Journal of Neurological Sciences [Turkish] **29:(3)**# 32; 510-517, 2012
http://www.jns.dergisi.org/text.php3?id=563
Research Article

The Synergistic Effect of Fotemustine and Genistein on Expressions of p53, EGFR and COX-2 Genes in Human Glioblastoma Multiforme Cell Line

Çığır Biray AVCI[1], Yavuz DODURGA[2], Nezih OKTAR[3], Sunde YILMAZ SÜSLÜER[1], Zeynep Özlem DOĞAN ŞIĞVA[1], Cumhur GÜNDÜZ[1]

[1]Ege University Medical Faculty, Medical Biology, Izmir, Turkey [2]Pamukkale University Medical Faculty, Medical Biology, Denizli, Turkey [3]Ege University Medical Faculty, Neurosurgery, Izmir, Turkey

Summary

GBM is the most common primary malignant neoplasm of the central nervous system in adults. Fotemustine (FTM) is a cytotoxic alkylating agent and a lipophilic chloroethylnitrosourea derivative. Its mechanism of action consists mainly in inducing DNA strand breaks and cross-linking. Genistein, one of the soy-derived isoflavones, exerts its anticancer properties via several mechanisms, including inhibition of tyrosine phosphorylation, weak estrogenic and anti-estrogenic properties, as an antioxidant, inhibition of topoisomerase II, inhibition of angiogenesis, and induction of cell differentiation in a number of human tumors. We aimed to investigate the anti-proliferative synergistic effect of genistein with fotemustine on human glioblastoma multiforme U87-MG cells. This study was also designed to answer the following question: Do the p53, EGFR, COX-2 genes' expression patterns differ in treatment of these both drugs alone and in combination?

Key words: Fotemustine, Genistein, U-87MG, p53, EGFR, COX-2

Fotemustin ve Genisteinin Glioblastom Multiforme Hücre Hattında p53, EGFR ve COX-2 Genlerinin Ekspresyonları Üzerine Sinerjistik Etkisi

Özet

GBM yetişkinlerde merkezi sinir sisteminin en yaygın primer malin neoplazmıdır. Fotemustin (FTM) sitotoksik bir ajan ve lifofilik kloroetilnitrozüri türevidir. DNA zincir kırıklarını ve çapraz bağlanmayı indüklemek ana mekanizmasını oluşturmaktadır. Soyadan elde edilen bir isoflavon olan Genistein, antikanser özelliğini tirozin fosforilasyonunun inhibisyonu, zayıf östrojenik ve anti-östrojenik özellikleri, antioksidan olarak, topoizomeraz II' nin inhibisyonu, anjiyogenezin inhibisyonu ve hücre faklılaşmasının başlaması gibi çeşitli mekanizmalar ile göstermektedir. Genisteinin fotemustin ile anti-proliferatif sinerjistik etkisinin insan glioblastoma multiforme U87-MG hücrelerinde incelenmesi amaçlanmıştır. Bu çalışma ayrıca p53, EGFR, COX-2 gen ekspresyon paternlerinin bu ilaçların ayrı ayrı veya kombine kullanılmasının ardından faklılık gösterip göstermediğine cevap verebilmek için tasarlanmıştır.

Anahtar Kelimeler: Fotemustin, Genistein, U-87MG, p53, EGFR, COX-2

INTRODUCTION

Glioblastoma multiforme (GBM) is a malignant primary brain tumor occurring in older people. The patients with glioblastoma usually have a short survival time which is less than a year. This tumor rarely metastasizes out of the central nervous system, but their invasion away from the tumor mass makes it difficult for surgical resection of the tumor and for the treatment of this tumor[14]. In spite of current therapies including surgery, radiation therapy, and chemotherapy, glioblastomas are associated with poor prognosis[16]. The major problem associated with treatment of glioblastomas is that total surgical removal of tumor is difficult as the tumor invades into brain tissue[12].

Comprehensive research has been carried out to study of invasion mechanisms and the effect of agents with brain tumors in recent years. Association with soy isoflavones of hormone dependent and independent cancers has led to the comprehensive research on isoflavones and their effect on cancer over two decades[27].

Soy isoflavones are natural compounds that exist in soy-based products in foods and infant formula. Derived from soybeans isoflavones are also a good candidate for the prevention and treatment of various types of human cancer.

Genistein (4′,5,7-trihydroxyisoflavone) is found in soy beans and other natural sources such as certain traditional Chinese medicinal herbs and tea leaves. In the past decade there have been some studies on the anti-tumor effects of genistein on cancers of the breast, prostate and colon in humans. It blocks receptor tyrosine kinases (RTKs) involved in signal transduction and has been shown to inhibit topoisomerase II activity and angiogenesis. In vitro and in vivo experiments with genistein have shown results in blocking growth, invasiveness and angiogenesis of a number of human tumors such as glioblastoma multiforme, leukemia, breast and colorectal cancer.

Fotemustine is a lipophilic chloroethylnitrosourea derivative whose therapeutic activity was demonstrated in primary brain tumors. Its mechanism consists in inducing DNA strand breaks and cross-linking, including DNA–protein cross-linking.

The p53 tumor suppressor is enclosed in cell cycle control, DNA repair, also replicates senescence, and programmed cell death. In normal cells p53 is expressed at a low constitutive level and is localized predominantly in cytoplasm. The latent form of p53 is stabilized and activated by post-translational modifications[23]. Genetic alterations in the p53 pathway contribute to more than 50% human cancers[21].

Cyclooxygenase (COX) is the key enzyme which is played an important role on the conversion of arachidonic acid to prostaglandins. There are known two isoforms that are called COX-1 and COX-2.

COX-2 is an enzyme which is regulated by cytokines, growth factors, tumor promoters and cellular stress conditions, is expressed in the uterus, ovary, kidney, stomach and brain[1,4,5,10,17,26].

Prostaglandins (COX-2 derived) are correlated with tumor cell proliferation, invasion, angiogenesis, metastasis and resistance to apoptosis. COX-2 overexpression has been shown for a variety of tumors (brain, head and neck, breast, cervix, prostate, bladder, liver, pancreas, skin, lung, colon, rectum and esophagus)[2,9,15,19]. EGFR gene overexpression was established to associate with higher malignancy and resistance to radiotherapy for brain tumors especially glioblastoma[3,11]. COX-2 gene upregulation was found to correlate with increased EGFR gene expression. In this respect, reactivity of COX-2 and EGFR gene expressions play important roles in glioma progression and also resistance to

radiotherapy. Some studies have shown that high COX-2 gene expression levels associated with poor survival after radiotherapy[25].

We aimed to investigate the anti-proliferative synergistic effect of genistein and fotemustine combination on human glioblastoma multiforme U87-MG cells. This study was also designed to answer the following question: Do the p53, EGFR, COX-2 gene expressions patterns differ in treatment of these both drugs alone and in combination?

MATERIAL AND METHODS

Chemicals and reagents

Genistein was obtained from Sigma Chemical Co., St Louis Missouri. The chemical was diluted in 0.5% dimethylsulphoxide (DMSO). Fotemustine was supplied from Servier Research International (Australia). Cell proliferation assay (XTT) was supplied from Roche Diagnostics. UPL Probes were used for determination of gene expressions. (Roche Applied Science, Mannheim, Germany). All other tissue culture supplies were obtained from Corning Incorporated (USA) unless otherwise specified.

Tumor cell line

U87MG glioma cell line was used as a model cell line which was obtained from ATCC.

Cell culture and preparation of cytotoxicity experiments

U87MG cell line was grown in BIO-AMF Basal medium containing 2mM L-glutamine supplemented with 10% fetal bovine inactivated serum (FBS) and 1% penicillin/ streptomycin were maintained at a density of 5x 10^5 cells/ml in a standard cell culture incubator at 37^{o}C, humidified 95% air, and 5% CO_2 atmosphere. Prior to any experiment, cells were split at 5 x10^5 cells/ml in the BIO-AMF Basal medium and cell suspensions were aliquoted into flasks for subsequent treatments. Genistein and Fotemustine diluted in RPMI 1640 medium and were used in treatments of 1, 10, 100 μM alone and in combination.

Cytotoxicity assay

Cytotoxic assays and determination of IC_{50} doses of genistein and fotemustine in glioma cells were performed by using trypan blue dye exclusion test and XTT assay as indicated in manufacturers' instruction.

XTT Assay

Cells were seeded in 96-well tissue culture plates and incubated for 24 hours without reagent. After addition of reagents, cells were incubated for 24, 48 and 72 hours and cell viability was assessed by using XTT-PMS mixture (XTT sodium salt; [2,3-bis (2-Methoxy-4-nitro-5-sulfophenyl) 2Htetrazolium- 5-carboxanilide inner salt], Phenazine Methosulfate (N-Methylphenazonium methyl sulfate salt)], as recommended by supplier. Formazan formation was quantified spectrophotometrically at 450 nM (reference wavelength 670 nM) using a microplate reader (Bio-Rad, Coda, Richmond, CA). Viability was calculated by using the background-corrected absorbance as follows:

Viability (%) = A of experiment well /A of control well × 100

Isolation of total RNA and cDNA synthesis

Fifty microliters of total RNA was isolated from cell culture of glioma cells treated with genistein, fotemustine and combination of these two chemicals in IC_{50} doses for 24, 48 and 72 hours and control cells by using High Pure RNA Isolation Kit (Roche, Germany). Reverse transcription procedure was performed for cDNA synthesis by using Transcriptor First Strand cDNA Synthesis Kit according to the manufacturers' instructions.

Relative quantification of p53, EGFR and COX-2 genes

Real-time quantitative RT-PCR analyses of p53, EGFR, and COX-2 genes were performed with Lightcycler instrument and software. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH "housekeeping" gene) was chosen as a standard to control the variability in amplification. The sequences of primers and probes used are shown in Table-1. PCR was performed by using TaqMan Master Kit (Roche Diagnostics) according to the manufacturer's instructions. Studied genes target probe was labeled at the 5' end with the reporter dye molecule 6-carboxyfluorescein (FAM). The GAPDH target probe was labeled with 6-carboxyfluorescein. Both probes were labeled with the quencher fluor 6-carboxytetramethylrhodamine (TAMRA) at the 3' end. To quantify genes mRNA from cell culture, a calibration curve was constructed (Error: 0.100 Efficiency: 1,790) using copy number variations (10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 and 10) of GAPDH. Relative ratio (RR) of gene expressions was calculated using the formula:

RR = Copy number of gene/Copy number of GAPDH x 1000

Table 1: Primers and probes of genes

Gene	Forward Primer	Reverse Primer	Probe (roche)
TP53	ccccagccaaagaagaaac	aacatctcgaagcgctcac	ggatggag
COX-2	tcacgcatcagtttttcaaga	tcaccgtaaatatgatttaagtccac	gggctggg
EGFR	cagccacccatatgtaccatc	aactttgggcgactatctgc	gctggatg
GAPDH	gaaggtgaaggtcggagtc	gaagatggtgatgggatttc	FAM-caagcttcccgttctcagcc-TAMRA

RESULTS

The expression profiles of p53, EGFR and COX-2 genes were evaluated by treating 1, 10 and 100 μM doses of fotemustine, genistein and fotemustine and genistein combination at the 24th, 48th and 72nd hours to U87MG cells. Expression analyses were performed by using the Real Time online PCR method and expression rates detected in time and dose dependent manner.

We have detected that fotemustine, genistein and fotemustine and genistein combination treatments suppress the metabolic potential of U87MG cells and then concentration-dependent experiments showed that fotemustine, genistein and fotemustine/genistein combination inhibited the metabolic abilities of U87MG cells.

As shown in the trypan blue test results in the first day of the study, IC_{50} doses of fotemustine, genistein and fotemustine/genistein combination were 1, 10 and 100 μM respectively. According to XTT test, in the first and second days of the study, the average cytotoxicity of fotemustine between 1-100 μM was 5%. In the second day, while the cytotoxicity of 100 μM genistein was 33%, it was 17% in 100 μM fotemustine/genistein combination (Fig 1.). Dose of 100μM genistein showed significant dose dependent linear cytotoxicity [$R=1$, $p<0.001$, (Fig 2.)].

There were no significant differences in the expressions of p53, EGFR and COX-2 genes in treatment of fotemustine, genistein alone or combination. A decrease was detected in the expression level of COX-2, with the treatment of genistein in time and dose dependent manner. In the first day, a distinct increase in the expressions of p53 and EGFR detected with 10 μM of genistein. In the second day, it was detected that expression of p53 gene increased with all the doses of genistein and fotemustine/genistein combination (Fig 3.).

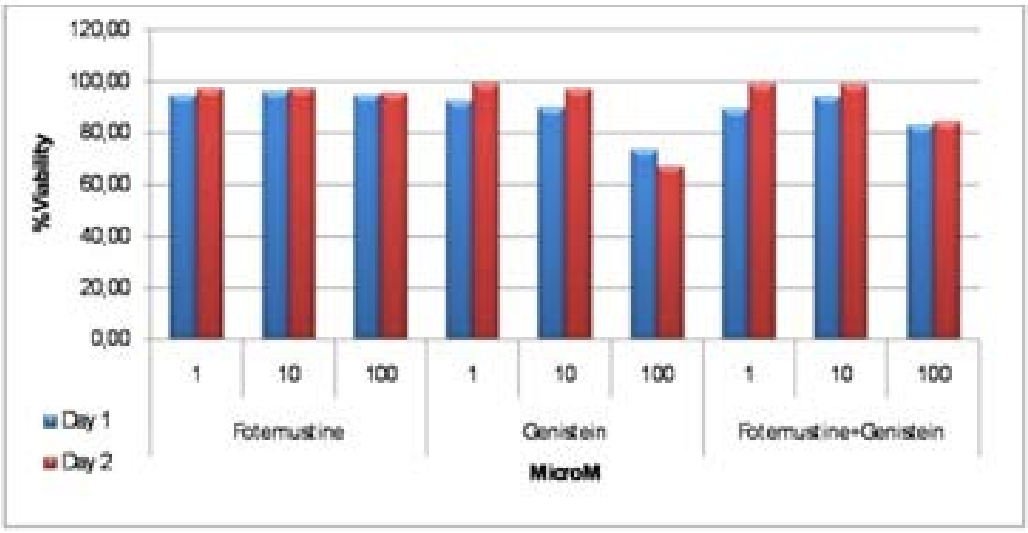

Figure 1: *Results of XTT assay*

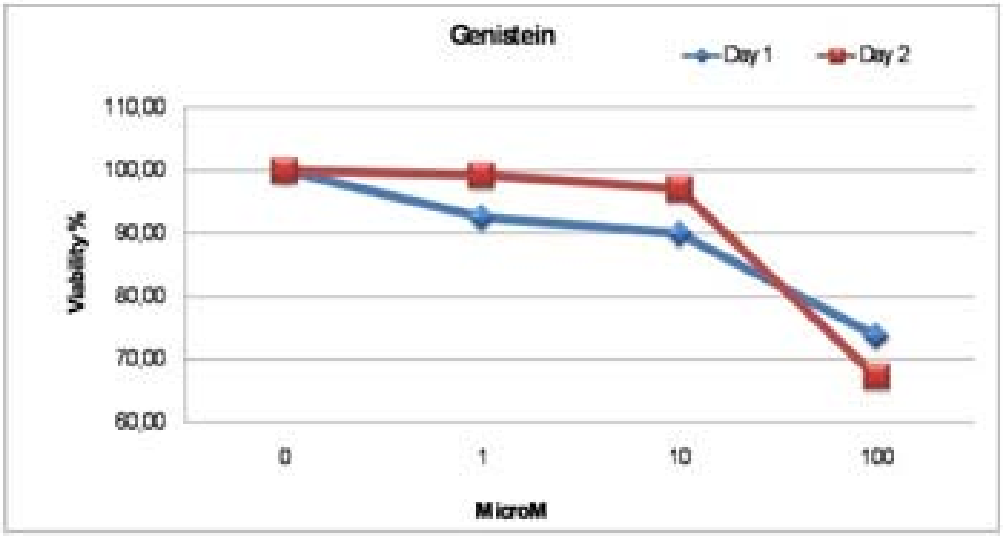

Figure 2: *Linear cytotoxicity of genistein*

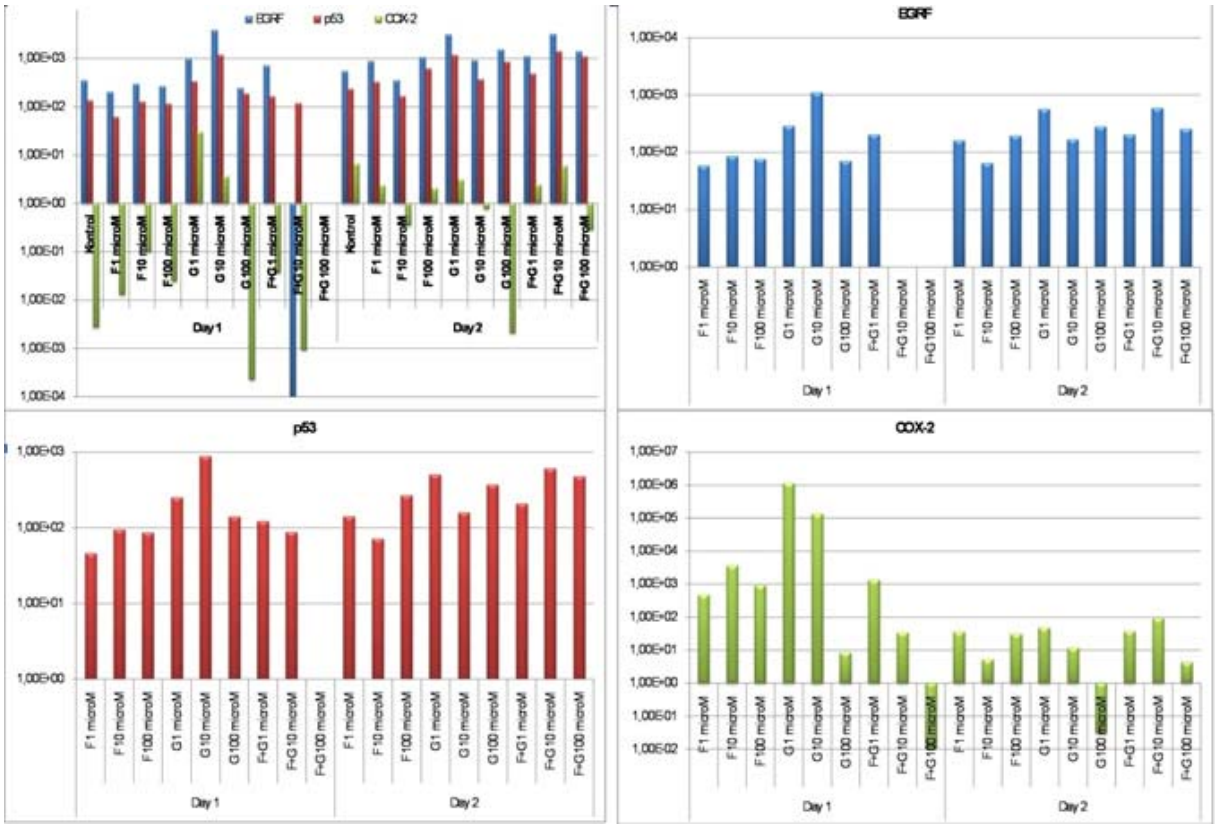

Figure 3: *Relative ratio of p53, EGFR, COX-2 gene expressions*

DISCUSSION

GBM is the most malignant tumor that invades normal brain tissue. The patients with glioblastoma usually have a short survival time which is less than a year. This tumor rarely metastasizes out of the central nervous system[14]. Soy isoflavones are natural compounds that exist in soy-based products in foods and infant formula. Few studies have investigated the effect of isoflavones on gliomas[20]. This study can help investigating the anti-proliferative synergistic effect of genistein with fotemustine on human glioblastoma multiforme U87MG cells.

Examined the studies done by fotemustine and genistein; Fabrini et al sustained second-line chemotherapy with fotemustine to 50 patients with relapsed malignant glioma. The group showed that fotemustine was safe and effective as second-line chemotherapy in recurrent glioblastoma and fotemustine was effective as second-line chemotherapy in recurrent glioblastoma[7]. Malhaire et al treated fotemustine (100 mg/m^2 days 1, 8 and 15) to patients during three weeks period. According to the results, it was seen that 4 patients responded to the treatment (18%), while 6 were stabilized (32%). So researchers didn't find any difference in survival according to the initial performance status of patients before treatment. Therefore, fotemustine seems to represent an interesting well-tolerated treatment possibility in patients with inoperable recurrent malignant gliomas of the brain[18]. Khoshyomn et al, indicated that genistein at typical adult dietary plasma levels can significantly enhance the anti-proliferative and cytotoxic action. 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 sequel for these patients[13]. Puli et al worked on 2 isoflavones that are genistein and biochanin A treated to U87MG cell line. Their results showed that genistein also induced a decrease in EGF-stimulated invasion thereby implicating an involvement of EGF-mediated signaling in invasion[22].

According to the expression results of other studies; Ruano et al analyzed 194 primary GBMs. Although most of the tumors showed a mutually exclusive pattern, concurrent alterations of EGFR and p53 were detected. Their results demonstrated the primary GBM tumors showing simultaneous EGFR and p53 alterations were significantly associated with worse survival ($p<0.01$) [24]. Halatsch et al found their study regardless of the underlying heterogeneity in EGFR mRNA expression and p53 status, MDM2 was similarly over-expressed among the cell lines. For results, they suggested overexpression of wild type EGFR and mutation of p53 in GBM, although considered mutually exclusive in vivo, are not reciprocally prohibitive[8]. Dong et al studied on EGFR expression in samples collected from 37 astrocytic gliomas and 6 normal brain tissue and p53 gene mutation and accumulation were detected simultaneously in the same specimens. According to their results the frequency of p53 mutation in diffuse astrocytomas, anaplastic astrocytomas, primary glioblastoma and secondary glioblastoma was 1/10, 4/19 (21.1%), 4/6 and 2/2, respectively and the frequency of EGFR overexpression was 5/10, 10/19 (52.6%), 5/6 and 2/2, respectively. Therefore they suggested EGFR overexpression and p53 gene mutation are not mutually exclusive in astrocytic gliomagenesis but synergistically to promote the glioma progression[6].

According to the results, there were no significant differences in the expressions of p53, EGFR and COX-2 in treatment of fotemustine and genistein and combination. A decrease was detected in the expression level of COX-2, with the treatment of genistein in time and dose dependent manner. In the first day, a distinct increase in the gene expression levels of p53 and EGFR detected with 10 μM of genistein. In the second day, it was detected that expression of p53 increased with all the doses of genistein and fotemustine and genistein combination.

In conclusion, our results demonstrated that genistein enhance the anti-proliferative and cytotoxic action of fotemustine and reduces the expression of COX-2 gene that is over-expressed and related with poor prognosis in gliomas.

Correspondence to:
Çığır Biray Avcı
E-mail: cigir.biray@ege.edu.tr

Received by: 21 February 2012
Accepted: 15 August 2012

The Online Journal of Neurological Sciences (Turkish) 1984-2012
This e-journal is run by Ege University Faculty of Medicine,
Dept. of Neurological Surgery, Bornova, Izmir-35100TR
as part of the Ege Neurological Surgery World Wide Web service.
Comments and feedback:
E-mail: editor@jns.dergisi.org
URL: http://www.jns.dergisi.org
Journal of Neurological Sciences (Turkish)
Abbr: J. Neurol. Sci.[Turk]
ISSNe 1302-1664

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