Cancer Letters 302 (2011) 100–108

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Green tea epigallocatechin gallate enhances therapeutic efficacy of temozolomide in orthotopic mouse glioblastoma models

Thomas C. Chen [a,*], Weijun Wang [a], Encouse B. Golden [b,1], Simmy Thomas [c], Walavan Sivakumar [a,2], Florence M. Hofman [b], Stan G. Louie [d], Axel H. Schönthal [c]

[a] Department of Neurosurgery, Keck School of Medicine, University of Southern California (USC), Los Angeles, CA, USA
[b] Department of Pathology, Keck School of Medicine, USC, Los Angeles, CA, USA
[c] Department of Molecular Microbiology & Immunology, Keck School of Medicine, USC, Los Angeles, CA, USA
[d] Department of Clinical Pharmacy and Pharmaceutical Economics and Policy, School of Pharmacy, USC, Los Angeles, CA, USA

ARTICLE INFO

Article history:
Received 29 September 2010
Received in revised form 13 November 2010
Accepted 17 November 2010

Keywords:
Green tea
EGCG
GRP78/BiP
CHOP/GADD153
Glioblastoma

ABSTRACT

The alkylating agent temozolomide, in combination with surgery and radiation, is the current standard of care for patients with glioblastoma. However, despite this extensive therapeutic effort, the inclusion of temozolomide extends survival only by a few short months. Among the factors contributing to chemoresistance is elevated expression of the endoplasmic reticulum (ER) chaperone GRP78 (glucose-regulated protein 78; BiP), a key pro-survival component of the ER stress response system. Because the green tea component EGCG (epigallocatechin 3-gallate) had been shown to inhibit GRP78 function, we investigated whether this polyphenolic agent would be able to increase the therapeutic efficacy of temozolomide in preclinical models of glioblastoma. Mice with intracranially implanted human U87 (p53 wild type) or U251 (p53 mutant) glioblastoma cells were treated with temozolomide and EGCG, alone and in combination. We found that EGCG alone did not provide survival benefit, but significantly improved the existing therapeutic effect of temozolomide, i.e., life extension was substantially greater under combination therapy as compared to temozolomide therapy alone. Immunohistochemical analysis of tumor tissue revealed increased expression levels of GRP78 in temozolomide-treated animals, which was diminished when temozolomide was combined with EGCG. Parallel in vitro experiments with siRNA targeting GRP78 or its major pro-apoptotic antagonist CHOP (CCAAT/enhancer binding protein homologous protein/GADD153) further established a critical role of the ER stress response system, where si-GRP78 sensitized cells to treatment with temozolomide, and si-CHOP provided protection from drug-induced toxicity. Thus, ER stress-regulatory components affect the chemotherapeutic response of glioblastoma cells to treatment with temozolomide, and inclusion of EGCG is able to increase the therapeutic efficacy of this DNA-damaging agent.

1. Introduction

Despite some improvements in the treatment of patients with malignant gliomas, the overall prognosis has remained frustratingly poor. Temozolomide (TMZ; Temodar®), an alkylating agent, is indicated for the treatment of adult patients with newly diagnosed glioblastoma multiforme concomitantly with radiotherapy and then as

Abbreviations: EGCG, epigallocatechin 3-gallate; TMZ, temozolomide; GRP78, glucose-regulated protein 78/BiP; CHOP, CCAAT/enhancer binding protein homologous protein/GADD153.

* Corresponding author. Address: Department of Neurosurgery, University of Southern California, 1200 N. State St., Suite 5046, Los Angeles, CA 90089, USA. Tel.: +1 323 226 7421; fax: +1 323 226 7833.
E-mail address: tcchen@usc.edu (T.C. Chen).
[1] Present address: NYU Clinical Cancer Center, New York, NY, USA.
[2] Present address: University of Utah, Salt Lake City, UT, USA.

0304-3835/$ - see front matter © 2010 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.canlet.2010.11.008

maintenance treatment. A randomized, multicenter, phase 3 trial with 573 patients presenting with glioblastoma had established a median survival benefit of 2.5 months when TMZ plus radiation (median survival: 14.6 months) was compared to radiotherapy alone (median survival: 12.1 months) [1]. However, despite this favorable improvement in treatment outcome, it is obvious that TMZ provides only for an overall small extension of survival, and therefore more efficient treatment modalities are urgently needed.

We have been studying approaches to increase the sensitivity of tumor cells towards chemotherapeutic treatments. In this context, we have recently reported [2] that treatment of malignant glioma cell lines with TMZ in vitro triggered the endoplasmic reticulum (ER) stress response, as indicated by elevated expression of the ER stress markers GRP78 (glucose-regulated protein 78; also called BiP) and CHOP (CCAAT/enhancer binding protein homologous transcription factor; also called GADD153). In general, ER stress is a cellular stress-response mode that pursues one of two objectives: (1) In case of moderate stress, such as hypoxia or hypoglycemia or accumulating misfolded proteins, the defensive module of this system serves to adapt the cell to the insult and restores proper homeostasis, with chaperone protein GRP78 playing a major protective role during these processes. One of the consequences of increased GRP78 activity is the suppression of caspase-mediated cell death pathways, which as a consequence leads to increased chemoresistance [3]. (2) On the other hand, if stress is too severe or extensively prolonged, the ER stress system switches to its pro-apoptotic function and initiates apoptosis. Here, elevated levels of the transcription factor CHOP play a key role by altering the transcriptional profile of cells to trigger a pro-apoptotic cell death program [4]. Altogether, the increased activity of GRP78 on one side, and the duration of elevated CHOP levels on the other, decisively contribute to the phenotypic outcome, i.e., they determine the balance between subsequent cell survival and cell death [5–7].

The major polyphenolic green tea component epigallocatechin 3-gallate (EGCG) is being intensively investigated for its potential chemopreventive and chemotherapeutic activity. Among its many biological effects [8,9] is the ability to bind to GRP78 and inactivate its anti-apoptotic function [10]. This interaction is thought to increase the chemosensitivity of tumor cells, and evidence in support of this view has been published. For example, EGCG has been shown to sensitize different types of tumor cells to a variety of pro-apoptotic agents, such as 5-fluorouracil, taxol, vinblastine, gemcitabine, or tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) in vitro [11–15] and to doxorubicin, paclitaxel, or interferon in mouse tumor models in vivo [13,16,17]. However, because of EGCG's recognized multifaceted biological activity profile, it has been difficult to determine whether inhibition of GRP78—or the ER stress response in general—contributed to these EGCG-enhanced cancer therapeutic outcomes, as several other known activities of this green tea component may have supported chemosensitization as well [8]. Moreover, it is unknown whether EGCG might be able to achieve chemosensitization across the blood–brain-barrier, as would be required for the treatment of malignant gliomas.

In the present study, we demonstrate that EGCG is able to enhance therapeutic efficacy of TMZ in orthotopic mouse models of malignant gliomas. In addition, the knockdown of GRP78 and CHOP levels in cell lines in vitro reveals that these ER stress components play an important role in mediating the antitumor effects of this combination treatment.

2. Materials and methods

2.1. Materials

Temozolomide (TMZ) was obtained from the pharmacy at the University of Southern California (USC) and dissolved in phosphate-buffered saline (PBS) to a concentration of 20 mg/mL. Immediately before use, this stock solution was diluted with 0.9% sodium chloride to 2 mg/mL. EGCG was purchased from Sigma–Aldrich (St. Louis, MO) and was dissolved in ddH_2O to 10 mg/mL immediately before use. We also used TEAVIGO-EGCG, which are capsules with caffeine-free, highly concentrated green tea extract ($\geqslant$94% EGCG) that are widely available at health food stores [18], the capsule contents were dissolved in ultra-pure water and used fresh (or stored at -80 °C).

2.2. Cell lines

The human glioblastoma cell lines U87, U251, and LN229 were propagated in DMEM (provided by the Cell Culture Core Lab of the USC/Norris Comprehensive Cancer Center and prepared with raw materials from Cellgro/MediaTech, Manassas, VA) supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, 100 U/mL penicillin, and 0.1 mg/mL streptomycin in a humidified incubator at 37 °C and a 5% CO_2 atmosphere.

2.3. MTT assay

Cells were seeded into 96-well plates in a volume of 50 μL per well at 2.0–5.0 $\times$ 10^5 cells/mL. The next day, an additional 50 μL of medium containing various concentrations of drug was added and the cells were incubated for 48 h. This was followed by the addition of 10 μL thiazolyl blue tetrazolium bromide (=methylthiazoletetrazolium, MTT; Sigma–Aldrich, St. Louis, MO) for 4 h (stock solution of MTT is 5 mg/mL in PBS). The reaction was stopped and the cell cultures lysed by the addition of 100 μL solubilization solution (10% sodium dodecyl sulfate, SDS, in 0.01 M hydrochloric acid, HCl). The 96-well plate was left in the cell culture incubator over night for complete solubilization of the MTT crystals, and the optical density (OD) of each well was determined in an ELISA reader at 560 nm. The background value (=OD of control well containing medium without cells + MTT + solubilization solution) was subtracted from all measured values. In individual experiments, each treatment condition was set up in quadruplicate, and each experiment was repeated several times independently.

2.4. Colony-formation assay

Two hundred cells were seeded into each well of a 6-well plate. After cells had fully attached to the surface of the culture plate, they were exposed to drug treatment for 48 h. Thereafter, the drugs were removed, fresh growth medium was added, and the cells were kept in culture undisturbed for 12–14 days, during which time the surviving cells spawned a colony of proliferating cells. Colonies were visualized by staining for 4 h with 1% methylene blue (in methanol), and then were counted.

2.5. Transfections

The different siRNAs were synthesized at the microchemical core laboratory of the USC/Norris Comprehensive Cancer Center. Cells were transfected in 6-well plates with the use of Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. To verify siRNA efficacy, cells were harvested for Western blot analysis 48 h after transfection; in parallel, cells were seeded for colony-formation assays.

2.6. Immunoblots and immunohistochemical staining

Total cell lysates were analyzed by Western blot analysis as described earlier [19]. Immunohistochemical analysis of protein expression in tumor tissues was performed with the use of the Vectastain avidin–biotin complex method kit (Vector Laboratories, Burlingame, CA) according to manufacturer's instructions. This procedure used biotinylated secondary antibodies and a preformed avidin/biotinylated enzyme complex. The primary antibodies were purchased from Cell Signaling Technology (Beverly, MA) or Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and used according to the manufacturers' recommendations. All immunoblots and immunohistochemical staining were repeated at least once to confirm the results.

2.7. TUNEL staining

Apoptosis in cultured glioma cells and tumor sections was measured and quantified with the use of the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay by using the ApopTag in Situ Apoptosis Detection kit (Chemicon, Temecula, CA) according to the manufacturer's instructions. The percentage of TUNEL-positive cells was determined from 10 random photomicrographs taken at 200× magnification.

2.8. Intracranial mouse model

All animal protocols were approved by the IACUC of USC, and all rules and regulations were followed during experimentation on animals. Athymic mice (Harlan, Inc., Indianapolis, IN) were anesthetized and fixed into a stereotactic head frame. A paramedian incision was made and a 1.5-mm burr hole was drilled 1 mm anterior to the coronal suture on the right hemisphere and 2 mm lateral from the midline. Two times 10^5 cells in a volume of 10 μL were injected into the right frontal lobe of the brain, and thereafter the skin incision was sutured with silk thread. Seven days after tumor cell implantation, drug treatment was initiated by gavage according to the following regimen: TMZ (5 mg/kg) was administered daily for seven consecutive days, which was followed by 7 days without TMZ; EGCG (50 mg/kg) was given daily in parallel to TMZ for the same 7 days on, 7 days off cycle. The entire 14-day cycle was repeated until the animals were sacrificed or had died.

2.9. Statistical analysis

All nonparametric data were analyzed using the Student t-test to calculate the significance values; a probability value (p) < 0.05 was considered statistically significant.

3. Results

3.1. EGCG enhances cytotoxic effects of TMZ

We performed our experiments in three different human glioma cell lines: U251, LN229 and U87. In order to investigate whether EGCG would be able to enhance cell killing by TMZ, we first determined the IC50 of these agents in two well-established in vitro cytotoxicity assays, namely the MTT assay (which measures short-term survival and metabolic activity of the entire cell culture) and the colony-formation assay (CFA; which establishes long-term survival of individual cells and their ability to spawn a colony of surviving cells). In both cases, cells were exposed to drug treatment for 48 h. As shown in Fig. 1A, EGCG at concentrations ranging from 1 to 100 μM did not substantially reduce cell survival in MTT assays. In contrast, when the effects of EGCG were studied in CFAs, we found that 100 μM completely prevented colony formation, and the IC50 was slightly below 50 μM (Fig. 1B). Similarly, TMZ concentrations up to 160 μM barely reduced cell survival in MTT assays (Fig. 1C), whereas this drug displayed much greater cytotoxic potency in CFAs, where 160 μM killed nearly all the cells and the IC50 was approximately 45 μM (Fig. 1D). Overall similar results were obtained with all three glioma cell lines (not shown) and indicated that CFAs were substantially more sensitive to reveal cytotoxic drug effects than MTT assays, which was similar to findings in a recently published study on EGCG in breast and prostate cancer cells in vitro [20]. Of note, very low concentrations of EGCG slightly increased colony survival above the 100% level of untreated controls (Fig. 1B); this effect was consistently observed in a variety of tumor cell lines, although the underlying mechanism is unclear. In any case, because CFAs are known to be more reliable predictors of in vivo drug activity [21], we continued our subsequent set of experiments primarily with this type of assay.

We next studied the effects of EGCG and TMZ when given in combination. As shown in Fig. 2A, EGCG at 10 or 20 μM, which by itself had no obvious effect on cell viability, significantly enhanced the cytotoxic efficacy of TMZ in CFAs; for example, 40 μM TMZ reduced colony formation to 50%, and inclusion of 10 or 20 μM EGCG further decreased survival to 30% and 25%, respectively. While Fig. 2A displays the average results from several measurements, Fig. 2B shows one representative cell culture plate with colonies grown after drug treatment. Calculation of the combination index (CI) established values of 0.89 and 0.85 for the combination of TMZ with 20 or 40 μM EGCG, respectively, and therefore indicated slight synergy of these effects.

Fig. 2C presents the results from two different measurements of cell death, where we used TUNEL and propidium iodide (PI) incorporation to determine the extent of apoptosis after combination drug treatment of cell cultures in vitro. Because low dosages of TMZ had revealed very little toxicity during the first 48 h of treatment, we increased this drug's concentration to 100 μM during these short-term experiments. Both types of assays demonstrated that the addition of 20 μM EGCG enhanced cell killing by TMZ, even though EGCG by itself did not exert noticeable toxicity under these conditions (Fig. 2C), similar to what was seen in the CFAs. Additionally, microscopic inspection of cell cultures revealed the presence of numerous apoptotic bodies after combination treatment, which were much less prominent after treatment with TMZ alone (Fig. 2D and E). Altogether, these results demonstrate the ability

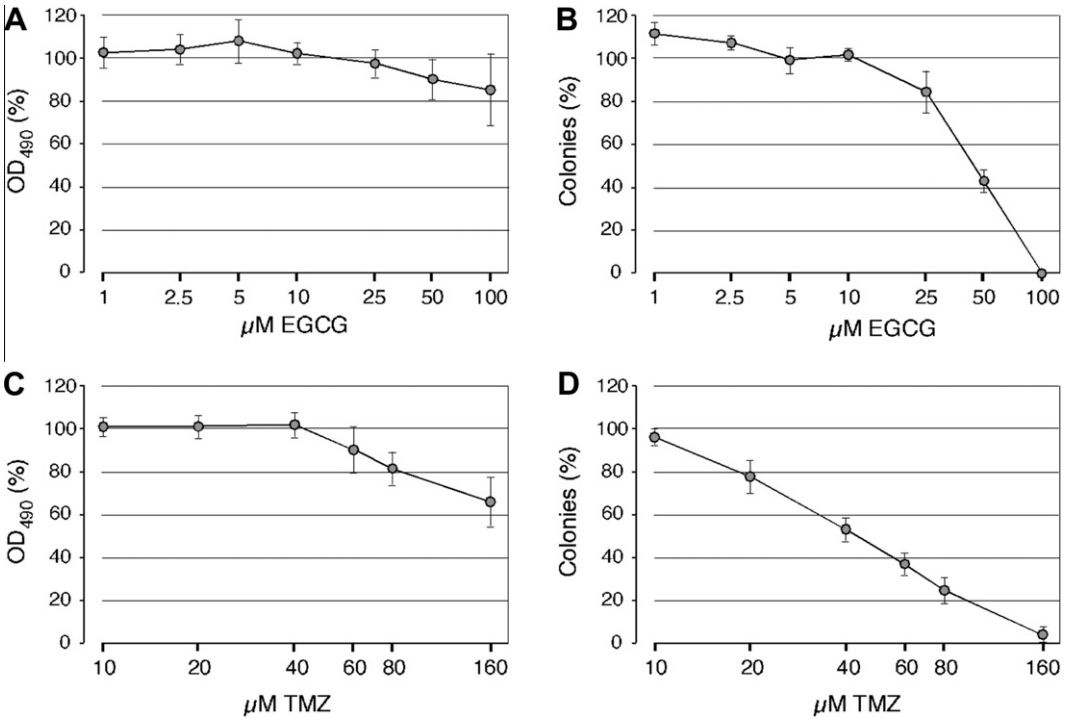

Fig. 1. Differential short-term and long-term drug toxicities. U251 glioblastoma cells were treated with the indicated concentrations of EGCG or TMZ for 48 h. Thereafter, cell viability and survival was determined by (A) short-term MTT assay and (B) long-term colony-formation assay (CFA), as described in Section 2. Shown is the mean from several repetitions (±SD). Note that *x*-axis is log-scale. Similar results were also obtained with other glioblastoma cell lines, including LN229 and U87.

of EGCG to enhance glioma cell killing by TMZ in vitro, and the green tea component was able to achieve this outcome at concentrations that had no obvious cytotoxic effect when administered individually by themselves.

3.2. GRP78 and CHOP participate in drug-induced cytotoxicity

We next investigated whether components of the ER stress response system played a role in the observed combination drug effects. In particular, we used siRNA to knockdown the expression of GRP78 (a critical protector of stressed tumor cells) or CHOP (a key pro-apoptotic trigger of ER stress-induced cell death). Thereafter, cells were treated with EGCG and TMZ, alone or in combination, and long-term survival was determined by CFA. As shown in Fig. 3A, si-GRP78 did not affect colony formation after treatment with EGCG alone or after combination treatment with EGCG plus TMZ; however, it did reduce the number of colonies after treatment with TMZ alone. Transfection of si-CHOP resulted in increased numbers of colonies under all treatment conditions, i.e., the knockdown of this protein significantly improved cell survival after drug treatment, in particular after combination drug exposure (Fig. 3A). This outcome indicated that ER stress played a critical role in mediating drug toxicity, with CHOP contributing substantial pro-apoptotic activity to EGCG-enhanced TMZ toxicity.

The efficiency of siRNA-mediated knockdown of GRP78 and CHOP was confirmed by Western blot analysis. Because non-stressed cells do not express detectable levels of CHOP protein, the cells were treated with chloroquine in order to stimulate CHOP expression; these conditions allowed us to visualize that si-CHOP was effective in repressing the increase in stress-induced CHOP levels (Fig. 3B). As well, si-GRP78 was effective at reducing the chronically elevated GRP78 levels in these cells, and at the same time produced increased expression of CHOP (Fig. 3B), which was consistent with the known role of GRP78 as a suppressor of CHOP expression [22].

3.3. EGCG enhances antitumor effects of TMZ in vivo

To determine whether EGCG would also enhance the antitumor effects of TMZ under in vivo conditions, we applied this treatment to an intracranial mouse tumor model. For this purpose, we used two glioma cell lines differing in their p53 tumor suppressor status: U87, which harbor wild type p53, and U251, which contain mutant p53. Both cell lines were implanted into the brains of nude mice, and seven days later the animals were treated with EGCG and TMZ, alone and in combination. In both experiments, all untreated mice quickly succumbed to disease within 27 days (in the case of U87 tumors) or within 33 days (in the case of U251 tumors) (Fig. 4). Similarly, animals receiving EGCG alone did not show improved survival and died within the same time frame. In contrast, and as expected, animals treated with TMZ alone displayed significantly prolonged survival times, where TMZ-treated mice died between 41 and 58 days (U87 tumors) and between 69 and 87 days (U251 tumors). Strikingly, animals receiving combination treatment survived significantly longer than TMZ-only treated animals, with mice succumbing to disease between 55 and 78 days (U87 tumors) and 70 and 140 days (U251 tumors) (Fig. 4). Thus, EGCG alone did not show therapeutic efficacy in this experiment, but when added to TMZ it was able to significantly ($p < 0.01$) prolong the survival of animals carrying malignant gliomas with either wild type p53 or mutant p53.

In order to investigate whether in vivo drug treatment would activate the ER stress response, we collected tumor tissues from animals and performed immunohistochemical analysis of GRP78 and CHOP expression. As shown in Fig. 5, CHOP expression was absent in tumor tissue from untreated animals, was slightly elevated in tumors from animals treated with EGCG alone, and was highest in tumors from animals treated with TMZ or with TMZ plus EGCG in combination. Basal level expression of GRP78 could be detected in tumors from untreated mice, and these levels were substantially increased by treatment with TMZ alone. In comparison, EGCG alone did not substantially increase GRP78 expression when given by itself, and when added to TMZ it appeared to prevent the

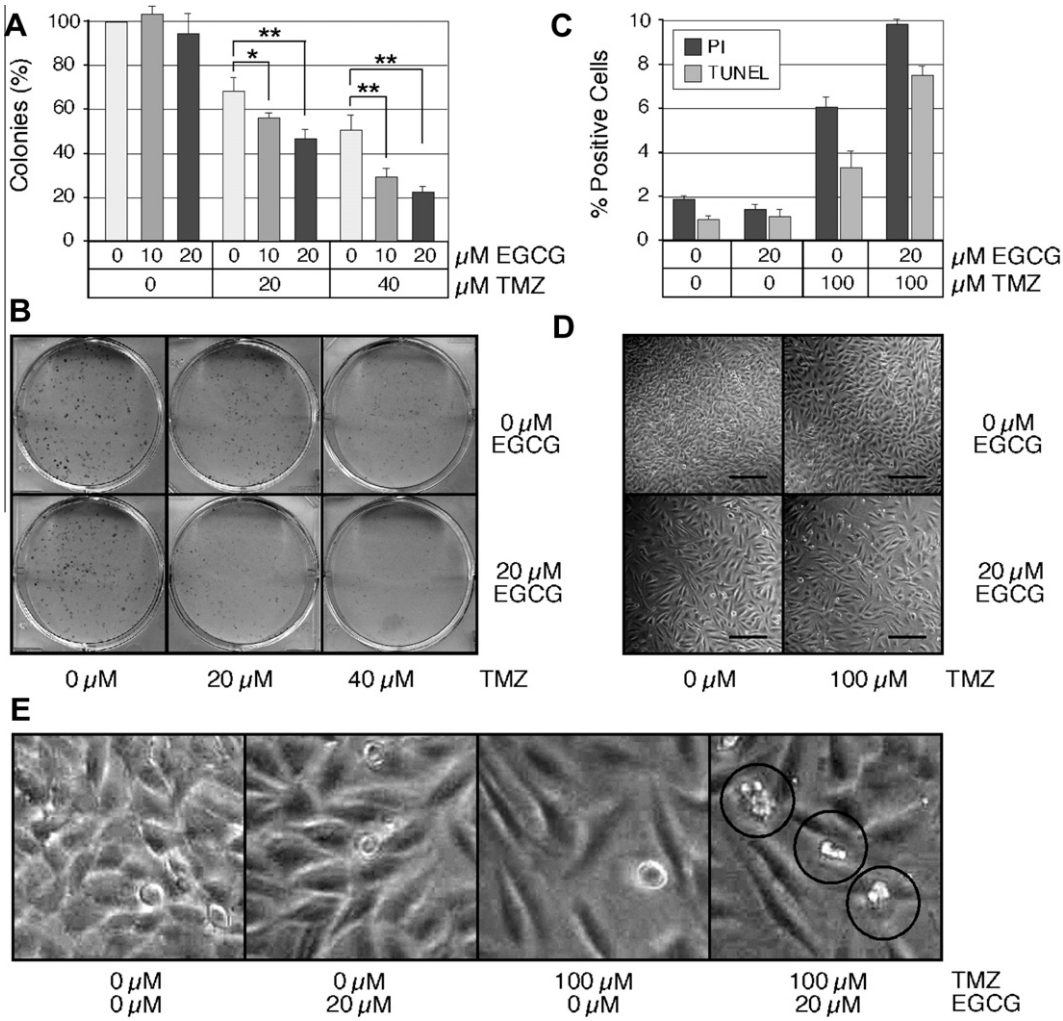

Fig. 2. Cell killing by combination treatment. U251 glioblastoma cells were treated with the indicated concentrations of EGCG and TMZ, either alone or in combination, for 48 h. (A) Long-term survival of individual cells was determined by CFA. Statistically significant differences are indicated by asterisks; one asterisk (*): $p < 0.05$; two asterisks: (**): $p < 0.01$. (B) Shown is a highly representative example of colony formation in a 6-well plate. (C) Cell death was determined by propidium iodide (PI) staining or by TUNEL. Shown is the percentage of PI-positive or TUNEL-positive cells (mean ± SD). The difference between TMZ-only and TMZ + EGCG treated cells was statistically significant ($p < 0.01$). (D) Photomicrographs of representative sections of monolayer cells during drug treatment. They show that in combination drug treatment there are not only fewer cells, but also numerous apoptotic bodies. (E) Shown are enlarged sections of D, revealing apoptotic bodies only under combination drug treatment (encircled). Overall similar results were also obtained with other glioblastoma cell lines.

TMZ-induced increase in GRP78 levels (Fig. 5). Taken together, these data established that TMZ treatment triggered the ER stress response in vivo; however, the addition of EGCG blocked the increase of TMZ-induced GRP78, thus eliminating a key pro-survival feature of the ER stress response and favoring its pro-apoptotic module CHOP.

4. Discussion

The use of temozolomide (TMZ) in combination with radiotherapy has become the gold standard treatment for patients with glioblastoma multiforme. However, although well tolerated, the drug has limited clinical efficacy [1], and therefore further improved therapeutic regimes are urgently needed. Among the factors contributing to increased chemoresistance of malignant glioma cells is the chaperone protein GRP78, which constitutes an important pro-survival component of the ER stress/unfolded protein response [3]. We and others have shown that GRP78 is highly expressed in glioblastomas, but not in oligodendrogliomas or normal brain tissue, and its expression is inversely correlated with median patient survival [2,23]. Thus, inhibition of GRP78 function may increase the therapeutic efficacy of TMZ. We now have tested this hypothesis in an orthotopic mouse glioblastoma model by using the green tea component EGCG, which had been reported earlier to bind to the ATP-binding site of GRP78 and thereby inactivate its pro-survival function [10].

As shown in this report, mice harboring intracranially implanted glioblastoma cells survive significantly longer when EGCG is added to TMZ therapy, and this outcome

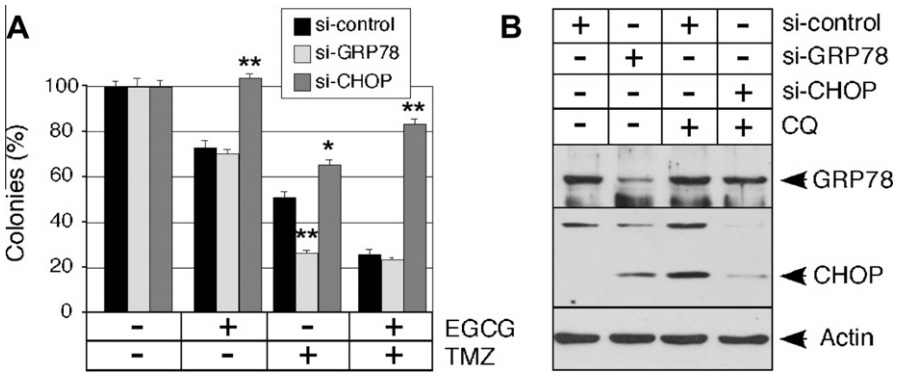

Fig. 3. Involvement of GRP78 and CHOP. U251 glioblastoma cells were transfected with siRNA against GRP78 (si-GRP78) or CHOP (si-CHOP), or with a scrambled siRNA (si-control). (A) Transfected cells were treated with TMZ and EGCG alone and in combination, and CFA was performed. Shown is the percentage of colonies formed under the different treatment conditions (mean ± SD). Statistically significant differences are indicated by asterisks; one asterisk (*): $p < 0.05$; two asterisks: (**): $p < 0.01$. (B) The efficiency of siRNA-mediated knockdown was established by Western blot analysis of transfected cells. Because non-stressed cells do not express CHOP, si-CHOP transfected cells were treated with chloroquine (CQ) in order to stimulate CHOP expression. As shown, si-CHOP was able to greatly diminish CQ-induced CHOP expression. Actin was used as a loading control.

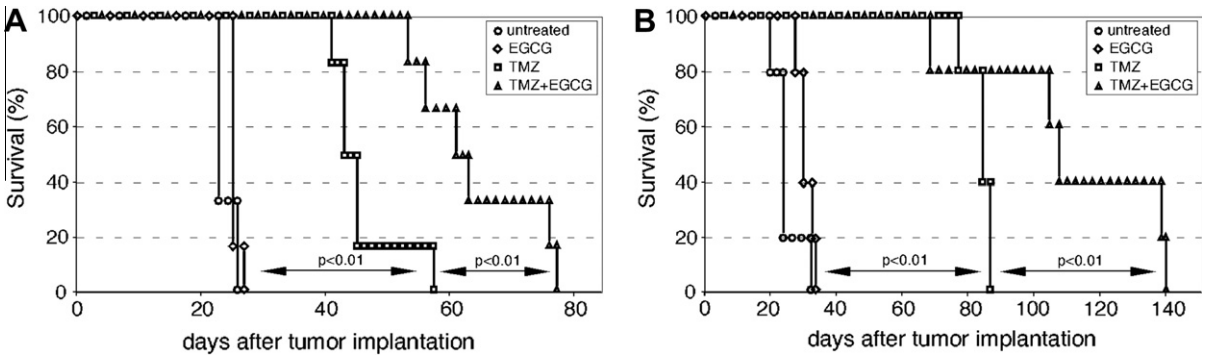

Fig. 4. Drug activity in orthotopic mouse tumor models. Mice were intracranially implanted with (A) U87 (wild type p53 status) or with (B) U251 (mutant p53 status) glioblastoma cells. Seven days later, the animals were separated into four groups, which received treatment with TMZ alone, EGCG alone, TMZ + EGCG in combination, or remained untreated, respectively. The survival of all animals was documented, and is presented here in a Kaplan–Meier plot. There was no statistical difference between the untreated groups of animals and those treated with EGCG. As expected, TMZ-treated mice lived significantly ($p < 0.01$) longer than untreated mice. Survival in the groups of animals receiving combination treatment (TMZ + EGCG) was significantly ($p < 0.01$) extended as compared to TMZ-only treated animals.

could be achieved independently of whether the tumor cells harbored p53 wild type (U87 cells) or p53 mutant (U251 cells) genes. This finding is of interest in view of p53's known ability to interfere with tumor cell responses to chemotherapy, although in the case of temozolomide there is conflicting evidence as to whether inactivation of p53 function makes glioblastoma more or less sensitive to temozolomide toxicity [24–26]. Similarly, in several other tumor cell types it has been reported that p53 may play a role in EGCG-induced apoptosis [27–30]. Compared to these previous studies, our results indicate that p53 status of glioblastoma does not appear to exert significant influence on therapeutic efficacy of the TMZ + EGCG combination in vivo, which bodes well for future clinical applications of this combination. However, other genes suspected or known to impinge on glioblastoma chemosensitivity, such as O-6-methylguanine DNA methyltransferase (MGMT) [31], may also play a role and will have to be investigated in future studies.

Before obtaining our results, it was unclear whether EGCG would be able to cross the BBB efficiently enough to cause chemosensitization, because earlier studies in rats had shown very low brain distribution ratio of this polyphenol, and it was thought that EGCG's bipolar functional group might have difficulty in penetrating the BBB efficiently [32,33]. However, our results indicate that EGCG indeed is active across the BBB and therefore must have passed through this barrier to an extent that was sufficient for chemosensitization towards TMZ to take place.

On the other hand, it is not entirely clear why EGCG, when given as monotherapy without TMZ, did not yield detectable therapeutic efficacy in our tumor model, i.e., did not extend survival of mice with intracranially implanted glioblastoma. One might be tempted to speculate that low brain distribution of systemically administered EGCG would prevent polyphenol accumulation to levels that are high enough to affect tumor growth in monotherapy fashion. Alternatively, tumor type-specific

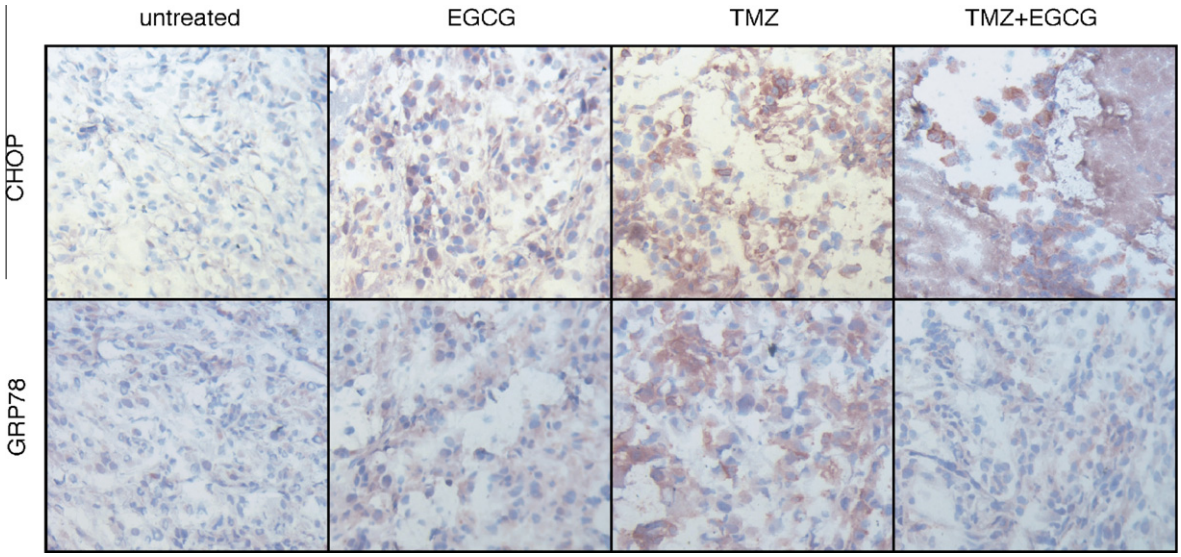

Fig. 5. Immunohistochemical analysis of tumor tissue. Intracranial tumors were collected from animals treated as described in the legend to Fig. 4 and were analyzed for the expression levels of GRP78 and CHOP by immunohistochemical means. Photomicrographs were taken at 200× magnification and representative sections are shown. CHOP expression was strongest in tumor tissue from animals treated with TMZ or TMZ + EGCG. GRP78 expression was strongest in response to TMZ treatment and was noticeable less when EGCG was added to TMZ (TMZ + EGCG).

responses may play a role, in that some tumor types appear to be sensitive to EGCG monotherapy, whereas others are not, and such differential antitumor outcomes indeed have been observed in subcutaneous xenograft mouse tumor models, where the issue of BBB penetration is irrelevant. For example, studies with mice carrying implanted melanoma, colon carcinoma, or pancreatic carcinoma cells have demonstrated significant reduction of tumor volume when mice were administered EGCG [13,34,35], whereas mouse models of hepatocellular or breast carcinoma did not respond to this polyphenol when administered in monotherapy fashion [16,17], even though similar dosages (30–45 mg/kg) were applied. Deduced from the results of our in vitro studies demonstrating that 20 μM EGCG is insufficient to affect glioblastoma cell proliferation and survival (Figs. 1 and 2), we surmise that glioblastoma represents one of those tumor types that is little responsive to EGCG alone.

Earlier studies had demonstrated that EGCG, despite its oftentimes very limited anticancer activity during monotherapy in vivo, was able to significantly enhance the therapeutic efficacy of other chemotherapeutic agents in mouse models of different tumor types. For instance, the polyphenol was shown to potentiate paclitaxel therapy of breast cancer, doxorubicin therapy of hepatocellular carcinoma, or interferon therapy of melanoma [13,16,17]. In comparison, our demonstration that EGCG can achieve chemosensitization towards TMZ is of particular importance, because TMZ chemotherapy of malignant glioma is among the least impressive therapeutic regimens for cancer patients in general, and there is a great medical need to improve the efficacy of TMZ-based treatments. Because we show that this enhanced therapeutic outcome can be achieved with an orthotopic brain tumor model, i.e., across the BBB, we are hopeful that this regimen might be effective in patients as well. The safety of both agents, TMZ and EGCG, has been extensively studied in humans, and therefore it should be possible to promptly design appropriate clinical trials to validate our in vivo results in the clinic.

Our molecular in vitro studies indicate that inhibition of pro-survival GRP78 might at least in part contribute to EGCG's ability to chemosensitize glioblastoma cells to TMZ, and several observations are consistent with this assessment. For instance, knockdown of GRP78 expression by si-RNA mimics some of the effects of EGCG; for example, colony formation after TMZ treatment is similar when cells were co-treated with EGCG (EGCG + TMZ) or when they were transfected with si-GRP78 (si-GRP78 + TMZ) (Fig. 3). Similarly, knock-down of GRP78 reduces colony survival after TMZ treatment (si-GRP78 + TMZ), but the addition of EGCG (si-GRP78 + TMZ + EGCG) is unable to reduce colony formation further, presumably because its target, GRP78, already is neutralized by si-RNA.

On the other side, knock-down of GRP78's antagonistic player in the ER stress response, pro-apoptotic CHOP, yields complementary results that further support a prominent role of the ER stress response system in these events. Among the pro-survival functions of GRP78 is its suppression of apoptotic pathways, in particular the repression of CHOP expression [6,22]. This latter effect becomes evident, for example, by increased expression of CHOP protein in response to si-RNA mediated knockdown of GRP78 (as displayed in Fig. 3B). In our experiments, we demonstrate that gliobastoma cells transfected with si-CHOP display increased chemoresistance, and are particularly well protected against EGCG's potency to enhance cell killing by TMZ (Fig. 3). In effect, while colony survival is decreased to only 25% after combination treatment (TMZ + EGCG) of control-transfected cells, the knock-down of CHOP

expression greatly improves survival to above 80%. Together, these results are consistent with a model where EGCG achieves chemosensitization via the inhibition of GRP78 and subsequent induction of the apoptosis trigger CHOP.

The above molecular observations are at least in part reflected by the immunohistochemical analysis of tumor tissue from drug-treated animals (Fig. 5). For example, tumors from animals treated with TMZ or with TMZ + EGCG reveal the highest levels of CHOP protein, and GRP78 levels are lower after TMZ + EGCG treatment than after treatment with TMZ alone. Thus, the balance of pro-survival GRP78 vs. pro-apoptotic CHOP is tilted in favor of CHOP in response to combination treatment, and is consistent with a key role of ER stress components during chemosensitization brought about by EGCG. Altogether, while these results are coherent with a model where EGCG achieves these outcomes through the inhibition of GRP78, they do not exclude the possibility that other known (or unknown) targets of EGCG [8,36–38] might participate in these processes as well and that the final anticancer outcome might represent a multitarget effect. But in any case, while some of the molecular details of EGCG's chemosensitizing properties may require further investigation, our study provides clear evidence that this polyphenol is able to enhance therapeutic efficacy of TMZ in an orthotopic glioblastoma mouse model, and thus proposes that clinical studies should be considered in the near future.

Conflict of interest

None declared.

Acknowledgements

Funding for this work was received from the National Brain Tumor Society (NBTS), specifically NBTS's Steven J. Bryant Chair of Research (to AHS), the L.K. Whittier Foundation via the USC/Norris Comprehensive Cancer Center (to TCC), and the Sounder Foundation (to TCC).

References

[1] R. Stupp, W.P. Mason, M.J. van den Bent, M. Weller, B. Fisher, M.J. Taphoorn, K. Belanger, A.A. Brandes, C. Marosi, U. Bogdahn, J. Curschmann, R.C. Janzer, S.K. Ludwin, T. Gorlia, A. Allgeier, D. Lacombe, J.G. Cairncross, E. Eisenhauer, R.O. Mirimanoff, Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma, New Engl. J. Med. 352 (2005) 987–996.
[2] P. Pyrko, A.H. Schönthal, F.M. Hofman, T.C. Chen, A.S. Lee, The unfolded protein response regulator GRP78/BiP as a novel target for increasing chemosensitivity in malignant gliomas, Cancer Res. 67 (2007) 9809–9816.
[3] A.S. Lee, GRP78 induction in cancer: therapeutic and prognostic implications, Cancer Res. 67 (2007) 3496–3499.
[4] S. Oyadomari, M. Mori, Roles of CHOP/GADD153 in endoplasmic reticulum stress, Cell Death Differ. 11 (2004) 381–389.
[5] M. Boyce, J. Yuan, Cellular response to endoplasmic reticulum stress: a matter of life or death, Cell Death Differ. 13 (2006) 363–373.
[6] D.T. Rutkowski, S.M. Arnold, C.N. Miller, J. Wu, J. Li, K.M. Gunnison, K. Mori, A.A. Sadighi Akha, D. Raden, R.J. Kaufman, Adaptation to ER stress is mediated by differential stabilities of pro-survival and pro-apoptotic mRNAs and proteins, PLoS Biol. 4 (2006) e374.
[7] A.H. Schönthal, Endoplasmic reticulum stress and autophagy as targets for cancer therapy, Cancer Lett. 275 (2008) 163–169.
[8] N. Khan, H. Mukhtar, Multitargeted therapy of cancer by green tea polyphenols, Cancer Lett. 269 (2008) 269–280.
[9] C.S. Yang, X. Wang, G. Lu, S.C. Picinich, Cancer prevention by tea: animal studies, molecular mechanisms and human relevance, Nat. Rev. Cancer 9 (2009) 429–439.
[10] S.P. Ermakova, B.S. Kang, B.Y. Choi, H.S. Choi, T.F. Schuster, W.Y. Ma, A.M. Bode, Z. Dong, (-)-Epigallocatechin gallate overcomes resistance to etoposide-induced cell death by targeting the molecular chaperone glucose-regulated protein 78, Cancer Res. 66 (2006) 9260–9269.
[11] M. Lang, R. Henson, C. Braconi, T. Patel, Epigallocatechin-gallate modulates chemotherapy-induced apoptosis in human cholangiocarcinoma cells, Liver Int. 29 (2009) 670–677.
[12] M. Masuda, M. Suzui, I.B. Weinstein, Effects of epigallocatechin-3-gallate on growth, epidermal growth factor receptor signaling pathways, gene expression, and chemosensitivity in human head and neck squamous cell carcinoma cell lines, Clin. Cancer Res. 7 (2001) 4220–4229.
[13] M. Nihal, H. Ahsan, I.A. Siddiqui, H. Mukhtar, N. Ahmad, G.S. Wood, (-)-Epigallocatechin-3-gallate (EGCG) sensitizes melanoma cells to interferon induced growth inhibition in a mouse model of human melanoma, Cell Cycle 8 (2009) 2057–2063.
[14] I.A. Siddiqui, A. Malik, V.M. Adhami, M. Asim, B.B. Hafeez, S. Sarfaraz, H. Mukhtar, Green tea polyphenol EGCG sensitizes human prostate carcinoma LNCaP cells to TRAIL-mediated apoptosis and synergistically inhibits biomarkers associated with angiogenesis and metastasis, Oncogene 27 (2008) 2055–2063.
[15] J. Wang, Y. Yin, H. Hua, M. Li, T. Luo, L. Xu, R. Wang, D. Liu, Y. Zhang, Y. Jiang, Blockade of GRP78 sensitizes breast cancer cells to microtubules-interfering agents that induce the unfolded protein response, J. Cell. Mol. Med. 13 (2009) 3888–3897.
[16] G. Liang, A. Tang, X. Lin, L. Li, S. Zhang, Z. Huang, H. Tang, Q.Q. Li, Green tea catechins augment the antitumor activity of doxorubicin in an in vivo mouse model for chemoresistant liver cancer, Int. J. Oncol. 37 (2010) 111–123.
[17] T. Luo, J. Wang, Y. Yin, H. Hua, J. Jing, X. Sun, M. Li, Y. Zhang, Y. Jiang, (-)-Epigallocatechin gallate sensitizes breast cancer cells to paclitaxel in a murine model of breast carcinoma, Breast Cancer Res. 12 (2010) R8.
[18] S. Wolfram, D. Raederstorff, Y. Wang, S.R. Teixeira, V. Elste, P. Weber, TEAVIGO (epigallocatechin gallate) supplementation prevents obesity in rodents by reducing adipose tissue mass, Ann. Nutr. Metab. 49 (2005) 54–63.
[19] P. Pyrko, N. Soriano, A. Kardosh, Y.T. Liu, J. Uddin, N.A. Petasis, F.M. Hofman, C.S. Chen, T.C. Chen, A.H. Schönthal, Downregulation of surviving expression and concomitant induction of apoptosis by celecoxib and its non-cyclooxygenase-2-inhibitory analog, dimethyl-celecoxib (DMC), in tumor cells in vitro and in vivo, Mol. Cancer 5 (2006) 19.
[20] P. Wang, S.M. Henning, D. Heber, Limitations of MTT and MTS-based assays for measurement of antiproliferative activity of green tea polyphenols, PLoS One 5 (2010) e10202.
[21] D.L. Trainer, T. Kline, F.L. McCabe, L.F. Faucette, J. Feild, M. Chaikin, M. Anzano, D. Rieman, S. Hoffstein, D.J. Li, et al., Biological characterization and oncogene expression in human colorectal carcinoma cell lines, Int. J. Cancer 41 (1988) 287–296.
[22] T. Suzuki, J. Lu, M. Zahed, K. Kita, N. Suzuki, Reduction of GRP78 expression with siRNA activates unfolded protein response leading to apoptosis in HeLa cells, Arch. Biochem. Biophys. 468 (2007) 1–14.
[23] H.K. Lee, C. Xiang, S. Cazacu, S. Finniss, G. Kazimirsky, N. Lemke, N.L. Lehman, S.A. Rempel, T. Mikkelsen, C. Brodie, GRP78 is overexpressed in glioblastomas and regulates glioma cell growth and apoptosis, Neuro Oncol. 10 (2008) 236–243.
[24] M.D. Blough, D.C. Beauchamp, M.R. Westgate, J.J. Kelly, J.G. Cairncross, Effect of aberrant p53 function on temozolomide sensitivity of glioma cell lines and brain tumor initiating cells from glioblastoma, J. Neurooncol. (2010).
[25] M. Hermisson, A. Klumpp, W. Wick, J. Wischhusen, G. Nagel, W. Roos, B. Kaina, M. Weller, O6-methylguanine DNA methyltransferase and p53 status predict temozolomide sensitivity in human malignant glioma cells, J. Neurochem. 96 (2006) 766–776.
[26] Y. Hirose, M.S. Berger, R.O. Pieper, p53 effects both the duration of G2/M arrest and the fate of temozolomide-treated human glioblastoma cells, Cancer Res. 61 (2001) 1957–1963.
[27] P.L. Kuo, C.C. Lin, Green tea constituent (-)-epigallocatechin-3-gallate inhibits Hep G2 cell proliferation and induces apoptosis

through p53-dependent and Fas-mediated pathways, J. Biomed. Sci. 10 (2003) 219–227.
[28] A.M. Roy, M.S. Baliga, S.K. Katiyar, Epigallocatechin-3-gallate induces apoptosis in estrogen receptor-negative human breast carcinoma cells via modulation in protein expression of p53 and Bax and caspase-3 activation, Mol. Cancer Ther. 4 (2005) 81–90.
[29] K. Hastak, M.K. Agarwal, H. Mukhtar, M.L. Agarwal, Ablation of either p21 or Bax prevents p53-dependent apoptosis induced by green tea polyphenol epigallocatechin-3-gallate, FASEB J. 19 (2005) 789–791.
[30] S. Manna, S. Banerjee, S. Mukherjee, S. Das, C.K. Panda, Epigallocatechin gallate induced apoptosis in Sarcoma180 cells in vivo: mediated by p53 pathway and inhibition in U1B, U4-U6 UsnRNAs expression, Apoptosis 11 (2006) 2267–2276.
[31] M.E. Hegi, L. Liu, J.G. Herman, R. Stupp, W. Wick, M. Weller, M.P. Mehta, M.R. Gilbert, Correlation of O6-methylguanine methyltransferase (MGMT) promoter methylation with clinical outcomes in glioblastoma and clinical strategies to modulate MGMT activity, J. Clin. Oncol. 26 (2008) 4189–4199.
[32] L.C. Lin, M.N. Wang, T.Y. Tseng, J.S. Sung, T.H. Tsai, Pharmacokinetics of (-)-epigallocatechin-3-gallate in conscious and freely moving rats and its brain regional distribution, J. Agric. Food Chem. 55 (2007) 1517–1524.
[33] K. Nakagawa, T. Miyazawa, Absorption and distribution of tea catechin, (-)-epigallocatechin-3-gallate, in the rat, J. Nutr. Sci. Vitaminol. (Tokyo) 43 (1997) 679–684.
[34] Y.D. Jung, M.S. Kim, B.A. Shin, K.O. Chay, B.W. Ahn, W. Liu, C.D. Bucana, G.E. Gallick, L.M. Ellis, EGCG, a major component of green tea, inhibits tumour growth by inhibiting VEGF induction in human colon carcinoma cells, Br. J. Cancer 84 (2001) 844–850.
[35] S. Shankar, S. Ganapathy, S.R. Hingorani, R.K. Srivastava, EGCG inhibits growth, invasion, angiogenesis and metastasis of pancreatic cancer, Front. Biosci. 13 (2008) 440–452.
[36] B. Annabi, M.P. Lachambre, N. Bousquet-Gagnon, M. Page, D. Gingras, R. Beliveau, Green tea polyphenol (-)-epigallocatechin 3-gallate inhibits MMP-2 secretion and MT1-MMP-driven migration in glioblastoma cells, Biochim. Biophys. Acta 1542 (2002) 209–220.
[37] S. Nam, D.M. Smith, Q.P. Dou, Ester bond-containing tea polyphenols potently inhibit proteasome activity in vitro and in vivo, J. Biol. Chem. 276 (2001) 13322–13330.
[38] M.D. Siegelin, A. Habel, T. Gaiser, Epigalocatechin-3-gallate (EGCG) downregulates PEA15 and thereby augments TRAIL-mediated apoptosis in malignant glioma, Neurosci. Lett. 448 (2008) 161–165.