNA over time was observed up to 90 min despite the large intertumoral differences in the net rate of [methyl-³H]-thymidine incorporation (Fig. 1).

Tissue mini-units generated from tumor specimens were incubated in DMEM containing 2 μCi/ml [methyl-³H]-thymidine plus DMSO alone (cases no. 1-25, controls/spontaneous DNA synthesis rate) or 100 μM genistein (cases no. 5-25) for 90 min. This concentration of genistein was chosen since it has been shown to inhibit cell proliferation (DNA synthesis) rate in different tissues (1,11,12). The standard incubation time of 90 min was chosen since this time gives a suitable difference in radioactive precursor incorporation between control and experimental samples. Furthermore, this short incubation time ensures minimal metabolic changes and allows monitoring of ongoing DNA synthesis rate (11,13,15). A high (around 250-fold) interspecimen variation of spontaneous DNA synthesis rate was observed (Table I). Genistein at 100 μM decreased the DNA synthesis rate in 18/21 of the specimens. The percentage of inhibition was 9-77% and was >50% in 12/18 of these cases. In the remaining 3 cases (13, 22 and 24) genistein increased the DNA synthesis rate. In the 18 cases where genistein decreased the DNA synthesis rate, the average percentage of inhibition was 55±20 % (mean ± SD, P<0.0001, Student's t-test) (Fig. 2). The inhibitory effect of genistein was >50% in 8/9 cases with spontaneous DNA synthesis rates >100 cpm/mg of protein/min while this was found for only 4/12 cases with spontaneous DNA synthesis rates <100 cpm/mg of protein/min (Table I). However, the quite heterogeneous and limited material does not allow any conclusion regarding possible relations between DNA synthesis rate and effect of genistein.

The concentration-dependent and temporal effects of genistein could, due to the limited amount of tumor tissue, only be studied in a few cases. Tissue mini-units generated from one tumor specimen (case no. 19) were also incubated with DMEM containing 2 μCi/ml [methyl-³H]-thymidine plus DMSO alone or 10, 50 and 100 μM genistein for 90 min. The results showed that genistein had a concentration-dependent
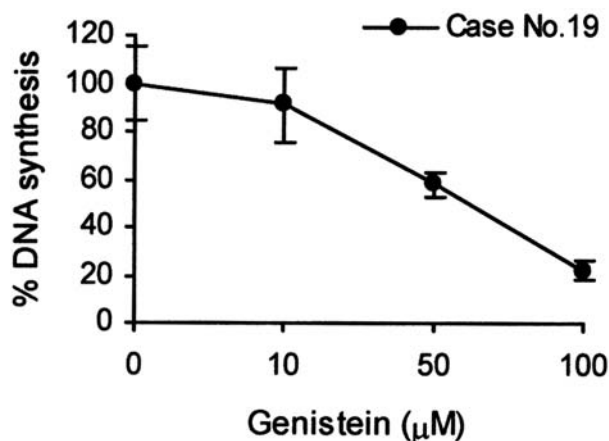
Figure 3. Concentration-dependent effect of genistein on DNA synthesis rate in mini-units prepared from one tumor specimen. Data are the mean ± SEM of one experiment performed in quadruplicate. No significant inhibition was detected in control mini-units treated with equivalent concentrations of vehicle [0.1 % (v/v) DMSO].
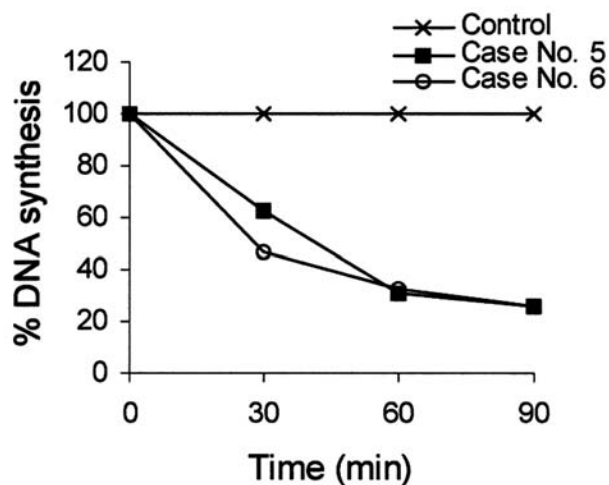

Figure 4. Temporal effect of 100 μM genistein on DNA synthesis rate in mini-units prepared from 2 different tumor specimens. Each point is the mean ± SEM of duplicates (0, 30 and 60 min) or triplicates (90 min). Control mini-units were treated with 0.1 % (v/v) DMSO.

effect on DNA synthesis rate (Fig. 3). Tissue mini-units generated from 2 tumor specimens (case no. 5 and 6) were incubated with DMEM containing 2 μCi/ml [methyl-^{3}H]-thymidine plus DMSO alone (controls) or 100 μM genistein for 0, 30, 60 and 90 min. The results showed that genistein decreased the DNA synthesis rate within 30 min (Fig. 4). Finally, in 4 cases (no. 10, 19, 21 and 23), the experiments included exposure to 10 μM herbimycin A [a potent tyrosine kinase inhibitor (16)] and/or 250 μM etoposide [a selective inhibitor of topoisomerase II (6)]. Herbimycin A had no effect on DNA synthesis rate while etoposide inhibited the DNA synthesis rate to a similar extent as genistein (data not shown).

Effect of genistein on ongoing DNA synthesis in tissue mini-units prepared from the RG2 rat glioma model. The tissue mini-units were prepared from tumor specimens obtained from the rat RG2 glioma model and incubated for 90 min in DMEM
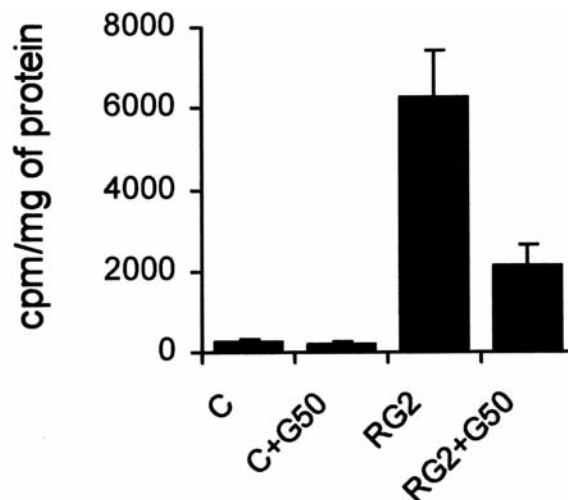

Figure 5. Effect of genistein on ongoing DNA synthesis in tissue mini-units prepared from the RG2 rat glioma model. The mini-units prepared from normal tissue (C) or from tumoral tissue (RG2) were incubated with DMEM plus 2 μCi/ml ^{3}H-methyl-tymidine alone (C, RG2) or DMEM + 2 μCi/ml [methyl-^{3}H]-thymidine + 50 (C+G50, RG2+G50) μM genistein for 90 min. Data are the mean ± SD of one (of two) representative experiment performed by quadruplicate.

containing 2 μCi/ml [methyl-^{3}H]-thymidine + 0 or 50 μM genistein. As control, tissue mini-units obtained from normal cerebral cortex of the opposite hemisphere of the same animal was used. Fig. 5 shows the percentage of DNA synthesis inhibition induced by 50 μM genistein in glioma cells as well as in adult rat cerebral cortex. The result show that the incorporation of [methyl-^{3}H]-thymidine in glioma cells is very high when compared to normal rat cerebral cortex and that 50 μM genistein produced a strong inhibitory effect in glioma cells with no significant effect on normal tissue probably due to the low proliferative acivity of the adult normal brain tissue.

Discussion

To the best of our knowledge, this is the first report on the effect of genistein on ongoing DNA synthesis rate in tissue specimen from human gliomas and from the rat RG2 glioma model. More than 50% inhibition of DNA synthesis rate by 100 μM genistein was observed in 12/21 of the tumor specimens (Table I). The inhibitory effect was shown to be concentration-dependent (Fig. 3) and of short-time onset (Fig. 4) as previously reported for normal rat cerebral cortex (11). Genistein increased the DNA synthesis rate in 3 cases. Although this enhancement is in contrast with the results for the major part of tumor specimens and the existing literature, an enhancement of experimental colon cancer by dietary genistein was reported by Rao *et al* (17). The authors emphasize that the biological effects of genistein may be organ specific and inhibit cancer development in some sites while showing no effect or an enhancing effect on the tumorigenesis at other sites. Our results suggest that the effect of genistein may also show interindividual variation even for tumors of the same histopathological type.

The tumors included in this study showed a considerable intertumoral variation in the spontaneous DNA synthesis rate

(Table I). This variation is unlikely to be due to a lack of reproducibility of the mini-unit method since i) the temporal course for [methyl-^{3}H]-thymidine incorporation into DNA was found to be linear up to 90 min despite a strong variation in the net radioactive incorporation into DNA (Fig. 1), ii) in a previous study, a similar intertumoral variation of spontaneous DNA synthesis rate has been found while the effect of another drug (roscovitine) on ongoing DNA synthesis was highly reproducible in mini-units prepared from human gliomas (13) and human cervical cancer (18), and iii) a high reproducibility of DNA synthesis rate determinations has been observed for mini-units prepared from normal rat cerebral cortex (11,15). In addition, other methods for measuring cell proliferation rate with preservation of the *in situ* metabolism and topology show a discrepancy between cell proliferation rate and cellular histopathology, e.g., the great variation in PCNA labeling index in glioblastomas (19) and the equivalent Ki-67 reactivity in tumors with significant malignancy grade differences (20).

Genistein could decrease the DNA synthesis rate of human glioma cells by i) a short-time onset mechanism of action as reported here, and ii) a long-time onset mechanism of action probably due to inhibition of the EGF-R associated tyrosine kinase activity and/or inhibiton of tyrosine phosphorylation of proteins important for signal transduction (8,12). We suggest that the early inhibition of glioma DNA synthesis rate by genistein observed in this study is independent of protein phosphorylation and probably due to inhibition of topoisomerase II activity: i) The short-time onset (within 30 min) of the inhibitory effect on DNA synthesis observed in this study was also found for normal rat cerebral cortex where the effect was found to be mediated by topoisomerase II inhibition (11,15). ii) More important, herbimycin A, a potent tyrosine kinase inhibitor, had no effect on DNA synthesis rate while the selective topoisomerase II inhibitor etoposide, inhibited similarly to that of genistein. iii) Ongoing DNA replication in normal rat cerebral cortex is relatively independent of protein kinase inhibition (15). iv) If the genistein effect was mediated through inhibition of protein phosphorylation related to mitogenic signalling pathways, a considerable lag would be expected until the fall in DNA synthesis rate (15). Consistent with this assumption, the inhibitory effect of genistein on ongoing DNA synthesis in mini-units prepared from rat cerebral cortex was not reversed by sodium orthovanadate, a potent inhibitor of phosphotyrosine phosphatases (15). Recent literature data support a topoisomerase II dependent mechanism in the arrest and induction of apoptosis in 4 different gliomas cell lines (21) at a concentration range used in our study (EC$_{50}$ concentrations of 20-80 μM).

It has been suggested that genistein or other isoflavones are related to the lower incidence of breast and prostate cancer in Asians with a high intake of soy products (22). Furthermore, it has been shown that genistein at 75-150 mg/kg/day reduces the formation of premalignant conditions in the colon of rats exposed to azoxymethane (23).

Although genistein at high doses may be toxic for rapidly proliferating normal neural cells (newborn rats), and adverse effect have been reported when genistein was administered during neonatal treatments (24) the toxicity seems to be less in cells derived from slowly proliferating tissue such as adult rat cerebral cortex (11). Genistein can be given *in vivo* with tolerable toxicity (12). Moreover, recent data indicate that genistein at pharmacological doses does not give any reproductive or developmental alterations even after chronic treatments (25-27). Asians consuming a traditional diet with high soy product content may show high plasma concentrations (up to 2.4 μM) of genistein (28). This concentration is close to the IC$_{50}$ (2.7 μM) for the autophosphorylation of the EGF-R associated tyrosine kinase and the IC$_{50}$ (5 μM) for topoisomerase II (6). In male Wistar rats, the plasma level of total genistein after an oral dose of 2,000 mg/kg was maintained at levels of ~40 μM for around 9 h. Even at these high levels no genotoxic effect was observed using different mutagenic and clastogenic tests (29). In a human prostate cell line genistein inhibits cell proliferation only at high concentration (50-100 μM) (30). New delivery methods of drugs such as intracerebroventricular injection (31,32), might allow the application of high concentration of genistein into the brain while preserving potential systemic toxicity in other high proliferating tissue. Considering the fact that the median survival time is 9-14 months in patients with high malignancy-grade gliomas (33), our results encourage further studies in order to investigate the chemotherapeutic and chemopreventive potential of genistein for these tumors.

Acknowledgements

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