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Assessment of temozolomide action encapsulated in chitosan and polymer nanostructures on glioblastoma cell lines

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Abstract

Purpose: Glioblastoma multiforme (GBM) remains one of the most devastating diseases known to mankind and affects more than 17,000 patients in the United States alone every year. This malignancy infiltrates the brain early in its course and makes complete neurosurgical resection almost impossible. Recent years have brought significant advances in tumor biology. Many cancers, including gliomas, appear to be supported by cells with stem-like properties. Nanoparticles are excellent candidates to serve as delivery vectors of drugs or biologically active molecules because of their unique chemical and physical properties that result in specific transportation and deposition of such agents in specific organs and tissues..

In the current study we have investigated the in vitro action of nanostructural systems (temozolomide encapsulated in chitosan and polymer nanostructures) on high-grade glioma-derived cancer stem cells (CSCs), with the intention of developing a new therapy to treat specific brain tumors with increased efficacy and minimal toxicity. In vitro cytotoxicity and apoptosis measurements indicated that the drug/vector combination facilitated the ability of the alkylating drug TMZ to alter the resistance of these cancer stem cells, suggesting a new chemotherapy strategy even for patients diagnosed with inoperable or recurrent malignant gliomas

Methods: At the National Institute for R & D of Isotopic and Molecular Technologies form Cluj Napoca were synthesized three types of nanostructures chitosan-TMZ, TMZ-chitosan-PEG (poly-ethylene glycol), TMZ-chitosan-PPG (polypropylene glycol). Three type of cell lines (Glioma-derived stem, HFL and HUVEC) were treated with the 3 types of nanostructures and the survival rate of the cells was compare to standard therapy (TMZ).

Results: The results showed a reduction in the rate of survival of the tumor cells. Cell proliferation assays clearly demonstrate the differences between

conventional chemotherapy (TMZ) and temozolomide encapsulated in chitosan and polymer nanostructures.

Conclusion: Nanostructures like chitosan, PEG, PPG are useful as vectors for drugs transport.

Despite combined therapy (surgery, radiotherapy, chemotherapy), currently median patient survival is reduced. The key to improving life expectancy could be an effective therapy targeted, customized for each case. An increasingly important role will be new methods of treatment such as immunotherapy, gene therapy or nanotherapy.

Key words: malignant gliomas, primary tumor cell culture, temozolomide.

Introduction

Malignant gliomas are responsible for the death of approximately 11,000 patients per year.[1] The standard care for patients diagnosed with high-grade central nervous systems (CNS) gliomas include, post-operatively, temozolomide (TMZ) concomitant and adjuvant to radiotherapy. This therapeutic strategy is, however, associated with high toxicity, limited efficiency and significant side effects. The median length of survival for patients with high-grade primary glial tumors ranges from 11 to 33 months after initial diagnosis and an average of 7 months following recurrence.[2,3]

The failure of current approaches to the treatment of malignant gliomas has been attributed to the existence of a sub-population of cancer cells malignant glioma cancer stem cells (CSCs), which have the ability to withstand chemotherapeutics and ionizing radiation based on certain of their unique properties: high expression of anti-apoptotic proteins, high expression of ABC pumps, and remarkable DNA repair capability. [4-6] Traditional therapies, such as DNA alkylating or methylating drugs along with radiation oncology treatments, have low treatment efficacy for these cell types. More complex treatments capable of overcoming the CSCs' ability to eliminate anti-cancer drugs and perform other protective functions are therefore critically needed. For this reason, a combination of traditional treatments and nanotechnology-based approaches offers attractive possibilities. More efficient and less toxic therapeutics that can cross the CSCs protective barriers are urgently needed. In this context, nanomaterials could play an important role based on their unique electronic, optical, magnetic, and structural properties that are found neither in bulk materials nor in single molecules and which are necessary to develop advanced cancer treatments.

Theoretical considerations of nanotherapy

Nanoparticles are biodegradable or bioresistence polymer matrix, with an average diameter of approx. 200 nm. Nanoparticles are obtained by polymerization of monomers or directly from the processed polymer.

The advantages of nanoparticles are: relatively simple preparation, they ensure protection of the active principles from

chemical and enzymatic degradation (nanocapsules) limiting side effects of active substances and provide transportation and release to the target by the biodegradable matrix. Disadvantages are the high costs of technology (equipment, raw materials) and toxicity of adjuvants [7].

A large area relative to their volume, increased bioavailability, provide controlled release of the active substance and support the intracellular and molecular vectors.

Due to the extremely small size, nanoparticles vectors easily cross biological barriers (blood-brain barrier in our case)[8].

Theoretical considerations of nanotherapy in brain tumors

Molecular nanodiagnostic. Nanoparticles increased sensitivity and specificity for high-resolution noninvasive medical imaging, at molecular and cellular level (ultrasound, CT, MRI, OI. PET). (PEG-chitosan-clorotoxin-fluoroscopic agent nanoparticles may improve the resolution by 10 times)[9].

Nanovaccines are useful for immunotherapy, nanoantibiotherapy offers a lower risk of developing resistance to antibiotics and some nanostructures can be used in neuronal nanoprotection [10].

Transport nanosystems for gene therapy (to replace viruses as vectors) are less likely immunologic and allow the transfer of large quantities of genetic material. A polyglycerol dendrimer polymer has been shown to improve RNA stability and accumulation in brain tumors in animals[7].

Antitumoral nanotherapy PEG dimethacrylate methyl ether methacrylate and iron oxide nanostructures can be used as a biomaterial for the thermal treatment of GBM. Many types of nanoparticles such as biodegradable polymers (PEG, PPG, PBCA) lactic acid loaded with different type of chemotherapy agents increased tumor cytotoxicity and survival in laboratory animals with decreased side effects of cytostatic. Use of nanoparticles can increase the effectiveness of radiotherapy with decrease of side effects [11].

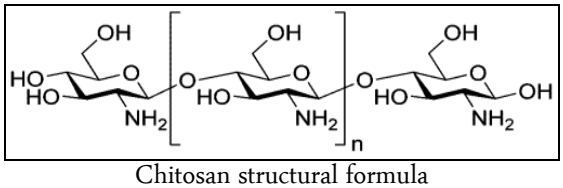
Methods

At the National Institute for R & D of Isotopic and Molecular Technologies form Cluj Napoca were synthesized three types of nanostructures chitosan-TMZ, TMZ-chitosan-PEG (poly-ethylene glycol), TMZ-chitosan-PPG (polypropylene glycol).

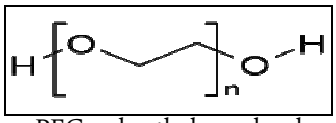
Chitosan [poly(b-(10/4)-2-amino-2-deoxy-Dglucose)] is a natural cationic polysaccharide derived from chitin, which is copolymer, a glucosamine and an N-acetyl glucosamine units, combined together [12]. Chitosan is being widely used as a pharmaceutical excipient, comprising a series of polymers varying in their degree of deacylation, molecular weight, viscosity, pKa etc. The presence of a number of amino groups permits CTS to chemically react with anionic systems, thereby resulting in alteration of

physicochemical characteristics of reactants and developing new properties of such combinations [1].

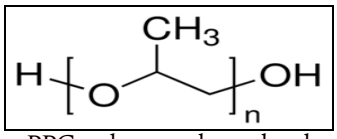
Temozolomide (brand names Temodar and Temodal and Temcad) is an oral alkylating agent used for the standard treatment of Grade IV astrocytoma.



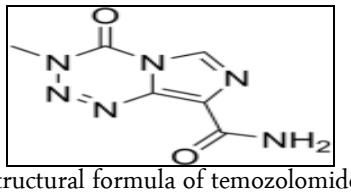
Chitosan structural formula



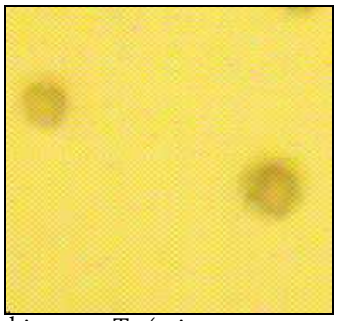
PEG poly-ethylene-glycol

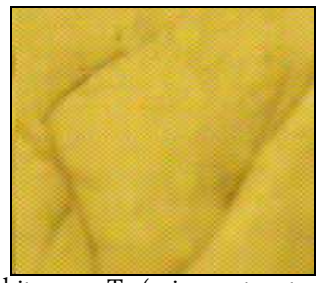


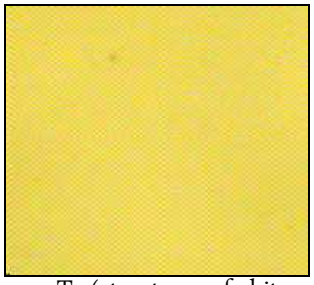
PPG poly-propylene-glycol



Structural formula of temozolomide


PEG + chitosan + Tz (micrometer structures of chitosan with poly-ethylene-glycol and temozolomide.. Spheres of 5-15 microns)


PPG + chitosan + Tz (micrometer structures of chitosan poly-propylene glycol and temozolomide filaments with a length of 10-50 microns)


chitosan + Tz (structures of chitosan and temozolomide, sub-micrometer (nanometer) spheroids)

Chitosan (CTS) from crab shells with 85% deacetylation degree, poly-ethylene-glycol, poly-propylene-glycol, were purchased from Sigma Aldrich.

CTS was dissolved in 1% (v/v) acetic acid aqueous solution stirring for 6 hours at 300C temperature, at final the solution being pale yellow, with a homogenous consistence and aspect. The CTS solution was split in 5 equal volumes, 50 ml each, and to each CTS sample was added 0,5 ml linking additive agent and 10 mg powder from Temodal. Calculations assured that each sample contains 10 mg TMZ.

We obtain a temozolomide concentration of 50 micromol/l .

The solutions were further stirred for 30 min and then ultrasonically treated in an

Elmasonic E60H ultrasonic bath for 360 min.

Microspheres of CTS-based polyelectrolyte complex containing TMZ/linker were characterized by FTIR electronic microscope connected to FTIR JASCO 6100 spectrometer.

The malignant glioma cancer stem cells (CSCs), used in this study were isolated from a glioblastoma multiforme biopsy. Briefly, after mechanical dissociation of tumor tissue, the fragments were placed in 1 ml of fetal calf serum (FCS). After three hours, 3 ml of DMEM/F-12 medium supplemented with 15% FCS was added to the dish. After reaching a subconfluent monolayer, cells were detached using trypsin/ EDTA and resuspended in a serum-free media: DMEM/F12 (1:1) medium, supplemented with 15 ng/ml basic fibroblast growth factor (bFGF), 20 ng/ml epidermal growth factor (EGF), 2mM/l L-glutamine, 4 U/l insulin growth factor-1 (IGF-1) and B 27 supplement (1:50) (Sigma Aldrich). Isolated and expanded cells revealed some stem-cell specific features, such as the expression of cellular markers (CD133, CD105, CD90, Nanog, Oct ¾ (immunocytochemistry) expression of specific genes, such as : CXCR4, nestin, glial fibrillary acidic protein (GFAP), and neurofilament protein (NF) (reverse transcription-PCR). Cells also displayed a high proliferative potential despite chemotherapy and irradiation and also had the ability to form spheroids in suspension.

The three type of cell lines (Glioma-derived stem, HFL-Normal fibroblasts human isolated from lung and endothelial cell line HUVEC-Human Umbilical Vein endothelial cell.) were treated with the 3 types of nanostructures and the survival rate of the cells was compare to standard therapy (TMZ).

We used three cell lines to study the difference between tumor cells and normal cell lines (fibroblasts and endothelial cells).

The four compounds (PEG + chitosan + Tz, Tz + PPG + chitosan, chitosan + Tz and TZ) after binding were filtered through a 220nm filter sterilized

Stem cells are in the exponential phase of cell growth they are detached by exposure for 5 min in 0.25% trypsin EDTA after 3 washes with PBS. Trypsin is inactivated by the addition of culture medium with 10% fetal calf serum, and the cell suspension centrifuged 5 min at 1100 rpm. Cell viability is checked by trypan blue 0.4%. The cells are counted with a Thoma chamber.

The 3 cell lines (GM 1, HFL and HUVEC) after being counted are seeded in 96-well plates each 7500 cells / well and suspended in 200 ml medium; after 24 h cells were subjected to treatment that joined us and after a further 24 hours MTT assay was performed

MTT test. Twenty-four hours after therapy culture medium was aspirated and the cells will be exposed to 100 ml solution of MTT 1mg/ml (tetrazolium Bromide Thyazolyl Blue) for 1 hour at 37 ° C. MTT is a tetrazolium salt which is converted in

cellular mitochondria of viable cells into a formazan compound dark blue colored, insoluble in aqueous solutions. After the incubation period, MTT solution is aspirated from the wells and formazan crystals were dissolved with DMSO 150µl/well (dimethyl sulfoxide) obtaining a color reaction. For measuring the optic density, boards are analyzed at 492 nm using a plate reader BioTek Synergy2. Each determination shall be made in triplicate.

Statistical analysis

Statistical significance values were obtained using a one-way analysis of variance (ANOVA), with 95% confidence (C.I.) level, using GraphPad Prism 5 statistics program (GraphPad Inc, San Diego, CA, USA). Bonferroni's multiple comparison test was considered statistically significant at $p < 0.05$. All experiments were performed in triplicate.

Results

In our experiments,. there was an impotant difference between TMZ alone (at the same concentration) and the control sample on the one hand and the three types of nanostructures studied (PEG + chitosan + Tz, Tz + PPG + chitosan, chitosan + Tz) on the other hand for each cell type (Glioma-derived stem, HFL and HUVEC). Figure 1

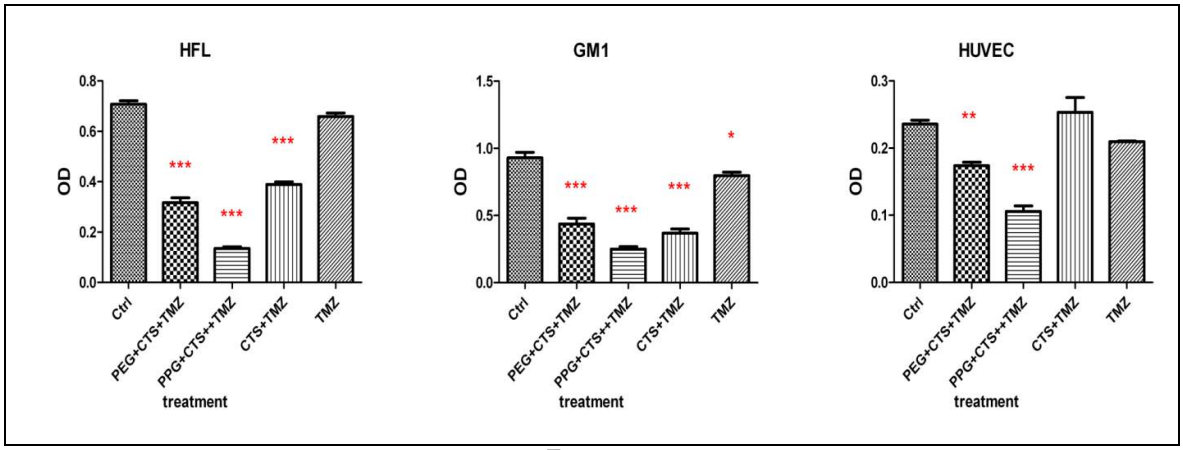


Figure 1

Survival chemotherapy graphics for the three type of cell lines (Glioma-derived stem, HFL-Normal fibroblasts human isolated from lung and endothelial cell line HUVEC-Human Umbilical Vein endothelial cell.) treated with the 3 types of nanostructures and standard therapy (TMZ). The vertical axis represents the optical density (remaining cell population) and the horizontal axis the various therapy options.

HFL

Bonferroni's Multiple Comparison Test	Mean Diff.	t	Significant? P < 0.05?	Summary	95% CI of diff
Ctrl vs PEG+CTS+TMZ	0.3903	20.68	Yes	***	0.3282 to 0.4523
Ctrl vs PPG+CTS+TMZ	0.5728	30.35	Yes	***	0.5107 to 0.6348

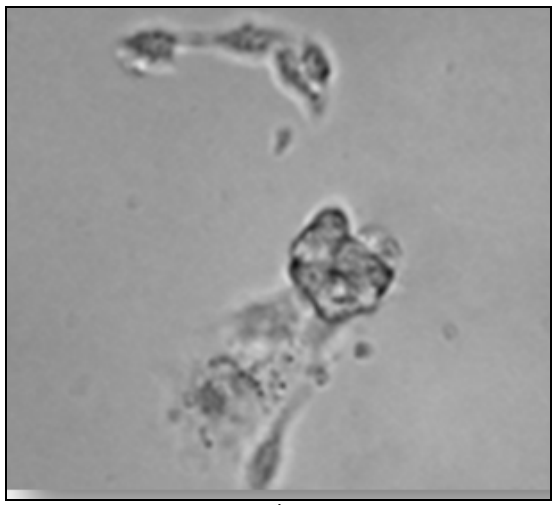
Ctrl vs CTS+TMZ	0.3183	16.86	Yes	***	0.2562 to 0.3803
Ctrl vs TMZ	0.04825	2.557	No	ns	-0.01376 to 0.1103
PEG+CTS+TMZ vs PPG+CTS+TMZ	0.1825	9.671	Yes	***	0.1205 to 0.2445
PEG+CTS+TMZ vs CTS+TMZ	-0.07200	3.815	Yes	*	-0.1340 to -0.009987
PEG+CTS+TMZ vs TMZ	-0.3420	18.12	Yes	***	-0.4040 to -0.2800
PPG+CTS+TMZ vs CTS+TMZ	-0.2545	13.49	Yes	***	-0.3165 to -0.1925
PPG+CTS+TMZ vs TMZ	-0.5245	27.79	Yes	***	-0.5865 to -0.4625
CTS+TMZ vs TMZ	-0.2700	14.31	Yes	***	-0.3320 to -0.2080

GM1

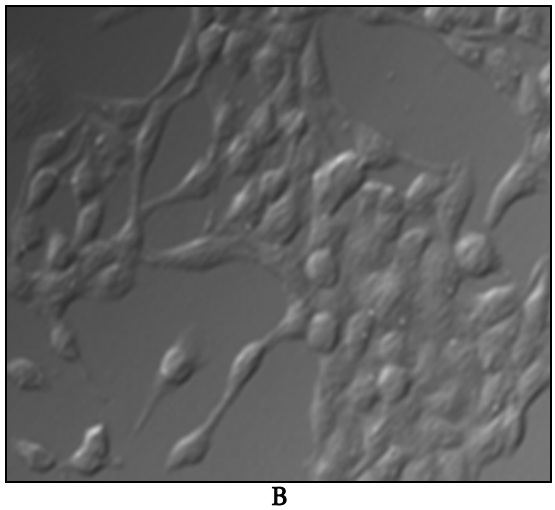
Bonferroni's Multiple Comparison Test	Mean Diff.	t	Significant? P < 0.05?	Summary	95% CI of diff
Ctrl vs PEG+CTS+TMZ	0.4933	10.53	Yes	***	0.3394 to 0.6471
Ctrl vs PPG+CTS+TMZ	0.6788	14.49	Yes	***	0.5249 to 0.8326
Ctrl vs CTS+TMZ	0.5603	11.96	Yes	***	0.4064 to 0.7141
Ctrl vs TMZ	0.1325	2.829	No	ns	-0.02138 to 0.2864
PEG+CTS+TMZ vs PPG+CTS+TMZ	0.1855	3.961	Yes	*	0.03162 to 0.3394
PEG+CTS+TMZ vs CTS+TMZ	0.0670	1.431	No	ns	-0.08688 to 0.2209
PEG+CTS+TMZ vs TMZ	-0.3608	7.704	Yes	***	-0.5146 to -0.2069
PPG+CTS+TMZ vs CTS+TMZ	-0.1185	2.531	No	ns	-0.2724 to 0.03538
PPG+CTS+TMZ vs TMZ	-0.5463	11.66	Yes	***	-0.7001 to -0.3924
CTS+TMZ vs TMZ	-0.4278	9.134	Yes	***	-0.5816 to -0.2739

HUVEC

Bonferroni's Multiple Comparison Test	Mean Diff.	t	Significant? P < 0.05?	Summary	95% CI of diff
Ctrl vs PEG+CTS+TMZ	0.06200	3.918	Yes	*	0.01001 to 0.1140
Ctrl vs PPG+CTS+TMZ	0.1303	8.232	Yes	***	0.07826 to 0.1822
Ctrl vs CTS+TMZ	-0.01775	1.122	No	ns	-0.06974 to 0.03424
Ctrl vs TMZ	0.02625	1.659	No	ns	-0.02574 to 0.07824
PEG+CTS+TMZ vs PPG+CTS+TMZ	0.06825	4.313	Yes	**	0.01626 to 0.1202
PEG+CTS+TMZ vs CTS+TMZ	-0.07975	5.040	Yes	**	-0.1317 to -0.02776
PEG+CTS+TMZ vs TMZ	-0.03575	2.259	No	ns	-0.08774 to 0.01624
PPG+CTS+TMZ vs CTS+TMZ	-0.1480	9.354	Yes	***	-0.2000 to -0.09601
PPG+CTS+TMZ vs TMZ	-0.1040	6.573	Yes	***	-0.1560 to -0.05201
CTS+TMZ vs TMZ	0.04400	2.781	No	ns	-0.007993 to 0.09599



A



B

Figure 2 (A)
A typical pre-apoptotic cell after 24h incubation with chitosan+TMZ (PlasDIC phase contrast, magnification 400x), and **(B)** in comparison with control (cells culture without chitosan+TMZ),(white light microscopy, PlasDIC contrast phase, magnification 400x)

After cell GM1 treatement with chitosan+TZM autophagic cells were observed.

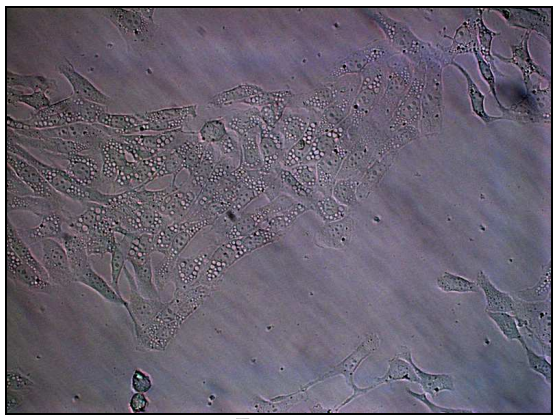


Figure 3
Autophagic cell GM1with intracytoplasmic vacuoles it was observed for chitosan+TZM nanoparticles. (phase contrast, magnification x400)

Discussion and conclusion

Long-term survival of patients diagnosed with high-grade gliomas remains poor, with population-based studies estimating that the 3-year survival rate is under 5%. [22-25]. Conventional treatment for newly diagnosed malignant gliomas was traditionally consisted of initial surgical resection followed by fractionated external beam RT, with or without chemotherapy usually using regimens containing alkylating agents. Until recently the benefit of chemotherapy in this setting remained controversial but TMZ, an oral alkylating agent, has proven to be efficient, primarily in the recurrent setting.

The failure of current approaches to the treatment of malignant gliomas has been attributed to the existence of a sub-population of cancer cells malignant glioma cancer stem cells (CSCs), which have the ability to withstand chemotherapeutics and ionizing radiation

based on certain of their unique properties: high expression of anti-apoptotic proteins, high expression of ABC pumps, and remarkable DNA repair capability.[13-15] Traditional therapies, such as DNA alkylating or methylating drugs along with radiation oncology treatments, have low treatment efficacy for these cell types. More complex treatments capable of overcoming the CSCs ability to eliminate anti-cancer drugs and perform other protective functions are therefore critically needed. For this reason, a combination of traditional treatments and nanotechnology-based approaches offers attractive possibilities. More efficient and less toxic therapeutics that can cross the CSCs protective barriers are urgently needed. In this context, nanomaterials could play an important role based on their unique electronic, optical, magnetic, and structural properties that are found neither in bulk materials nor in single molecules and which are necessary to develop advanced cancer treatments.

Similarities between the self-renewal mechanisms of stem cells and cancer cells have led to the new concept of cancer stem cells (CSCs). Over the course of the past 10-15 years, there has been increasing evidence to support the cancer stem cell hypothesis, which postulates that CSCs are responsible for tumor initiation, metastasis, and resistance to treatment. It is now generally believed that a tumor has its origin in CSCs, which come either from transformed tissue stem cells or from transformed progenitor cells that have regained self-renewal activity.[16] These rare CSCs could be crucial in controlling and curing cancer: through asymmetric division, CSCs drive tumor growth and evade therapy with the help of traits shared with normal stem cells such as quiescence, self-renewal ability, and multidrug resistance pump activity.[17] These cells were first identified in hematologic cancers, but recently have been isolated from solid tumors. CSCs are tumor initiating cells in immunocompromised mice and have the ability to generate heterogeneous cancer cell populations.[18]

The gold standard assay to determine whether a stem cell is or not a CSC involves serial transplantation in animal models. Potential surface markers of CSCs include the following: CD133, aldehyde dehydrogenase 1 (ALDH1), CD44, and CD24. Efflux of Hoechst or Rhodamine dyes, also referred to as Side Population (SP), have also been used to identify putative CSCs. However, these markers have certain limitations in that they fail to identify all CSCs and merely designate a subpopulation that is enriched for clonogenic and tumorigenic activity. Also, not all cells with a CSC marker phenotype behave as CSCs. Most markers for separating CSCs were chosen due to their expression on normal stem cells of certain tissues, and, interestingly, there are a number of molecules that are commonly expressed in normal and cancer stem cells that lead to different phenomena depending on the local environment.[19]

Using Bonferroni's Multiple Comparison Test, we found statistically significant results ($P<0.05$) between the CSCs (control) and the CTR+ PEG+CTS+TMZ (95% CI of 0,3394 to 0,6471), CTR+PPG-CTS-TMZ (95% Cl of 0,5249 to 0,83260), CTR+CTS-TMZ (95% of 0,4064 to 0,7141). When one or more initiating genetic changes appeared at the progenitor level, all of the downstream cells continued this change. In one particular case, it is possible that a daughter cell acquired not only the properties of the stem cell, but also the additional alterations that allow the glioma to progress to the next step and invade surrounding tissues. Due to the small population of glioblastoma-derived stem cells, the malignant gliomas have a negative response to various conventional treatments.[20] The killing efficiency of the PEG+CTS+TMZ, PPG-CTS-TMZ, CTS-TMZ nanostructures on glioblastoma-derived stem cells is better compared with the drug temozolomide, alone.

Our proposed nanoscaled drug delivery system offers also a new chemotherapy strategy for patients diagnosed with unresectable or recurrent malignant gliomas. Current therapies are not yet curative, as CSCs may survive as a result of the increased efflux of chemotherapy agents due to ABCG2 cell membrane proteins and increased DNA repair.[21]

Cancer cells are very complex biological structures that perform functions ranging from invasion or metastasis to the elimination of anticancer drugs from the cell membrane. Although the exact mechanisms need to be explored further, combining advances in fundamental oncology and nanotechnology offers the opportunity to significantly impact future diagnostics and therapeutics. We have shown that drug delivery vectors based on chitosan and polymers have the ability to deliver temozolomide (TMZ), a cytostatic drug, to treat malignant gliomas. Our studies have shown that a novel drug delivery has low toxicity and the ability to internalize TMZ.

Malignant gliomas are highly infiltrative and lethal cancers of the central nervous system. The highly infiltrative nature of glioma cells often renders a complete surgical removal impossible and inevitably will lead to tumor recurrence.

The attention of the scientific community is currently focused on nanoparticles, a novel vector for the delivery of anticancer drugs to target cancer cells. These particles have many advantages which recommend it over classically administered drugs. Due to their submicroscopic size and modifiability, nanoparticles have an enhanced access to cancer cells, being able to maintain high concentrations of drugs in target tissues. Because of their special distribution, drug-loaded nanoparticles may even have a decreased risk of systemic adverse effects which normally occur at increased drug doses, while locally maintaining effective concentrations. Nanoparticles loaded with various cytostatics may prove to have numerous additional advantages. The

increased drug concentrations may also be explained by the facilitated penetration of the particles in the tumor through endocytosis, which has the advantage of bypassing the transporter- mediated drug internalization systems. Moreover, the intracellular drug concentration increases in spite of tumor cell multidrug resistance protein activity, which may result in better tumor-level effect of the drug in spite of multidrug resistant tumor phenotype. Another positive viewpoint of using nanoparticles is their capacity to cross the blood brain barrier, which shapes new directions for drug delivery into the brain. Most drugs partially cross the blood brain barrier, but its incorporation into nanoparticles may enhance its passage and increase the relative amount of drug reaching brain tissue. Therefore, the use of loaded nanoparticles could be of paramount importance for glioblastomas, aggressive tumors with a very dismal prognosis, for which temozolomide has been entitled "the most clinically relevant drug ever reported for targeting of glioma-initiating cells".[25]

Nanostructures like chitosan, PEG, PPG are useful as vectors for temozolomide transport.

Our study shows a net decrease of cell population by treating them with drug-nanostructures. Cell population decrease was more important in tumor cell cultures (GM1) compared with normal cells fibroblasts (HFL) or endothelial cells (HUVEC). The best response was obtained for chitosan-Tz, considering that drug chemotherapy should be aggressive on tumor cells and less aggressive on normal cells.

Our study tries to find new drugs for the treatment of glioblastoma. Results must be confirmed by in vivo studies.

Despite combined therapy (surgery, radiotherapy, chemotherapy), currently median patient survival is 10-12 months. The key to improving life expectancy could be an effective therapy targeted, customized for each case. An increasingly important role will be new methods of treatment such as immunotherapy, gene therapy or nanotherapy.

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