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Intranasal Administration of Temozolomide Delayed the Development of Brain Tumors Initiated by Human Glioma Stem-Like Cell in Nude Mice

Pineda JR[1,6*$], Jeitany M[1$], Andrieux A[2], Junier MP[3-5#], Chneiweiss H[3-5#] and François D Boussin[1*]

[1]CEA DSV iRCM SCSR, INSERM, Laboratory of Radiopathology, UMR967, F-92265 Fontenay-aux-Roses, France
[2]CEA, IRTSV-GPC, INSERM, U836-GIN i Health and Beauty Health in La Tronche, BP170, 38042 Grenoble, Cedex 9, France
[3]CNRS UMR8246 Neuroscience Paris Seine-IBPS, Team Glial Plasticity, 7 Quai Saint-Bernard, 75005 Paris, France
[4]INSERM U1130, Neuroscience Paris Seine-IBPS, Team Glial Plasticity, 7 Quai Saint-Bernard, 75005 Paris, France
[5]University Pierre and Marie Curie UMCR18, Neuroscience Paris Seine-IBPS, Team Glial Plasticity, 7 Quai Saint-Bernard, 75005 Paris, France
[6]Achucarro Basque Center for Neuroscience Fundazioa, Sede Building 3rd Floor, Barrio Sarriera s/n. 48940 Leioa, Vizcaya, Spain
[$]Contributed equally to this work, [#]Contributed equally to this work.

Abstract

Objective: Intranasal route is an emerging option for brain cancer treatment to infuse directly telomerase inhibitors and/or viruses into the brain. Paradoxically, the standard chemotherapeutic Temozolomide (TMZ) widely used to treat glioma tumors is orally given. Here, we tested for the first time the intranasal administration of TMZ in nude mice xenograft models carrying human glioblastoma tumors generated from the human glioma stem-like cells TG16, TG1N and TG20.

Methods: The resistance to TMZ of the different glioma stem-like cells was determined by WST-1 cell proliferation and cell viability assay. Tumour cells were stereotaxically injected intrastriatally and one month after graft, mice were anesthetized using Isofluorane and TMZ was infused into the nostrils three times a week during two weeks with a nano-injector using Hamilton syringe coupled to a cannula. Buried food pellet test was carried out to check the sense of smell. Animals were weighted and surveilled once a week and separated into two cohorts, one for histopathological analysis and the other for Kaplan-Meier survival analysis.

Results: Intranasal administration of TMZ did not induce major adverse effects on the sense of smell of the animals. TMZ administred intranasally delayed tumour growth and significantly extended the lifespan of mice engrafted with TG16 and TG1N cells, which are sensitive *in vitro* to TMZ. By contrast, TMZ at the dose tested had no effects on the tumors generated by TG20 cells that are resistant to TMZ *in vitro*.

Conclusion: Our results demonstrate that the intranasal route should be further considered as an option for TMZ delivery into the brain to treat intrastriatal brain tumours. Moreover, it consists of an easy, fast, and cost-less method to gain direct access to the brain.

Keywords: Nasal absorption; Cancer stem-like cells; Nasal drug delivery; Alkylators; Stereotaxia; Cancer chemotherapy; Drug effects

Introduction

Glioblastoma multiforme (GBM) is the most frequent and severe malignant tumour of the adult central nervous system [1]. The standard treatment consists of maximal surgical resection, radiotherapy and adjuvant chemotherapy with temozolomide (TMZ) [2]. TMZ is a second-generation alkylating agent with good penetration across the blood brain barrier when administered orally. Tumor relapses are frequently associated to glioma stem-like cells with the emergence of tumor cells resistant to TMZ [3,4]. TMZ exhibits hematological adverse effects including a statistically significant increased risk of leukocytopenia and/or thrombocytopenia [2], which limit the possibility of increasing the dose given orally to prevent the generation of tumor resistant cells.

Intranasal route became recently a center of attention as an alternative way to treat brain solid tumours [5]. The intranasal route has been previously used to deliver hormones, Perillyl alcohol, telomerase inhibitors, neuropeptides and viruses into the brain [6-11], Recent works described the feasibility of the route to direct lipid-based nanoparticles of TMZ or treat glioma in rats [12,13]. The molecules intranasally infused are driven through the trigeminal and olfactory nerves anatomic connection between the nose and the brain. Intranasal delivery could thus increase the dose effectively delivered in the brain and reduce the adverse effects associated with systemic delivery.

In the present study, we have assessed for the first time, the effects of intranasal administration of TMZ on slow-progressing tumors generated in immunodeficient mice by intracerebral grafts of three human glioma stem cell lines (GSC), two of which being sensitive to TMZ *in vitro* and one being resistant [14].

Material and Methods

Intracerebral grafts of glioma stem cells

The human glioma cell lines TG16, TG1N and TG20 have been previously described [15,16]. GSC were intrastriatally injected in

***Corresponding authors:** François D Boussin, CEA, DSV, iRCM, SCSR, Laboratoiry of Radiopathology BP n°6 F-92265 Fontenay-aux-Roses, Cedex, France, Tel: 33146549791; Fax: 33146549180; E-mail: boussin@cea.fr

Jose R Pineda, Achucarro Basque Center for Neuroscience Fundazioa, Sede Building 3rd Floor, Barrio Sarriera s/n. 48940 Leioa, Vizcaya, Spain, Tel: 34946018139; E-mail: jr.pineda@achucarro.org

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ten-week-old female Swiss nu/nu mice (Charles River Laboratories, L' Arbresle, France) in compliance with the European Communities Council Directive of November 24, 1986 (86/609/EEC), the principles of laboratory animal care (NIH publication No. 85-23, revised 1985) using a protocol approved by our institutional committee on animal welfare (CETEA-CEA DSV IdF saisine number #7746). Briefly, mice were anesthetized with ketamine (75 mg/kg, Imalgen, Merial, Lyon, France) and medetomidine (1 mg/kg, Domitor, Pfizer, Paris, France). Two microliters containing 300.000 cells were stereotaxically injected with a nanoinjector (KOPFT, KD Scientific, Holliston, MA) using a 33-gauge stainless Hamilton syringe at the following coordinates from bregma: anteroposterior= +0.5 mm, lateral ± 1.5 mm, dorsoventral -3 mm. Paracetamol 1.64 mg/mL (Doliprane, Sanofi, France) was administered in drinking water for 5 days following the graft to ensure post-operational analgesia.

Intranasal administration and Kaplan-Meier survival analysis

One month after graft, mice were anesthetized using Isofluorane. Ten µL of TMZ (100 mM, Sigma-Aldrich, France, to ensure a delivery of 7 mg/Kg) or 10 µL of vehicle (DMSO, Sigma-Aldrich, France) were infused into the nostrils at the rate of 12 µL/minute with a nano-injector using Hamilton syringe coupled to a cannula of 0.2 mm inner diameter, as previously described [17] (Figure 1A). Paracetamol (1.64 mg/mL, Dolipran, Sanofi, France) was administered in drinking water during the period of intranasal administrations as a palliative treatment for nasal discomfort. Intranasal drug administrations were repeated three times a week during two weeks (Figure 1A). Possible acute adverse effects (stop of feeding, increased grooming, prostration) were examined during the 48 hours after administrations as recommended previously [18]. Mice were weighed once a week and end-point time was determined when loss of weight reached 20% of initial weight, and/or when mice showed moribund state (lethargy, failure to ambulate, hunched posture, failure to groom) and euthanized. Animals were deeply anesthetized in a CO_2 chamber and transcardially perfused with a 4% paraformaldehyde solution in 0.1 M sodium phosphate, pH 7.2.

Histopathological analysis

Brains were post-fixed for 2 h in 4% PFA, cryoprotected in increasing concentration of sucrose (10% to 30% in PBS). Serial coronal cryostat sections of 20 µm (200 µm apart) were made and mounted on polysine slides (Thermo Scientific, Braunschweig, Germany) for Haematoxylin-Eosin staining. Tumour volumes were calculated by multiplying the sum of all sectional areas (square millimeters) by the distance between successive sections as described previously [19]. Images acquisition were obtained using a UPlanFI 4X dry objective of 0.13 numerical aperture using a motorized microscope Olympus BX51 equipped with a Sony ExwaveHAD 3CCD DSP camera and the software Histolab 7.6.1 (Microvision Instruments, Evry France).

Buried food pellet test

Olfactory functions of mice were assessed as previously described [20]. Animals were food starved for 12 hours prior to being transferred into a clean cage containing an approximately 4 g pellet of rodent food buried under the mouse bedding. The time (in seconds) taken by mice to grab the food was then measured. The trial was repeated 3 times, the food being each time hidden into different positions.

WST-1 assay

TG16, TG1N or TG20 cells were plated on laminin-coated 96-well plates at 7 000 cells/well. Temozolomide was added the next day at different concentrations and the WST-1 assay (11644807001, Roche) was performed 72 hours later according to the manufacturer's instructions.

Statistical Analysis

Log-rank and non-parametric tests, Mann-Whitney, Kruskal-Wallis, Student's T and Scheffe's test for pair comparisons were conducted using StatView5 software (SAS Institute Inc., Cary, NC). Statistical significance was set at $p<0.05$.

Results

The sensitivity of the GSC lines to TMZ was first determined *in vitro* using the WST-1 assay. As shown Figure 1B, TG16 and TG1N cells were sensitive to high concentration of this alkylating agent, whereas TG20 cells were resistant (Figure 1B).

Mice engrafted with TG16 were sacrificed 142 days post graft, revealing a significant reduction of tumour volume in TMZ treated-mice versus controls consistent with the sensitivity of these cells to TMZ observed *in vitro* (p=0.05, n=3 per groups, Figure 1C). By contrast, no effect of TMZ on TG20 tumor growth was observed in animals sacrificed at 171 days post graft, confirming the resistance to TMZ of TG20 cells (Figure 1D).

Kaplan-Meier survival analysis of animals grafted with TG16 cells determined a median of survival of 184 days for controls. TMZ significantly increased the survival of the animals since the median of survival in the TMZ-treated group was more than 625 days for TMZ-treated mice. Indeed sixty six percent of TMZ-treated mice were still alive at 625 days corresponding to the end of the experiment (Figure 1D, p=0.0002 Log-rank test). Similar results were obtained in mice engrafted with TG1N cells: the median survival for the DMSO control group was 266 days post graft and more than 357 days for TMZ-treated mice since 85% of TMZ-treated animals were still surviving at that time.

Since the DMSO was used to dissolve TMZ, we have assessed the damage its administration may induce to the olfactive mucosa. For this purpose, we used the buried food pellet test which relies on the sense of smell to locate food. Ungrafted mice that were treated with either DMSO or Saline solution were tested 24 hours after the last treatment. The results showed that mice treated with DMSO needed more time to locate buried food than mice treated with saline solution (372 s vs. 157 s respectively, p= 0.0078, Mann-Whitney, test Figure 2B). However, 7 and 21 days after the last treatment, no difference remained between the two groups (Figures 2C and 2D), indicating that intranasal infusions of DMSO had only temporary short-term side effects on the sense of smell of the animals.

Finally, we assessed olfactory functions of mice engrafted with TG16 that survived at 195 days post graft. We did not find any statistical differences between control and TMZ-treated mice for the time spent to locate buried food (177 s ± 139 s vs.157 s ± 133 s respectively, p=0.6905, Figure 2E). These data indicate therefore that intranasal treatments with TMZ did not induce a long-term impairment of the olfactory function.

Discussion

We report here for the first time, that intranasal administration of TMZ delayed tumour growth and significantly extended the lifespan of mice engrafted with two human GSC lines. Importantly, we have also shown that intranasal administration of TMZ did not induce major adverse effects on the sense of smell of the animals, confirming previous reports of minimal side effects related to intranasal administration of other compounds like Perillyl alcohol [14].

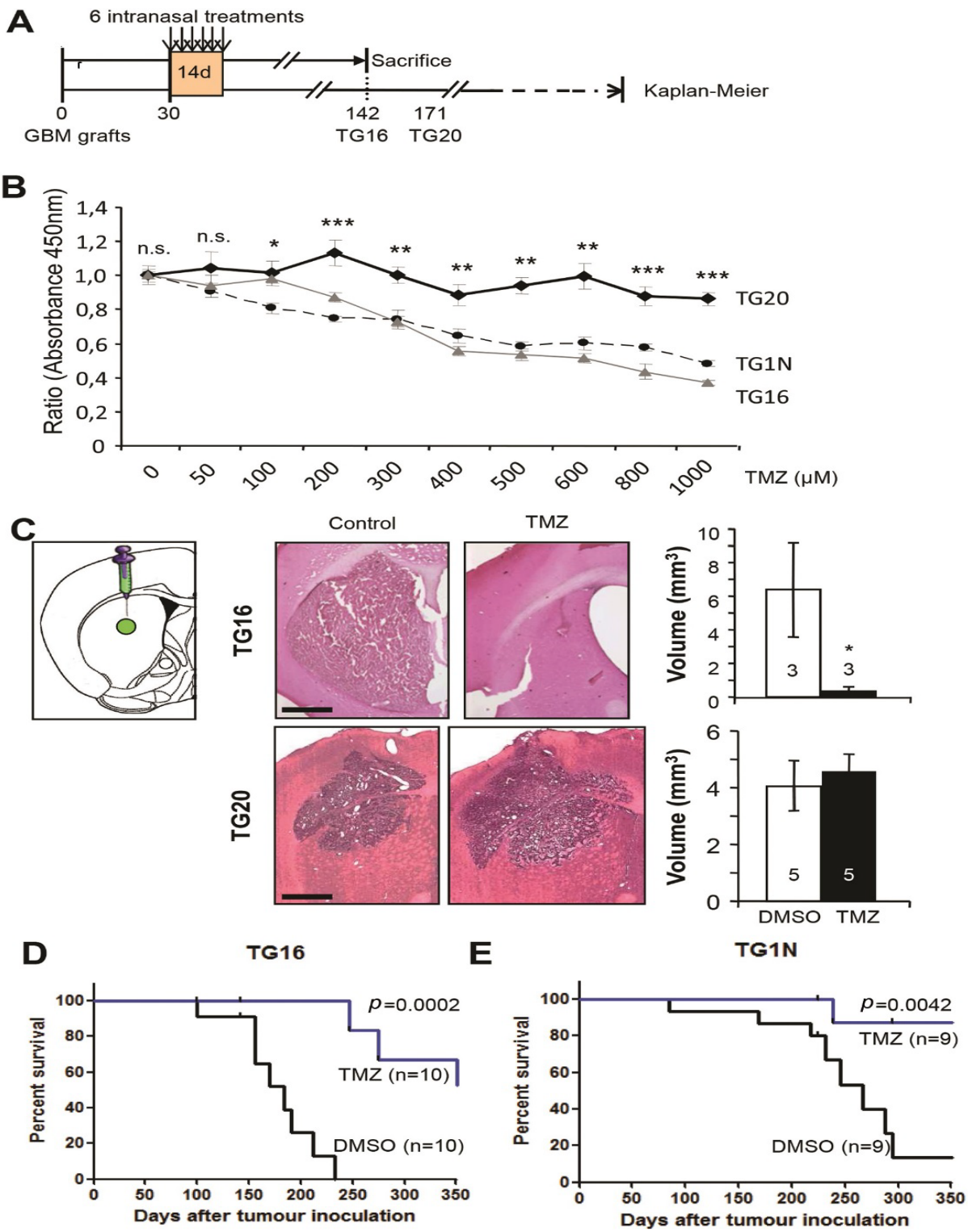

Figure 1: A) Experimental design for the Kaplan-Meier assays. Intranasal treatments with TMZ were delivered 30 days after cell injection. Mice were sacrificed at 142 or 171 days for histological staining. B) *In vitro* WST-1 proliferation assay for TG20, TG1N and TG16 cells, 72 hours after treatment with increasing concentrations of Temozolomide. Values were calculated relative to control cells treated with DMSO. Error bars represent the SEM from 6 replicates (*p<0.05, **p<0.01, ***p<0.001, Kruskal-Wallis test). C) The site of injection of cells is represented on the left. On the right, the graphs represent the mean percentages ± SD of TG16 or TG20 tumor volumes in DMSO or TMZ-treated mice. In the middle, representative Hematoxilin-eosin stained brain sections are shown for each condition (scale bars=2 mm) (*p<0.05, Anova's test). D-E) Kaplan Meier survival plots showing overall survival of mice grafted with TG16 or TG1N cells and treated with either DMSO or TMZ. Statistical analysis was performed using log rank Mantel-Cox test.

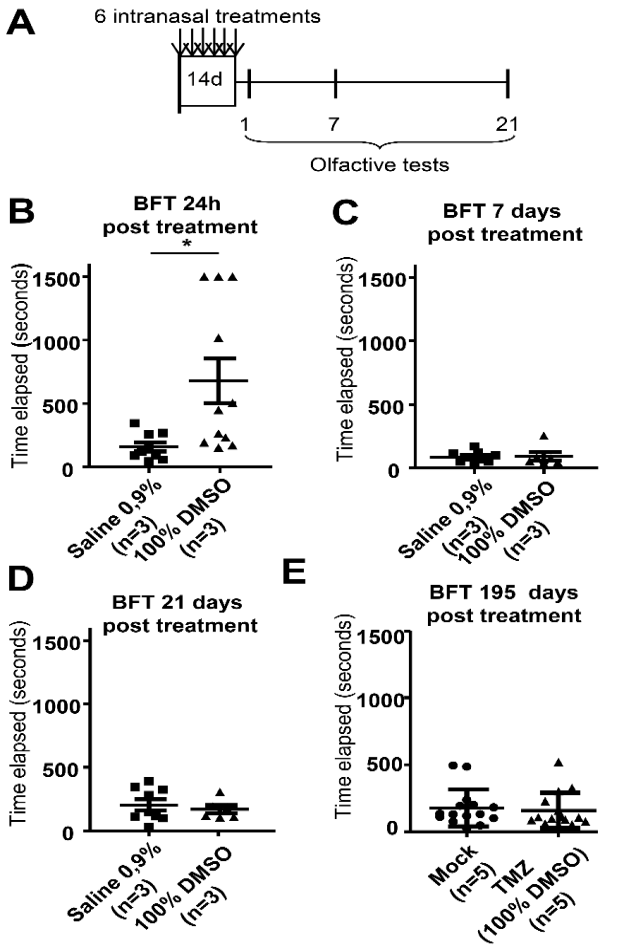

Figure 2: A) Experimental design for the olfactive tests after intranasal administration of DMSO in ungrafted mice. Buried food tests (BFT) were carried out at 1, 7, 21 after the last treatment. B-D) Buried Food Tests showing the food-finding time (in seconds) of the mice treated with Saline or DMSO. (E) Buried Food Tests of mice grafted with TG16 and treated with DMSO or TMZ at 195 days post-graft. Three replicates are represented for each mouse. n represents the number of mice used for each condition, *p<0.05.

GBM are associated with high rates of relapse, particularly due the emergence of glioma cells resistant to TMZ. We showed that intranasal administration of TMZ was efficient only on tumors generated by TG1N and TG16 cells, two GSC lines that have been isolated from samples of untreated GBM tumors. By contrast, intranasal administration of TMZ had no effect on the tumors initiated by TG20 cells, which have been isolated from a second-relapse GBM in a patient that received the Stupp protocol associating surgery, radiation and TMZ [15,16]. These results are in accordance with *in vitro* data showing that TG20 cells were resistant to high concentrations of TMZ *in vitro* unlike TG1N and TG16. Therefore our study indicates that the intranasal route does not allow TMZ to overcome the resistance already acquired by tumor cells at the dose tested. Future studies should thus determine if increasing the dose delivered by this alternative route to reach the brain is able to reduce or delay the development of resistance to TMZ by glioma cells in comparison to sytemic routes.

More generally, this work not only corroborates the feasibility of the use of intranasal route for the administration of anti-cancer drugs in preclinical models of glioma, but suggests that the intranasal route could be considered as an alternative method to deliver easily, fast and direct to brain, the standard anticancer drug TMZ in patients suffering of incurable glioblastoma.

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Disclosures

None.

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