JBUON 2016; 21(1): 199-207
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
E-mail: editorial_office@jbuon.com

ORIGINAL ARTICLE

Sensitizer drugs for the treatment of temozolomide-resistant glioblastoma

Adriana Baritchii [1,2], Anca Jurj[3], Olga Soritau[4] , Ciprian Tomuleasa[1,4], Layos Raduly[3], Oana Zanoaga[3], Dana Cernea[5], Cornelia Braicu[3], Ioana Neagoe[3], Ioan Stefan Florian[1,2]

[1]Iuliu Hatieganu University of Medicine and Pharmacy, Cluj Napoca; [2]Department of Neurosurgery - Emergency University Hospital, Cluj Napoca; [3]Functional Genomics , Biomedicine and Translational Medicine Research Center -Iuliu Hatieganu University of Medicine and Pharmacy, Cluj Napoca; [4]Department of Cancer Immunology - Ion Chiricuta Oncology Institute, Cluj Napoca; [5]Department of Radiation Oncology - Ion Chiricuta Oncology Institute, Cluj Napoca, Romania

Summary

Purpose: *Glioblastoma multiforme (GBM) is the most aggressive primary brain tumor. Despite maximal cytoreductive surgery followed by chemoradiotherapy the prognosis is still poor. Although surgery and radiotherapy may have reached maximal effectiveness, chemotherapy has the potential to improve survival. The aim of this study was to evaluate in vitro the antitumor efficacy of tamoxifen (TAM), raloxifen (RAL), pyrimethamine (PYR) and alphanizomenon flos-aquae (AFA) in combination with temozolomide (TMZ) plus ionizing radiation against cultured glioblastoma stem-like cells and primary glioblastoma cells , as possible way to increase the treatment options in patients with therapy-refractory GBM.*

Methods: *Stem-like tumor cells and glioblastoma cells isolated from two GBM biopsies were established by cell proliferation assays. TAM, RAL, PYR and AFA were added prior to TMZ and ionizing irradiation.*

Results: *All tested drugs enhanced the cytotoxic effect of TMZ and sensitized cancer cells to radiotherapy as demonstrated by MTT assay and different staining reagents (TMRE, Hoechst dye and Annexin V) used for monitoring of several events in apoptosis.*

Conclusion: *The administration of certain selective estrogen receptor modulators (SERMs) (TAM and RAL), PYR and AFA before conventional postoperative chemoradiotherapy for glioblastoma might increase therapy efficiency compared to standard oncological treatment. These results need to be extended in vivo on laboratory animals in order to test the encouraging results obtained in vitro.*

Key words: *alphanizomenon flos-aquae, glioblastoma, in vitro model, pyrimethamine, raloxifen, tamoxifen*

Introduction

GBM is the most aggressive primary brain tumor. Standard treatment is surgical resection followed by radiotherapy plus concomitant adjuvant chemotherapy in case of total surgical resection [1]. Despite the continuous effort to improve the multidisciplinary therapeutic approach of these tumors the overall prognosis is still poor, the medial survival being less than one year [2]. Although surgery and radiotherapy may have reached maximal effectiveness, chemotherapy has the potential to improve survival and many studies in the literature are focused on using different drugs that sensitize cancer cells to chemoradiotherapy.

High grade gliomas contain subpopulations of cancer stem cells (CSCs) which are critical in the initiation, maintenance and progression of tumors and in the development of resistance to therapy [3]. The identification and proper characterization of stem cells-like precursors could be the key of developing new tailored therapeutic

Correspondence to: Adriana Baritchii, MD. Department of Neurosurgey-Emergency University County Hospital Cluj Napoca, Victor Babes Street 43, 400012 Cluj Napoca, Romania. Tel: +40 264592771, E-mail: adriana.baritchii@gmail.com, she_adriana@yahoo.com
Received: 03/07/2015; Accepted: 22/07/2015

strategies capable of eradicating these devastating tumors [4].

Some studies showed the prognostic role of CSCs in patients with glioblastoma [5,6]. Prognostic markers of clinical outcomes like neurosphere formation, expression of stem cell markers, such as podoplanin (PDPN), CD133, and nestin have been identified [7-9]. The American Association for Cancer Research suggests that modern anticancer therapies should target these cells via inhibition of their self-renewing capacity or by inducing differentiation of brain CSCs into non-tumorigenic cells, more sensitive to conventional therapy [10].

Due to the disappointing results of the current treatments there is an urgent need of new treatment exploration. Although TMZ crosses the blood brain barrier and has synergistic effect with radiotherapy, the clinical response is in most cases poor. The actual effort is to find a medication with cheap price, higher efficiency and lower toxicity that could sensitize cancer cells to conventional chemoradiotherapy. TAM, an anti-estrogen, is a very important protein kinase C (PKC) inhibitor that can pass through the blood brain barrier and also has radiosensitization effect [11,12]. Second generation SERMs such as RAL, have been shown to improve functional recovery after bilateral cortical contusion injury [13], has well-documented antioxidant effects [14,15] and is able to decrease the inflammatory response caused by lipopolysaccharide (LPS) in mouse and rat microglia cells in vitro [16]. The antifolate activity of pyrimethamine was demonstrated to enhance TMZ efficiency in melanoma and glioma cells [17]. AFA is commonly used in alternative medicine. A study published in 2011 showed that algae extracts may inhibit acute myeloid leukemia (AML) cell lines and leukemia blasts [18].

The purpose of this study was to evaluate the combined therapeutic effect of TMZ, TAM, RAL, PYR and AFA on glioblastoma cells.

Methods

Drug preparation

TMZ, TAM and RAL were dissolved in dimethylsulphoxide (DMSO) while pyrimethamine was dissolved in 70% ethanol. All drugs were stored at -20°C. Preparation of algal extract was made by adding one gram of algae to 10 ml of 70% ethanol and incubated at 4°C on a shaker for 24 hrs. The slurry was centrifuged at 400 g for 10 min at 4°C and the supernatant was filtered through 413-grade filter paper. The resulting extract was kept in the dark at -80°C. All drugs were stored as a stock solution and dilutions were made using bidistilled water.

Cell culture

CSCs and primary glioma cells were isolated from two glioblastoma biopsies. One hour after tumor removal the tumors material was minced with fine scissors into fragments of 1×1×1 mm^3 and cultured in 1 ml of fetal calf serum (FCS) in Petri dishes. Four hrs later, 2 ml of Dulbecco's Modified Eagles Medium (DMEM; Sigma-Aldrich, St. Louis, USA) were added, supplemented with fetal calf serum (FCS), antibiotics (penicillin 100 IU/ml, streptomycin 100 µg/ml), non-essential aminoacids and glutamine (all were purchased from Sigma Aldrich, St Louis, MO, USA). The explants were stored in a 37°C incubator with 95% air and 5% CO_2. One week after, a monolayer of tumor cells was formed. CSCs were proven to be CD133+, Oct ¾+, CD 90+ and Nanog +.

Survival assay

Cell survival was assessed using the MTT assay. The CSCs were seeded at 5000 cells per well in 96-well plates, whereas primary glioblastoma cells were plated at a density of $1x10^4$. They were incubated at 37°C overnight to allow them to attach. The drugs were added to cell cultures either alone or in combination with 50 µg/mL TMZ or 1 µg/mL methotrexate (MTX). The drug concentrations used were: TAM 2.75 µg/mL, RAL 15 µg/mL, PYR 15 µg/mL and AFA 1.75 mg/mL. After 48 hrs half of the plates were irradiated with 2 Gy as used in the clinic [19], at the Radiotherapy Department of "Prof. Dr. Ion Chiricuta" Oncology Institute, using a high megavoltage source (Theratron 1000 ^{60}Co source).

Determination of apoptosis

Caiman's Multi-Parameter Apoptosis Assay Kit (Michigan, USA) was used as a measure of early apoptosis induction in CSCs samples. This includes 4 different reagents for monitoring several events in apoptosis and it employs FITC-conjugated Annexin V as a probe for phosphatidylserine on the outer membrane of apoptotic cells, TMRE as a probe for mitochondrial membrane potential, 7-AAD as an indicator of membrane permeability/cell viability, and Hoechst dye to demonstrate nuclear morphology. The staining protocol provided by the company was used.

Statistics

Statistical analyses were done using Prism 6.0 statistics program for Windows (GraphPad, San Diego, USA). All data were analyzed using one-way ANOVA with the Bonferroni multiple comparison test (Kruskal-Wallis as nonparametric). A p value of <0.05 was required in order to determine a statistically significant value.

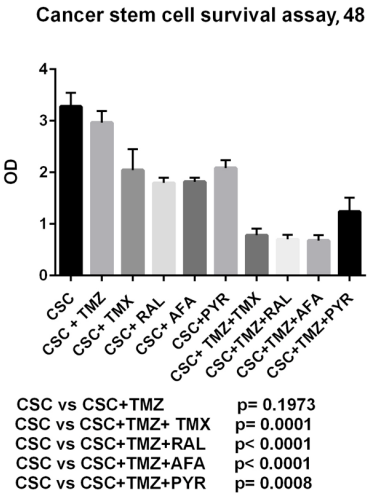

Figure 1. Cancer stem cells were resistant to temozolomide and were partially killed by the other tested drugs. The effect was more enhanced when combined with temozolomide. OD: optical density.
For other abbreviations see text.

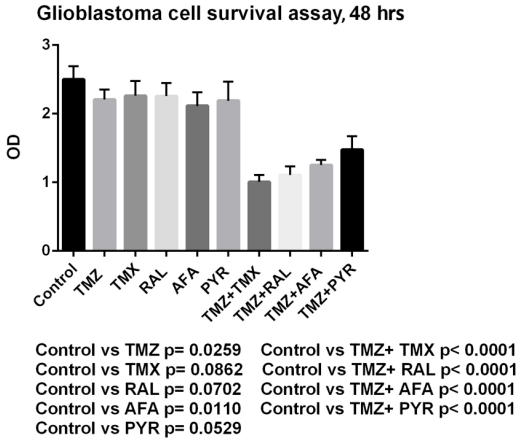

Figure 2. Even if the primary glioblastoma cells were initially resistant to all tested drugs, when combined with temozolomide the survival curves shifted downward dramatically. OD: optical density. For other abbreviations see text.

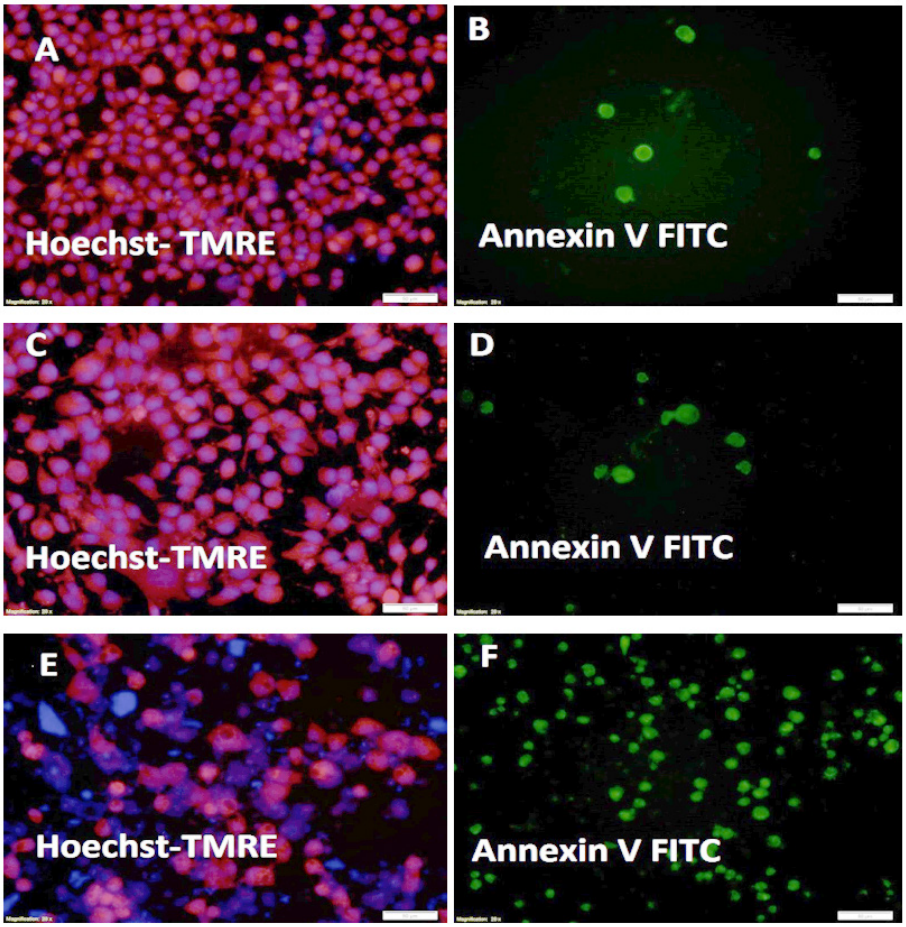

Figure 3. TMZ-RAL induced apoptosis in CSC as measured by a decrease in mitochondrial membrane potential, nuclear morphology and an increase of Annexin V FITC positive cells. **A-B** control cells; **C-D** TMZ treated cancer stem cells; **E-F** TMZ-RAL treated cancer stem cells.

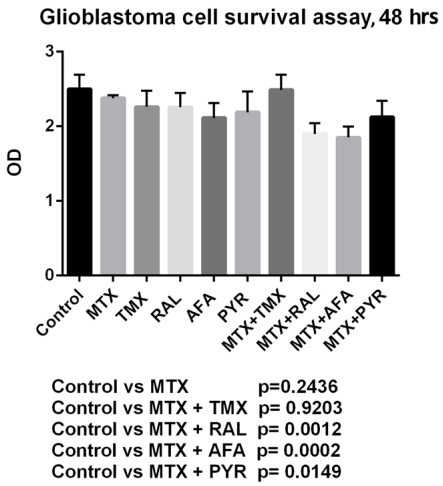

Figure 4. Primary glioblastoma cells proved to be also resistant to methotrexate and also to the combination of MTX/TMX, MTX/PYR. A significant statistical difference was noted when combining MTX with RAL and AFA.

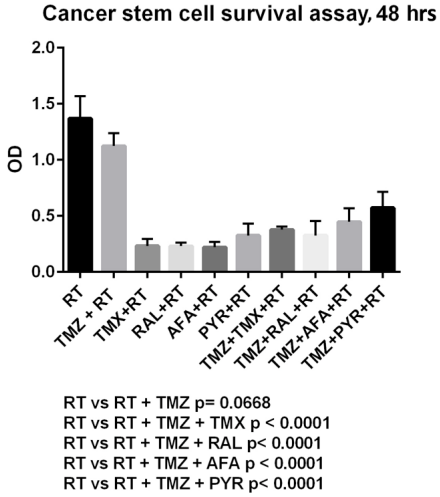

Figure 5. Cancer stem cells were sensitized by all drugs and combinations -except temozolomide- to standard radiotherapy (2 Gy).

Results

We examined the effects of TMZ, MTX, TMX, RAL, AFA and PYR alone or in combinations (TMZ/TMX, TMZ/RAL, TMZ/AFA, TMZ/PYR; MTX/TAM, MTX/RAL, MTX/AFA, MTX/PYR) on glioblastoma cell proliferation. We have tested both CSCs and primary glioblastoma cells.

When adding each of the tested drugs prior to treatment the results showed a reduced survival of CSCs for TMX, RAL AFA and PYR but not for TMZ (Figure 1). On the other hand, primary glioblastoma cells were resistant to all tested drugs (Figure 2). Statistical analyses showed no significant survival in the case of control vs TMZ (95% CI from -0.1653 to 0.7990), control vs TAM (95% CI from -0.2200 to 0.7442), control vs RAL (95% CI from -0.2158 to 0.7485), control vs AFA (95% CI from -0.07429 to 0.08900) and control vs PYR (95% CI from -0.1485 to 0.8157). Bonferroni's multiple comparison test showed statistically significant survival benefit ($p<0.05$) between control vs TMZ+TAM (95% CI from 1.035 to 2.00), and control vs TMZ+ RAL (95% CI from 0.9337 to 1.898), control vs TMZ+ AFA (95% CI from 0.7890 to 1.753), control vs TMZ+ PYR (95% CI from 0.5640 to 1.528). In CSC survival statistically beneficial data were obtained between CSC vs CSC+TMZ+TAM (95% CI from 0.9526 to 4.604), CSC vs CSC+TMZ+RAL (95% CI from 1.342 to 4.472), CSC vs CSC+TMZ+AFA (95% CI from 1.317 to 4.532) and CSC vs CSC+TMZ+PYR (95% CI from 0.1775 to 4.468)

CSCs were processed for staining of nuclei with Hoechst dye, mitochondrial membrane potential with TMRE and Annexin V FITC. In Figure 3, images A and B show the cells from the control group, images C and D show the CSCs treated with TMZ, whereas images E and F represent the cells treated with combination of TMZ and RAL. Hoechst staining showed that most control and TMZ-treated cells had round and intact nuclei while the nucleus of the cells treated with the combination TMZ-RAL were fragmented. TMRE staining revealed that most control and TMZ-treated cells had undisrupted mitochondrial membrane potential, whereas TMZ+RAL-treated cells were not stained because they had diminished mitochondrial membrane potential. Most control and TMZ-treated cells were Annexin V negative (Figure 3 B,D) while CSCs treated with TMZ-RAL were mostly Annexin V positive, indicating that cells were undergoing apoptosis.

In order to evaluate the response of primary glioblastoma cells to other chemotherapeutic drug we tested MTX alone and in combination with TAM, RAL, AFA and PYR (Figure 4). The cancer cells were resistant to MTX and statistical

Table 1. Mean difference and 95% CI for primary glioblastoma cells treated with MTX alone or in combination with TAM, RAL, AFA and PYR

Bonferroni multiple comparisons test	*Mean diff*	*95% CI of diff*	*Significant?*
Control vs. MTX	0,1228	-0,2887 to 0,5343	No
Control vs. TMX	0,2410	-0,1704 to 0,6525	No
Control vs. RAL	0,2453	-0,1662 to 0,6568	No
Control vs. AFA	0,3868	-0,02470 to 0,7983	No
Control vs. PYR	0,3125	-0,09895 to 0,7240	No
Control vs. MTX+TAM	0,01254	-0,3989 to 0,4240	No
Control vs. MTX+RAL	0,6023	0,1493 to 1,055	Yes
Control vs. MTX+AFA	0,6513	0,2398 to 1,063	Yes
Control vs. MTX+PYR	0,3775	-0,03395 to 0,7890	No

For abbreviations see text

analyses showed a significant survival benefit in case of control vs MTX/RAL (95% CI from 0.1493 to 1.055) and control vs MTX/AFA (95% CI from 0.2398 to 1.063) (Table 1).

CSCs treated with TAM, RAL, AFA and PYR were sensitized to radiotherapy but the combination of these drugs with TMZ showed no additional benefit (Figure 5). Primary glioblastoma cells were killed when treated with TMZ, TAM, RAL and AFA prior to radiotherapy. Primary glioblastoma cells were killed when treated with TMZ,TAM,RAL and AFA prior to radiotherapy (Table 2). PYR showed no significant statistical difference comparing with control cells but when combined with TMZ the survival curves shifted downward dramatically (Figure 6).

Discussion

Today's standard therapy for glioblastoma is maximal tumor resection followed by chemoradiotherapy [19]. TMZ, a DNA methylating agent, was proved to provide some survival advantage, enhancing the radiosensitivity of cancer cells [20] and therefore is the front line drug for the treatment of glioblastoma. Resistance to chemoradiotherapy and the aggressive growth and infiltration of these tumors have led to little improvement in the treatment of this brain cancer. CSCs were demonstrated to influence the tumor growth and resistance to treatment [21] and to have a prognostic role in predicting the clinical outcome in primary glioblastoma patients [6]. Since the pre-Röentgen era, cancer recurrences have been attributed to CSCs [22]. Overcoming the resistance of CSCs is a major priority on the way of achieving clinical cure of cancer.

A series of studies have demonstrated that the proliferation of high grade gliomas relies partially on the activation of protein kinase C (PKC)

Table 2. Mean difference and 95% CI for primary glioblastoma cells treated with TMZ alone or in combination with TMX, RAL, AFA, PYR and radiotherapy

Bonferroni multiple comparisons test	*Mean diff*	*95% CI of diff*	*Significant?*
Control + RT vs. TMZ+RT	0,5687	0,2398 to 0,8975	Yes
Control + RT vs. TMX+RT	0,4964	0,1676 to 0,8252	Yes
Control + RT vs. RAL+RT	0,6552	0,3263 to 0,9840	Yes
Control + RT vs. AFA+RT	0,4742	0,1453 to 0,8030	Yes
Control + RT vs. PYR+RT	0,2684	-0,06040 to 0,5972	No
Control + RT vs. TMZ+TMX+RT	0,08142	-0,2474 to 0,4102	No
Control + RT vs. TMZ+RAL+RT	0,08167	-0,2472 to 0,4105	No
Control + RT vs. TMZ+AFA+RT	-0,1963	-0,5252 to 0,1325	No
Control + RT vs. TMZ+PYR+RT	0,8839	0,5551 to 1,213	Yes

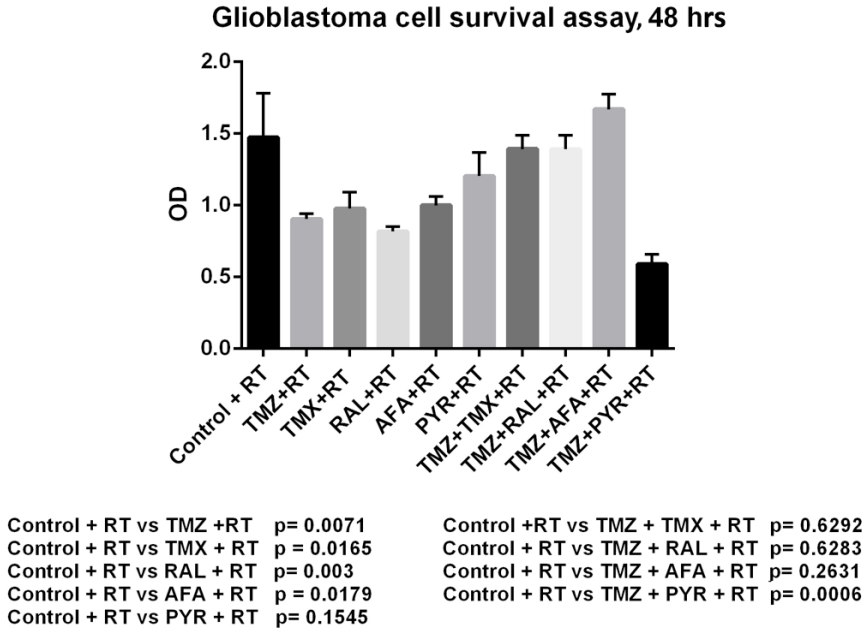

Figure 6. Primary glioblastoma cells showed a better response to temozolomide when combined with radiotherapy. The only drug combination that proved to be efficient with radiotherapy was TMZ/PYR in this case.

mediated pathways [23-26], and blocking this enzyme could provide a novel approach for inhibition of glioma cell growth. This led to increasing attention of glioma researchers for TAM, an antiestrogen that has a significant PKC inhibition activity. TAM was proved to block glioma cells *in vitro* at concentration several fold higher than the dose used for its traditional role as an estrogen receptor–binding agent [27-30]. This drug is now being evaluated in clinical trials in patients with malignant gliomas [31]. Even if the role of TAM in the treatment of high grade gliomas was studied in the last 2 decades, the efficacy of RAL, a second generation of SERMs is not documented.

RAL is a drug registered for the treatment of osteoporosis [32]. SERMs have both estrogen-agonistic and antagonistic properties depending on the target tissue, cell or even gene. *In vitro*, RAL has antiestrogen activity on breast tumor cells and it was suggested that is associated with a decreased incidence of invasive breast cancer [33]. Some new researches showed that RAL induces autophagy via the activation of AMPK by sensing decreases in ATP and the overactivation of autophagy promotes cell death and thereby mediates the anti-cancer effects of this drug in breast cancer cells [34]. A study published in 2006 suggested that RAL is a very promising drug for the treatment of multiple myeloma, inducing myeloma cell cycle arrest and apoptosis partly through NF-κB-dependent mechanisms [35]. The neuroprotective actions of RAL have been assessed in different experimental models. A study showed that TAM and RAL are able to decrease the inflammatory response caused by lipopolysaccharide (LPS) in mouse and rat microglia cells *in vitro* [16]. Another study suggested that RAL decreases the number of astrocytes and microglia in the brain of aged animals [36]. Several SERMs - including RAL - have shown to reduce neuronal loss in the hippocampus of rats after administration of the excitotoxin kainic acid [37]. A randomized controlled clinical trial showed that treatment with 120 mg RAL for 3 years may successfully delay the onset of mild clinical cognitive impairment and possibly Alzheimer's disease in elderly women [38]. The effect of RAL on glioma cells need to be explored.

In our study we have shown that RAL exhibited the same inhibitory effect on CSCs as TAM. In case of primary glioblastoma cells that proved to be resistant to both SERMs, the survival curves shifted downward dramatically when adding TMZ. The two drugs also acted as radiosensitizers for CSCs alone but the combination with TMZ failed to prove an additional benefit.

PYR is an effective dihydrofolate reductase inhibitor, especially in protozoa such as toxoplasma and malaria. Antifolates have been studied for many years for their anticancer effect. Dihydro-

folate reductase inhibitors have selective toxicity on rapidly dividing cells, such as tumor cells, due to disruption of folic acid metabolism which is essential for the *de novo* synthesis of the nucleoside thymidine. PYR, a lipophilic antifolate, in addition to antiprotozoal action also has a strong proapoptotic activity. Some PYR analogues -DDEP and DDMP- have been studied as antitumor agents since 6 decades ago [39]. In various cells lines PYR can produce apoptosis and S phase accumulation as shown in different studies [40-44]. In cell culture, folate deficiency induces an excess of strand breaks in DNA [44,45]. 06 methylguanine-DNA transferase (MGMT), by directly removing O^6meG, plays a key role in the repair of DNA damage induced by TMZ [46]. The MGMT level was proved to be susceptible to epigenetic silencing [47]. A study published in 2009 showed that in melanoma, the enhancement of TMZ efficacy by PYR is independent of MGMT level and therefore PYR-induced sensitization of cells to TMZ treatment was observed in both MGMT-expressing and MGMT-negative cells [17].

This study has shown that the combination of TMZ with PYR was effective for both CSCs and primary glioblastoma cells. PYR could be an attractive option to improve the efficiency of TMZ in glioblastoma patients due to its bioavailability after oral administration and also the fact that PYR has been safely administered to malarial patients for prolonged periods with limited side effects. Moreover, because it is a lipophilic antifolate agent it can easily cross the blood brain barrier.

There is a growing interest among patients to utilize traditional complementary medicinal compounds, and cancer patients make no exception from this rule. This inspired us to test an algae extract with claims of boosting the immune system and preventing cancer. Spirulina and AFA are the most common edible cyanobacteria [48] which contain phycocyanin, a molecule that was proved to induce apoptosis in the chronic myeloid leukemia cell line K562 [49] and other types of cancer [50-52]. AFA, a freshwater species of cyanobacteria, is known to produce hepatotoxin cylindrospermopsin (CYN) [53] and paralytic shellfish poisons, a potent neurotoxin [54,55]. A study of Hart et al. suggested that the extract of AFA is a potent *in vitro* activator of NK cells that are capable of killing some tumor cells without prior sensitization to antigen [48]. Another study of Pugh and Pasco showed that AFA extract activated the monocyte cell line THP-1 [56]. In the present study, we demonstrated that ethanol extracts from AFA inhibited CSCs and primary glioblastoma cells when combined with TMZ.

Conclusion

The disappointing results of current malignant glioma therapy compel to continuous research and improvement of today's knowledge over high grade gliomas biology and treatment. On this long road every new study represents a small step on the way of achieving clinical improvement of this brain cancer. Our data suggest that there are some drugs not so well documented so far for the treatment of high grade gliomas that can sensitize glioblastoma cells to chemoradiotherapy. These studies need to be extended not only in vitro but also in vivo in order to prove the long expected efficiency on malignant gliomas' treatment.

Acknowledgements

This work was financially supported by Functional Genomics, Biomedicine and Translational Medicine Research Center -Iuliu Hatieganu University of Medicine and Pharmacy, Cluj Napoca.

References

1. Stupp R, Mason WP, van den Bent MJ et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 2005;352:987-996.
2. Robins HI, Peterson CG, Mehta MP. Combined modality treatment for central nervous system malignancies. Semin Oncol. 2003;30 (Suppl 9):11-22.
3. Sulman E, Aldape K, Colman H. Brain tumor stem cells. Curr Probl Cancer 2008;32:124-142.
4. Tomuleasa C, Soritau O, Rus-Ciuca D et al. Functional and molecular characterization of glioblastoma multiforme derived cancer stem cells. J BUON 2010;15:583-591.
5. Kim KJ, Lee KH, Kim HS et al. The presence of stem cell marker-expressing cells is not prognostical-

ly significant in glioblastomas. Neuropathology 2011;31:494-502.

6. Kong BH, Moon JH, Huh YM et al. Prognostic Value of Glioma Cancer Stem Cell Isolation in Survival of Primary Glioblastoma Patients. Stem Cells Int 2014, Article ID 838950.
7. Laks DR, Masterman-Smith M, Visnyei K et al. Neurosphere formation is an independent predictor of clinical outcome in malignant glioma. Stem Cells 2009;27:980-987.
8. Mishima K, Kato Y, Kaneko MK, Nishikawa R, Hirose T, Matsutani M. Increased expression of podoplanin in malignant astrocytic tumors as a novel molecular marker of malignant progression. Acta Neuropathologica 2006;11:483-488.
9. Dahlrot RH, Hermansen SK, Hansen S, Kristensen BW. What is the clinical value of cancer stem cell markers in gliomas?" Int Clin Exper Pathol 2013;6:334-348.
10. Clarke MF, Dick JE, Dirks PB et al. Cancer stem cells-perspectives on current status and future directions: AACR workshop on cancer stem cells. Cancer Res 2006;66:9339-9344.
11. Pollack IF, DaRosso RC, Robertson PL et al. A phase I study of high-dose tamoxifen for the treatment of refractory malignant gliomas of childhood. Clin Cancer Res 1997;3:1109-1115.
12. Zhang W, Couldwell WT, Song H et al. Tamoxifen induced enhancement of calcium signaling in glioma and MCF-7 breast cancer cells. Cancer Res 2000;60:395-400.
13. Kokiko ON, Murashov AK, Hoane MR. Administration of raloxifene reduces sensorimotor and working memory deficits following traumatic brain injury. Behav Brain Res 2006;170:233-240.
14. Biewenga E, Cabell L, Audesirk T. Estradiol and raloxifene protect cultured SN4741 neurons against oxidative stress. Neurosci Lett 2004;373:179-183.
15. Konyalioglu S, Durmaz G, Yalcin A. The potential antioxidant effect of raloxifene treatment: a study on heart, liver and brain cortex of ovariectomized female rats. Cell Biochem Funct 2007;25:259-266.
16. Suuronen T, Nuutinen T, Huuskonen J, Ojala J, Thornell A, Salminen. Anti-inflammatory effect of selective estrogen receptor modulators (SERMs) in microglial cells. Inflam Res 2005;54:194-203.
17. Chen M, Osman I, Orlow SJ. Antifolate activity of pyrimethamine enhances temozolomide-induced cytotoxicity in melanoma cells. Mol Cancer Res 2009;7:703-712.
18. Bechelli J, Coppage M, Rosell K, Liesveld J. Cytotoxicity of algae extracts on normal and malignant cells. Leuk Res Treat 2011;2011:373519.
19. Stupp R, Mason WP, van den Bent MJ et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 2005;352:987-996.
20. Taphoorn MJ, Stupp R, Coens C et al. EORTC, Brain Tumor Group; EORTC Radiotherapy Group; NCI of Canada, Clinical Trials Group. Health-related quality of life in patients with glioblastoma: a randomised controlled trial. Lancet Oncol 2005;6:937-944.
21. Sulman E, Aldape K, Colman H. Brain tumor stem cells. Curr Probl Cancer 2008;32:124-142.
22. Cohnheim J. Uber Entzündung und Eiterung (About inflammation and suppuration) Virchows Arch 1867;40:1-79.
23. Baltuch GH, Dooley NP, Villemure JG, Yong VW. Protein kinase C and growth regulation of malignant gliomas. Can J Neurol Sci 1995;22:264-271.
24. Gelmann EP. Tamoxifen for the treatment of malignancies other than breast and endometrial carcinoma. Semin Oncol 1997;24 (Suppl):S 65-70.
25. Leslie KK, Keefe D, Powell S, Naftolin F. Estrogen receptors are identified in the glioblastoma cell line U138MG. J Soc Gynecol Invest 1994;1:238-244.
26. Weller M, Trepel M, Grimmel C et al. Hypericin-induced apoptosis of human malignant glioma cells is light-dependent, independent of bcl-2 expression, and does not require wild-type p53. Neurol Res 1997;19:459-470.
27. Couldwell WT, Hinton DR, Surnock AA et al. Treatment of recurrent malignant gliomas with chronic oral high-dose tamoxifen.Clin Cancer Res 1996;2:619-622.
28. Chang SM, Barker FG, Huhn SL et al. High dose oral tamoxifen and subcutaneous interferon alpha-2a for recurrent glioma. J Neurooncol 1998;37:169-176.
29. Mastronardi L, Farah JO, Puzzilli F, Ruggeri A. Tamoxifen modulation of carboplatin cytotoxicity in a human U-138 glioma cell line. Clin Neurol Neurosurg 1998a;100:89-93.
30. Li C, Zhou C, Wang S et al. Sensitization of glioma cells to tamoxifen-induced apoptosis by Pl3-kinase inhibitor through the GSK-3β/β-catenin signaling pathway. PLoS One 2011;6(10):e27053.
31. Patel S, DiBiase S, Meisenberg B et al. Phase I clinical trial assessing temozolomide and tamoxifen with concomitant radiotherapy for treatment of high-grade glioma. Int J Radiat Oncol Biol Phys 2012;82:739-742.
32. Delmas PD, Ensrud KE, Adachi JD et al. Efficacy of raloxifene on vertebral fracture risk reduction in postmenopausal women with osteoporosis: four-year results from a randomized clinical trial. J Clin Endocrinol Metab 2002;87:3609-3617.
33. Martino S, Cauley JA, Barrett-Connor et al. Continuing outcomes relevant to Evista: breast cancer incidence in postmenopausal osteoporotic women in a randomized trial of raloxifene. J Natl Cancer Inst 2004;96:1751-1761.
34. Kim DE, Kim Y, Cho DH et al. Raloxifene Induces Autophagy-Dependent Cell Death in Breast Cancer Cells via the Activation of AMP-Activated Protein Kinase. Mol Cells 2015;28:138-144.
35. Olivier P, Close P, Emilie Castermans E et al. Raloxifene-Induced Myeloma Cell Apoptosis: A Study of Nuclear Factor-κB Inhibition and Gene Expression Signature. Mol Pharmacol 2006; 69:1615-1623.
36. Lei DL, Long JM, Hengemihle J et al. Effects of estrogen and raloxifene on neuroglia number and morphology in the hippocampus of aged female mice. Neuroscience 2003;121:659-666.

37. Ciriza I, Carrero P, Azcoitia I, Lundeen SG, Garcia-Segura LM. Selective estrogen receptor modulators protect hippocampal neurons from kainic acid excitotoxicity: differences with the effect of estradiol. J Neurobiol 2004;61:209–221.
38. Yaffe K, Krueger K, Cummings SR et al . Effect of raloxifene on prevention of dementia and cognitive impairment in older women: the multiple outcomes of raloxifene evaluation (MORE) randomized trial. Am J Psychiatry 2005;162:683-690.
39. Murphy ML, Ellison RR, Karnofsky DA, Burchenal JH. Clinical effects of the dichloro and mono-chlorophenyl analogues of diamino pyrimidine: antagonists of folic acid. J Clin Invest 1954;33:1388-1396.
40. James SJ, Pogribny IP, Pogribna M, Miller BJ, Jernigan S, Melnyk S. Mechanisms of DNA damage, DNA hypomethylation, and tumor progression in the folate/methyl-deficient rat model of hepatocarcinogenesis. J Nutr 2003;133:S3737–3740.
41. Lin H, Chen C, Tsai W, Yen J, Liu H. In vitro folate deficiency induces apoptosis by a p53, Fas (Apo-1, CD95) independent, bcl-2 related mechanism in phytohaemagglutinin-stimulated human peripheral blood lymphocytes. Br J Nutr 2006;95:870-878.
42. Huang RF, Ho YH, Lin HL, Wei JS, Liu TZ. Folate deficiency induces a cell cycle-specific apoptosis in HepG2 cells. J Nutr 1999;129:25-31.
43. Giammarioli AM, Maselli A, Casargrande A et al. Pyrimethamine induces apoptosis of melanoma via a caspase and cathepsin double-edged mechanism. Cancer Res 2008;68:5291-5300.
44. Branda RF, Blickensderfer DB. Folate deficiency increases genetic damage caused by alkylating agents and γ-irradiation in Chinese hamster ovary cells. Cancer Res 1993;53:5401-5408.
45. Courtemanche C, Huang AC, Elson-Schwab I, Kerry N, Ng BY, Ames BN.. Folate deficiency and ionizing radiation cause DNA breaks in primary human lymphocytes: a comparison. FASEB J 2004;18:209-211.
46. Jackson SP. Sensing and repairing DNA double-strand breaks. Carcinogenesis 2002;23:687-696.
47. Donson AM, Addo-Yobo SO, Handler MH, Gore L, Foreman NK. MGMT promoter methylation correlates with survival benefit and sensitivity to temozolomide in pediatric glioblastoma. Pediatr Blood Cancer 2007;48:403-407.
48. Hart AN, Zaske LA, Patterson KM, Drapeau C, Jensen GS. Natural killer cell activation and modulation of chemokine receptor profile in vitro by an extract from the cyanophyta Aphanizomenon flos-aquae. J Medicinal Food 2007;10:435-441.
49. Subhashini J, Mahipal SVK, Reddy MC, Reddy MM, Rachamallu A, Reddanna P. Molecular mechanisms in C-Phycocyanin induced apoptosis in human chronic myeloid leukemia cell line-K562. Biochem Pharmacol 2004;68:453-462.
50. Li B, Chu X, Gao M, Li W. Apoptotic mechanism of MCF-7 breast cells in vivo and in vitro induced by photodynamic therapy with C-phycocyanin. Acta Biochim Biophys Sinica 2010;42:80-89.
51. Basha OM, Hafez RA, El-Ayouty YM, Mahrous KF, Bareedy MH, Salama AM. C-Phycocyanin inhibits cell proliferation and may induce apoptosis in human HepG2 cells. Egypt J Immunol 2008;15:161-167.
52. Pardhasaradhi BV, Ali AM, Kumari AL, Reddanna P, Khar A. Phycocyanin-mediated apoptosis in AK-5 tumor cells involves down-regulation of Bcl-2 and generation of ROS. Molec Cancer Ther 2003;2:1165-1170.
53. Preußel K, Stüken A, Wiedner C, Chorus I, Fastner J. First report on cylindrospermopsin producing Aphanizomenon flos-aquae (Cyanobacteria) isolated from two German lakes. Toxicon 2006;47:156-162.
54. Liu Y, Chen W, Li D, Shen Y, Liu Y, Song L. Analysis of paralytic shellfish toxins in Aphanizomenon DC-1 from Lake Dianchi, China. Environ Toxicol 2006;21:289-295.
55. Liu Y, Chen W, Li D, Shen Y, Li G, Liu Y. First report of aphantoxins in China-waterblooms of toxigenic Aphanizomenon flos-aquae in Lake Dianchi. Ecotoxicol Environ Safety 2006;65:84-92.
56. Pugh N, Pasco DS. Characterization of human monocyte activation by a water soluble preparation of Aphanizomenon flos-aquae. Phytomedicine 2001;8:445-453.