Original Paper

# The Effect of B-Arrestin2 Overexpression Regarding Viability and Temozolomide Treatment in High-Grade Glioma Cells

ALEXANDRU OPRITA[1], GEORGIANA ADELINE STAICU[1], CARINA BALOI[1], ANICA DRICU[1], STEFAN ALEXANDRU ARTENE[1]

[1]*Units of Biochemistry, Faculty of Medicine, University of Medicine and Pharmacy of Craiova, Romania*

**ABSTRACT:** The β-arrestins (β-arr) family are proteins that regulate the signaling and trafficking of various G protein-coupled receptors. Out of the four members, β-arr 1 and 2 have been proven as essential actors behind different processes that lead to the progression of cancer as cell proliferation, migration, invasion and metastasis. In addition to this, these proteins are also capable of transmitting anti-apoptotic signals, influence tumor growth rate and drug resistance. Several studies have demonstrated that β-arr 2 overexpression corelates with an impaired overall survival and also showed that it may mediate multidrug resistance in certain types of cancer. In the current study we analyzed the effect of β-arr 2 overexpression on proliferation and how it affects Temozolomide (TMZ) response on the CL2:6 High Grade Glioma (HGG) cell line. We observed contradictory results after transfection, with β-arr 2 overexpressing cells having a superior proliferation rate after 24 and 48h, when compared to untransfected cells, while the opposite was noted after 72h. In terms of response to TMZ, we observed a similar contradictory pattern with modest differences between doses being observed at 24h, while the smallest and largest doses in our experiment produced opposite effects after 48h and 72h. This further underscores the scarcity of information regarding the exact roles and the importance of β-arrs in the intrinsic mechanisms which govern cancer cells.

***KEYWORDS:*** *Glioblastoma, temozolomide, β-arrestin2.*

## Introduction

In the last decade, several cancer treatment options have been made available to the current stand of care (SOC) which saw an immense improvement in survival and quality of life for patients suffering from a great number of cancer types.

Unfortunately, the vast majority of brain tumors, more specifically, HGGs, have seen little to no benefit from the addition of new therapeutic agents.

The standard of care (SOC) for the most aggressive type of HGGs, glioblastomas (GBM), has remained mostly unchanged since 2005 and consists of surgery followed by concomitant radiotherapy and chemotherapy with TMZ.

While long term survivals have been observed, over 99% of patients suffering from GBMs have an median overall survival (mOS) of just 14.7 months, with a 5-year survival of under 5% [1].

This abysmal prognosis can be down to a plethora of factors but in many cases, it is strongly related to inherited or acquired drug resistance.

For the vast majority of GBMs, resistance may be a consequence of increased O (6)-Methylguanine - DNA - methyltransferase (MGMT) activity.

One study comparing activity MGMT in brain tumors, found a value of 37fmol/mg protein in untreated tumor samples versus 182fmol/mg protein in the recurrent sample, a result of either selection of MGMT-expressing cells or MGMT induction caused by the use of alkylating agents [2].

Even though the incidence of microsatellite instability (MSI) is low in HGGs, there have been studies which showed high levels of MSH6 gene deficiency in recurrent GBMs which were previously treated with an alkylating agent [3].

Multidrug resistance (MDR) has also been observed in HGGs with high levels of MDR proteins such as (MRP)1, MRP3, MRP5, and glutathione-S-transferase (GST) π [4] being present.

The arrestins are a protein family consisting of four members [5], two of them being visual arrestins, respectively arrestin 1 and arrestin 4, the former being localized in the retinal rods and cones while the latter is found exclusively in the retinal cones.

The other two members: arrestin 2 (β-arr 1) and arrestin 3 (β-arr 2) are ubiquitous to the human body, where they regulate signaling and trafficking of various G protein-coupled receptors (GPCRs) [6].

Their role in cancer progression is accomplished through multiple molecular signal pathways including GPCRs, transforming growth

10.12865/CHSJ.48.04.07

factor-β (TGF-β), Hedgehog, Wingless, Notch pathways, mitogen activated protein kinases (MAPK) and AKT/phosphatidylinositol 3 kinase (PI3K) cascades [7].

One study by Woom-Yee Bae et. al. demonstrated that β-arr 2 binds to the hypoxia-inducible factor-1 (HIF-1), a transcription factor that facilitates adaptation to low oxygen concentrations and more specifically HIF-1α which is responsible for hypoxic specific functions.

The β-arr 2-HIF-1α combination stimulated proteasome-mediated degradation of HIF-1α by recruiting the von Hippel-Lindau tumour suppressor protein (pVHL) and prolyl hydrohydroxylase domain (PHD) proteins in GBM proving that a high β-arr 2 expression correlates with reduced tumorigenesis and angiogenesis in GBM [8].

Another study by Sandeep K. Raghuwanshi et. al. used β-arr 2-deficient mice (β-arr 2-/-) to prove its' role in tumorigenesis by inoculating them with Lewis lung cancer (LLC) cells.

he results showed that β-arr 2-/-mice exhibited a significant increase in LLC tumor growth and metastatic capacity in comparison to the control group.

Additionally, the β-arr 2-/-presented a decreased number of tumor-infiltrating lymphocytes but elevated levels of ELR+chemokines, vascular endothelial growth factor (VEGF), and increased microvessel density, thus showing that β-arr 2 depletion may promote angiogenesis, tumour development and metastasis [9].

The aim of this study is to assess how β-arr 2 transfection would influence proliferation of HGG cells and, subsequently, their response to the alkylating agent TMZ.

## Materials and Methods

### Cell cultures

This experiment was conducted on the CL2: 6 HGG cell line.

The cell line was cultured and maintained in Eagles' minimum essential medium (MEM) using additional 10% FBS and 1% Streptomycin/Penicillin.

The cell line culture was maintained in a humidified incubator at 95% air/5% carbon dioxide atmosphere and 37ºC in 75 $cm^2$ flasks.

### Transfection

Cell lines were maintained in accordance with the manufacturer's instructions until reaching an 80% confluency and transfected using β-arr 2 plasmid alongside Lipofectamine 2000 (Invitrogen).

Afterwards, the transfected HGG cells were incubated for a minimum of 24H before being treated with TMZ in progressive doses.

### Temozolomide treatment

The untransfected HGG cells were cultivated in 96-well plates (1.24x10 4 cells/well) and afterwards exposed to increasing concentrations of TMZ (Sigma-Aldrich), 200μM, 250μM and 300μM.

All cell lines were treated with a single dose at the beginning of the experiment.

### Cell viability

Cell line cultures were incubated in 96-well plates and viability was measured using a Prestoblue (Life Technologies, Carlsbad, CA, USA) cell viability assay.

The cells were incubated at 37ºC for 30 minutes with the Prestoblue reagent.

Florescence was measured using a Tecan Infinite 1000 plate reader.

A standard curve was used to correlate fluorescence to cell number.

Each experiment was performed in triplicates and independent experiments.

### Statistical analysis

The study was performed as independent, triplicate experiments.

We used a Student's T test and an ANOVA analysis of variance to compare the differences and significance of each study group.

A $p \leq 0.05$ was considered an acceptable level of statistical significance and the experimental data was presented as mean±standard deviation (SD).

## Results

### β-arrestin2 transfection increased CL2: 6 proliferation

Very little is known about the role β-arr 2 in the highly aggressive phenotype of HGGs.

While we previously discussed how normal β-arr 2 levels correlate with angiogenesis impairment through HIF degradation and or how β-arr knockdown in mice triggers high levels of VEGF, pro-angiogenic chemokines and enhanced microvascular density [8,9].

More recently, a study by Yun-Hua Kuo et al. has shown that the inhibition of the brain-enriched myelin-associated protein 1 (*BCAS1*) determined an increase in β-arr 2 levels alongside impaired angiogenesis.


10.12865/CHSJ.48.04.07

This translated into reduced proliferation and migration of HGG cells and an increased survival for murine models with subcutaneous and intracranial HGG xenografts [10].

Despite this evidence, there is no information available regarding the role of β-arrs 2 in initial tumoral development and treatment response.

In this experiment we transfected cell from the CL2: 6 line with a β-arr 2 plasmid.

After the incubation period, we compared the transfected cells with their untransfected counterparts to observe how β-arr 2 transfection can influence proliferation. After 24 h, β-arr 2 transfection determined a small increase of 8.7% in proliferation in comparison to the control group.

After 48h the transfected cells provided an increase of almost 12% compared to the control group.

After the 72h point, however, the proliferation percentage was down to about 6% when compared to the control group.

All the results were statistically significant ($p>0.05$) (Figure 1).

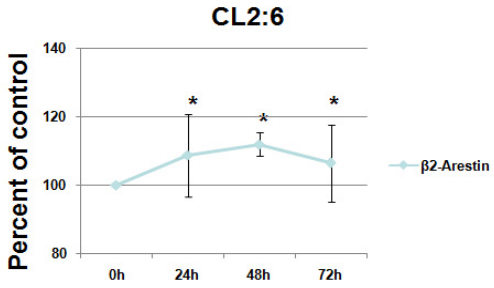


***Figure 1. The effect of β-arr 2 transfection on the CL2: 6 cells viability. The cells were grown six-well plates at 80% confluency and then transfected with β-arr 2 plasmid. Viability was evaluated by Prestoblue assay and 24h, 48h and 72h. Results are the mean between three independent experiments±SD, *p<0.05 vs. untransfected control cells.***

## The effect of β-arrestin2 transfection on CL2: 6 cells response to TMZ

TMZ is a small (194 Da) lipophilic molecule, orally and intravenously available, monofunctional DNA alkylating agent of the imidazotetrazine class that can cross the blood-brain barrier.

After absorption TMZ undergoes nonenzymatic breakdown to produce 5-aminoimidazole-4-carboxamide (AIC) and a highly reactive methyl diazonium cation.

The methyl diazonium cation methylates DNA at the N7 position of guanine (N7-MeG), N3 position of adenine (N3-MeG) and the O6 position of guanine (O6-MeG).

Whereas N7-MeG and N3-MeG are rapidly repaired by the base excision repair pathway the repair of O6-MeG requires the MGMT enzyme to remove the methyl group and restore guanine.

If not removed, O6-MeG mispairs with thymine thus activating the DNA mismatch repair (MMR) pathway to remove the thymine resulting in eventual DNA strand breaks leading to apoptosis [11].

TMZ resistance is a widely studied phenomenon and apart from MGMT promoter methylation and MMR deficiency which we have previously mentioned, other mechanisms such as proliferation of glioma stem cells [12] or enhanced autophagy [13] make TMZ monotherapy non-viable after first line treatment.

In this part of the study, we analyzed the response to TMZ treatment in the CL2:6 HGG cell line, using progressive doses.

The first dose of 200μM TMZ determined a slightly less cytotoxic effect in the untransfected group compared to the control with the difference in proliferation being 4% between the two groups.

The 250μM TMZ dose determined a similar response between the two groups with a 2% more cytotoxic effect observed in the transfected cell lines.

In regards to the maximum administered TMZ dose of 300μM, the difference in proliferation was only 0.5% between the two groups.

At the 24h time point the only statistically significant results in regards to the transfected β-arr 2 group were using the 200μM TMZ dose, whereas the untransfected group showed results of statistical significance throughout all three dose increments ($p<0.05$) (Figure 2).

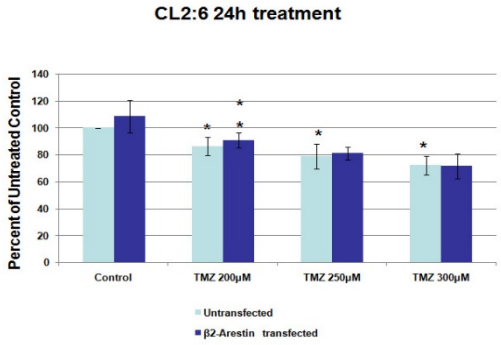


***Figure 2. The influence of TMZ treatment on the CL2: 6 HGG cells versus β-arr 2 transfection at 24h. Cells were seeded in 96-well plates and treated with 200μM, 250μM and 300μM TMZ. The viability was evaluated by Prestoblue assay and 24h. Results are the mean between three independent experiments±SD, *p<0.05 vs. untransfected control cells.***

At the 48h mark, differences in viability between the transfected and untransfected cells started to emerge at different doses.

For the 200µM dose, the untransfected group saw nearly a 14% higher cytotoxicity to TMZ compared to the experimental group.

At the 250µM dose the proliferation was similar between the two groups, the difference from the start point being lower than 1%.

Lastly, the highest 300µM dose produced an opposite effect compared to the lowest one, where β-arr 2 transfection helped produce a higher cytotoxic effect with proliferation at an 18% lower level.

At the 48h time point all results were of statistical significance ($p<0.05$) (Figure 3).

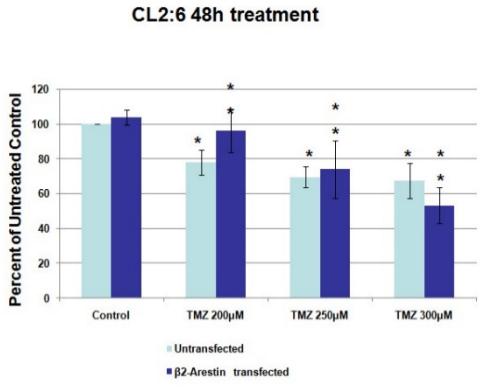


***Figure 3. The influence of TMZ treatment on the CL2: 6 HGG cells versus β-arr 2 transfection at 48h. Cells were seeded in 96-well plates and treated with 200µM, 250µM and 300µM TMZ. The viability was evaluated by Prestoblue assay and 48h. Results are the mean between three independent experiments±SD, *p <0.05 vs. untransfected control cells.***

The final 72h experimental time point produced comparable results between the two groups for the lowest two TMZ doses.

The 200µM showed a 4% higher cytotoxic effect in the β-arr 2 transfected group, a 10% cytotoxicity increase at the 250µM TMZ dose.

And the highest 300µM dose presented a notable 23% higher decrease in proliferation in the experimental transfected group compared to the control.

All the results at the 72h time point were of statistical significance ($p<0.05$) (Figure 4).

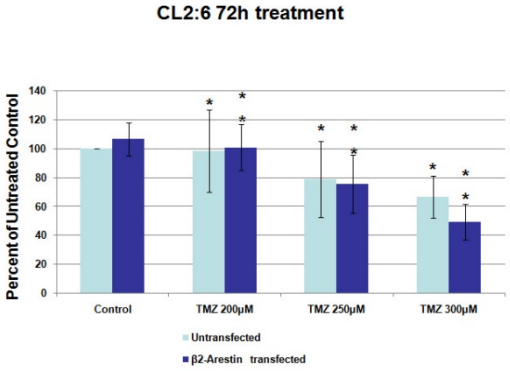


***Figure 4. The influence of TMZ treatment on the CL2: 6 HGG cells versus β-arr 2 transfection at 72h. Cells were seeded in 96-well plates and treated with 200µM, 250µM and 300µM TMZ. The viability was evaluated by Prestoblue assay and 48h. Results are the mean between three independent experiments±SD, *p<0.05 vs. untransfected control cells.***

## Discussion

β-arr 1 and 2 are intracellular adaptor proteins, best known for their capacity to regulate the activity of GPCRs [14].

These ubiquitous proteins were reported to control a wide array of cellular functions, including proliferation, motility, mitogenic responses etc. (ref) Interestingly, β-arr 1 and 2 were also reported to be involved in the development and progression of several types of tumors [15,16].

Overexpression, downregulation or manipulation of β-arr were also reported to influence the response to the therapy in non-small cell lung cancer (NSCLC) and breast cancer [17].

There are currently very few studies that evaluate the effect of β-arr 2 overexpression on the effect of TMZ in a malignant glioma cell line and also very little data regarding the role of β-arr as TMZ sensitizer in patients with GBM.

One study reviewed in depth the roles of β-arr 1 and β-arr 2, stating that depending on the cancer site, the two actors can produce opposite effects in cancer progression.

Whereas in NSCLC, EP4/β-arrestin1/c-Src-mediated PGE2 activation induces the migration of lung cancer cells whilst homology β-arr 2 exerts the opposite effect [18].

Regarding breast cancer, one publication observed that the expression of β-arr 2 in a multidrug resistant cell line, namely ADM (derived from MCF-7 cell line) is evidently higher than other chemotherapy sensitive cells MDA-MB-231 and MCF-7, which was consistent with the expression of MDR1/p-gp.


10.12865/CHSJ.48.04.07

Thus, the silencing of β-arr 2 by small interfering RNA would down-regulate the expression of MDR1/p-gp, and consequently, sensitivity to anti-cancer drugs could be partially restored [19].

In our previous work, we analyzed how β-arr overexpression influenced proliferation and response to treatment of glioma cell lines.

In one experiment, our group used β-arrestin 1 transfection on the 11HGG cell line, with similar doses of TMZ being used on both the transfected and untransfected cells.

At the 24h point, TMZ treatment produced a more cytotoxic effect in the experimental group, the second time point only the 300μM dose produced a statistically significant drop in proliferation, whereas at the final 48h timepoint there were only minor differences between the transfected group and the control for the 200μM and 250μM doses and even a statistically insignificant effect for the 300μM dose [20].

Another experiment conducted on 18 HGG glioma cell line showed that β-arr 1 transfection did have an influence on TMZ treatment.

The most intense cytotoxic effect was present at 24h, became less pronounced at 48h and statistically insignificant by the 72h time point [21].

An opposite effect was observed in regards to transfection of U-343MGa and CL 2:6 with β-arr 1, where treatment with TMZ showed that the transfection actually reduced the cytotoxic effect of the experimental cell lines.

This result demonstrates that different cells families from the same cancer type may react differently to the same type of treatment [22].

We also showed that β-arr 2 transfection of U-343MGa GBM cell line yielded contradictory results related to the response to TMZ treatment.

The minimal 200μM dose produced a small drop in proliferation at 24h, no effect at 48h and even a rise in proliferation at 72h.

At 250μM seems to produce a higher cytotoxic effect on the transfected cells at the 24 and 72h mark but presented resistance to treatment after 48h and the highest dose produced comparative results with a drop in proliferation at all three time points [23].

In this paper we have observed a similar pattern of seemingly contradictory results.

As such, overexpression of β-arr 2 had a positive effect on proliferation at the 24h and 48h time points with this trend being reversed after 72h, when untransfected cells presented a superior proliferation when compared to transfected cells.

Regarding the influence on treatment response, after 24h, only incremental differences between the untrasfected and transfected cells were observed, the only statistically significant ones being observed for the 200μM dose (4%).

As for the 48h and 72h, the smallest doses (200μM) were more toxic for the transfected cells while the highest doses (300μM) had a more profound effect on the untransfected cells.

## Conclusion

The current study showed that β-arr 2 overexpression does play a role in HGG cell proliferation and it could also be involved in treatment resistance.

However, further studies are needed to be conducted in order to fully elucidate the mechanisms through which β-arr overexpression mediates cancer growth and drug resistance.

These proteins as well as the effect of their overexpression seem to have different effects in different sites.

The endpoint may help the medical field in developing new biomarkers that can improve diagnosis and treatment of not only CNS tumors but also other types of cancers as it is evident that these proteins do play an important role in cancer development.

### Acknowledgements

Work supported by The European Association for Cancer Research granted by Travel Fellowship, 2016; European Association for Cancer Research.

Oprita A. and Staicu G.A. share equal contributions to this work.

### Conflict of interest

The authors declare no conflict of interest.

### References

1. Stupp R, Mason WP, Van Den Bent MJ, Weller M, Fisher B, Taphoorn MJ, Belanger K, Brandes AA, Marosi C, Bogdahn U. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. New England journal of medicine, 2005, 352(10):987-996.
2. Wiewrodt D, Nagel G, Dreimüller N, Hundsberger T, Perneczky A, Kaina B. MGMT in primary and recurrent human glioblastomas after radiation and chemotherapy and comparison with p53 status and clinical outcome. International journal of cancer, 2008, 122(6):1391-1399.
3. Cahill DP, Levine KK, Betensky RA, Codd PJ, Romany CA, Reavie LB, Batchelor TT, Futreal PA, Stratton MR, Curry WT. Loss of the mismatch repair protein MSH6 in human glioblastomas is associated with tumor progression during temozolomide treatment. Clinical cancer research, 2007, 13(7):2038-2045.

4. Calatozzolo C, Gelati M, Ciusani E, Sciacca F, Pollo B, Cajola L, Marras C, Silvani A, Vitellaro-Zuccarello L, Croci D. Expression of drug resistance proteins Pgp, MRP1, MRP3, MRP5 AND GST-π in human glioma. Journal of neuro-oncology, 2005, 74(2):113-121.
5. Kovacs JJ, Hara MR, Davenport CL, Kim J, Lefkowitz RJ. Arrestin development: emerging roles for β-arrestins in developmental signaling pathways. Developmental cell, 2009, 17(4):443-458.
6. Luttrell LM, Lefkowitz RJ. The role of β-arrestins in the termination and transduction of G-protein-coupled receptor signals. Journal of cell science, 2002, 115(3):455-465.
7. Hu S, Wang D, Wu J, Jin J, Wei W, Sun W. Involvement of β-arrestins in cancer progression. Molecular biology reports, 2013, 40(2):1065-1071.
8. Bae W-Y, Choi J-S, Nam S, Jeong J-W. β-arrestin 2 stimulates degradation of HIF-1α and modulates tumor progression of glioblastoma. Cell Death & Differentiation, 2021, 28(11):3092-3104.
9. Raghuwanshi SK, Nasser MW, Chen X, Strieter RM, Richardson RM. Depletion of β-arrestin-2 promotes tumor growth and angiogenesis in a murine model of lung cancer. The Journal of Immunology, 2008, 180(8):5699-5706.
10. Kuo YH, Hung HS, Tsai CW, Chiu SC, Liu SP, Chiang YT, Shyu WC, Lin SZ, Fu RH. A Novel Splice Variant of BCAS1 Inhibits beta-Arrestin 2 to Promote the Proliferation and Migration of Glioblastoma Cells, and This Effect Was Blocked by Maackiain. Cancers (Basel), 2022, 14(16)
11. Zhang J, FG Stevens M, D Bradshaw T. Temozolomide: mechanisms of action, repair and resistance. Current molecular pharmacology, 2012, 5(1):102-114.
12. Ciurea ME, Georgescu AM, Purcaru SO, Artene SA, Emami GH, Boldeanu MV, Tache DE, Dricu A. Cancer stem cells: biological functions and therapeutically targeting. Int J Mol Sci, 2014, 15(5):8169-8185.
13. Dong Q, Wang D, Li L, Wang J, Li Q, Duan L, Yin H, Wang X, Liu Y, Yuan G, Pan Y. Biochanin A Sensitizes Glioblastoma to Temozolomide by Inhibiting Autophagy. Mol Neurobiol, 2022, 59(2):1262-1272.
14. Gimenez LE, Kook S, Vishnivetskiy SA, Ahmed MR, Gurevich EV, Gurevich VV. Role of receptor-attached phosphates in binding of visual and non-visual arrestins to G protein-coupled receptors. J Biol Chem, 2012, 287(12):9028-9040.
15. Xiao K, McClatchy DB, Shukla AK, Zhao Y, Chen M, Shenoy SK, Yates JR, 3rd, Lefkowitz RJ. Functional specialization of beta-arrestin interactions revealed by proteomic analysis. Proc Natl Acad Sci U S A, 2007, 104(29):12011-12016.
16. Karin M. Nuclear factor-kappaB in cancer development and progression. Nature, 2006, 441(7092):431-436.
17. Lundgren K, Tobin NP, Lehn S, Stal O, Ryden L, Jirstrom K, Landberg G. Stromal expression of beta-arrestin-1 predicts clinical outcome and tamoxifen response in breast cancer. J Mol Diagn, 2011, 13(3):340-351.
18. Song Q, Ji Q, Li Q. The role and mechanism of β-arrestins in cancer invasion and metastasis. International Journal of Molecular Medicine, 2018, 41(2):631-639.
19. Jing X, Zhang H, Hu J, Su P, Zhang W, Jia M, Cheng H, Li W, Zhou G. β-arrestin 2 is associated with multidrug resistance in breast cancer cells through regulating MDR1 gene expression. International journal of clinical and experimental pathology, 2015, 8(2):1354.
20. Horescu C, Artene S-A. The effect of beta-arrestin 1 transfection on proliferation and temozolomide treatment response in HGG cells. Medico Oncology, 2020, 1(1):1-9.
21. Folcuti C, Horescu C, Barcan E, Alexandru O, Tuta C, Vatu BI, Artene SA, Dricu A. beta-arrestin 1 transfection induced cell death in high grade glioma in vitro. J Immunoassay Immunochem, 2020, 41(6):1021-1032.
22. Artene SA, Folcuti C, Dricu A. beta-arrestin 1 Overexpression Increases Temozolomide Resistance in Human Malignant Glioma Cells. Curr Health Sci J, 2017, 43(2):112-119.
23. Cioc C-E, Barcan E, Artene S. The Interference of B-Arrestin2 Expression and the Response to Tmz Treatment in High Grade Glioma. Medico Oncology, 2021, 2(2):60-70.

***Corresponding Author: Carina Baloi, Units of Biochemistry, Faculty of Medicine, University of Medicine and Pharmacy of Craiova, Str. Petru Rares nr. 2-4, 710204 Craiova, Romania, e-mail: carina_baloi@yahoo.com***