Encapsulation of temozolomide in a calixarene nanocapsule improves its stability and enhances its therapeutic efficacy against glioblastoma

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Abstract

The alkylating agent temozolomide (TMZ) is the first-line chemotherapeutic for glioblastoma (GBM), a common and aggressive primary brain tumour in adults. However, its poor stability and unfavourable pharmacokinetic profile limit its clinical efficacy. There is an unmet need to tailor the therapeutic window of TMZ, either through complex derivatization or by utilizing pharmaceutical

excipients. To enhance stability and aqueous solubility, we encapsulated TMZ in a p-sulphonatocalix[4]arene (Calix) nanocapsule and employed ^{1}H-NMR, LC-MS and UV-Vis spectroscopy to chart the stability of this novel TMZ@Calix complex according to FDA and EMA guidelines. LC-MS/MS plasma stability assays were conducted in mice to further explore the stability profile of TMZ@Calix *in vivo*. The therapeutic efficacy of TMZ@Calix was compared to that of unbound TMZ in GBM cell lines and patient derived primary cells with known O6-methylguanine-DNA methyltransferase (*MGMT*) expression status and *in vivo* in an intracranial U87 xenograft mouse model. Encapsulation significantly enhanced the stability of TMZ in all conditions tested. TMZ@Calix was more potent than native TMZ at inhibiting the growth of established GBM cell lines and patient derived primary lines expressing *MGMT* and highly resistant to TMZ. *In vivo*, native TMZ was rapidly degraded in mouse plasma, whereas the stability of TMZ@Calix was enhanced 3-fold with increased therapeutic efficacy in an orthotopic model. In the absence of new effective therapies, this novel formulation is of clinical importance serving as an inexpensive and highly efficient treatment that could be made readily available to GBM patients and warrants further pre-clinical and clinical evaluation.

Key words: GBM; glioma; temozolomide; calixarene; LC-MS/MS

Introduction

Temozolomide (TMZ) is used for the treatment of primary brain tumours and brain metastases. It is the first-line chemotherapeutic for glioblastoma (GBM), which is the most common and aggressive malignant primary brain tumour. Radiotherapy with concomitant TMZ following surgical resection of the tumour is considered standard therapy for GBM and in this combination the contribution of TMZ is an increase in the median overall survival of 2.5 months (1, 2). TMZ is a pro-drug for the

metabolite 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide (MTIC), which acts as an alkylating agent to deliver a methyl group to the N-7 or O-6 position of guanine residues in the DNA. This DNA damage leads to inhibition of DNA replication and ultimately cell-cycle arrest (3). However, the O6-methylguanine adduct can be repaired by O6-methylguanine-DNA methyltransferase (MGMT), which confers TMZ resistance to approximately 60% of GBM tumours that express it (4-6). Despite this high incidence of inherent or acquired resistance, TMZ remains the main chemotherapeutic used against GBM. This is largely due to its pharmacokinetic characteristics and biodistribution. It has 100% oral bioavailability, a rapid absorption with a t_{max} of approximately one hour and due to its small size (194 Da) it is able to cross the blood brain barrier (BBB) (7-9).

Unfortunately, TMZ has some unfavourable characteristics that greatly limit its clinical potential. Although it is stable at acidic pH, under slightly alkaline conditions it is rapidly hydrolysed to MTIC and this active alkylating agent rapidly degrades to the methyl diazonium cation and the metabolite 5-amino-imidazole-4-carboxamide (AIC) (Fig 1). Compared to TMZ, MTIC has poor BBB penetration and reduced cellular uptake (10). Therefore, the accumulation of therapeutically effective amounts of MTIC at the site of the tumour relies on the stability of TMZ and its delivery past the BBB. However, due to the rapid elimination rate and short half-life of only around 1.8 hours the majority of the administered TMZ never reaches the tumour. In fact, only around 20% of the administered dose of TMZ is generally detectable in the cerebrospinal fluid at peak concentrations (11). High doses of TMZ need to be repeatedly administered to achieve the desirable anti-tumour effect, which leads to severe side-effects of which the most clinically significant is myelosuppression.

Stabilisation of TMZ's core to achieve a longer plasma half-life could enable a dosing regimen with the same treatment benefits, but without negatively impacting the patient's quality of life. TMZ has previously been stabilised by loading it onto functionalized nanoparticles, nanoliposomes or nanotubes resulting in a more effective chemotherapeutic in various tested models (12-20). However, nanoparticles have numerous drawbacks that reduce their clinical potential, such as generally low drug loading efficiencies, undefined toxicity profiles and a high production cost (21). To achieve stabilisation of TMZ while avoiding these drawbacks, we explored the water-soluble p-sulfonatocalix[4]arene (Calix) as a carrier due to its low toxicity, lack of immunogenicity and its high

aqueous solubility (22-25). Furthermore, Calix has a hydrophobic core that can accommodate the methyl group of the imidazotetrazine ring of TMZ and thereby protect it from rapid hydrolysis. This is important because this methyl group is key for the DNA damaging activity of TMZ. Calix is known to improve the stability of guest molecules, as well as their solubility (26, 27). It has been used to enhance the stability, aqueous solubility and efficacy of anti-cancer agents' paclitaxel, imatinib and of dinuclear platinum complexes (28-30). Its applications in cancer chemotherapy have been reviewed previously (31). Here, we developed a formulate of TMZ and Calix with the aim of enhancing the stability of TMZ and improving its absorption profile to ultimately enhance its therapeutic efficacy.

Materials and methods

Preparation of TMZ@Calix inclusion complex

10 mg (1 eq) of p-sulphonatocalix[4]arene (Sigma Aldrich, USA) was dissolved in 3 mL phosphate buffer pH 7.0 (10 μM). 4 mg (1.5 eq) of TMZ (Sigma Aldrich, USA) was diluted in 300 uL MeOH. The solutions were mixed and magnetically stirred at room temperature for 1 h. The contents were filtered through nylon filter with 0.45 μm pore size. Aqueous phase was evaporated under high vacuum giving the TMZ@Calix complex. Determination of encapsulated TMZ was performed using UV-Vis spectroscopy as described by Ishaq BM et al (32). Generally 1mg TMZ@Calix complex contained 100ug TMZ.

NMR spectroscopy

^{1}H-NMR spectra of calixarene, TMZ and the complex were recorded in a Bruker 400 MHz Advance spectrometer using D_2O and DMSO-d_6 as solvents. Samples were dissolved in 500 uL D_2O and transferred to 5 mm NMR tubes warmed to 28 ^{o}C. Topspin 3.1 was used to control the NMR system.

UV-Vis spectroscopy

The UV-Vis spectra of the TMZ@Calix complex were recorded with a Perkin Elmer Lambda 25 spectrometer (slit=1, speed 240 nm/min) at room temperature. The samples were dissolved in LC-MS grade H_2O and incubated under shaking at 37 ± 0.1 ^{o}C at 600 rpm. Samples were centrifuged for 5 minutes and precipitated TMZ was filtered off through regenerated cellulose syringe filters of 0.20 μm pore size.

UHPLC-MS/MS buffer stability assay

Reversed phase liquid chromatography was performed using an Advance Ultra High Performance Liquid Chromatography (UHPLC) system (Bruker, Germany). For the ionization and detection of TMZ and IS, EVOQ Elite ER triple quadrupole mass spectrometer (Bruker, Germany) was operated in positive ionization electrospray mode (ESI) in multiple reaction monitoring (MRM). To evaluate the stability of TMZ in its encapsulated form, stability studies were conducted at pH 2.1, 4.5 and 7.1

at 37 ^{o}C according to the FDA and EMA guidelines (33). 10 µL of TMZ@Calix (30 µM) was added to 280 µL hydrochloric acid (HCl, pH 2.1) and incubated at 37^{o}C in a shaking bath. At time intervals of 0, 2, 4, 6, 8, 16 and 24 h, the samples were removed from the bath and 10 µL of IS (27 µM) was added, vortex-mixed and transferred to LC-MS vials for analysis. Same procedure was followed for the samples incubated in ammonium formate (pH 4.5) and phosphate buffer (pH 7.1). To compare the stability of TMZ@Calix and TMZ, the same experiments were also conducted for TMZ. All samples were studied in triplicate and the % fraction remaining of TMZ against incubation time was plotted.

Cell culture

This study was approved by Imperial College London Research and Ethics Committee (REC 14/EE/0024).

GBM cells were maintained at 37^{o}C in a 5% CO_2 humidified incubator. Established GBM cell lines (8MG passage 8, U87 passage 8) were cultured in DMEM supplemented with 10% FBS. Cell lines were originally purchased from ATCC in 2014, expanded and stored as master stocks at low passage number (between 1-4). Master stocks were expanded into working stocks for use in experiments and did not exceed passage numbers beyond 20. Cells were maintained in cultures for less than 6-8 continuous weeks and tested for mycoplasma contamination on a regular basis using the Mycoplasma Detection Kit quick test (Stratech Scientific UK). Patient derived primary GBM cell cultures (GBM31, GBM59, GBM77) were established from fresh tumour tissue obtained from first surgical debulking or stereotactic biopsies at Charing Cross Hospital. Tissue samples were provided by the Imperial College Healthcare NHS Trust Tissue Bank, which is supported by the National Institute for Health Research (NIHR) Biomedical Research Centre based at Imperial College Healthcare NHS Trust and Imperial College London. Tumours were washed in DMEM:F12 (1:1) (Thermo Fisher Scientific, USA) and minced through a 100 µm cell strainer (Corning, USA) to obtain a single cell suspension. Cells are then centrifuged at 300 x g for 5 minutes and resuspended in sterile dH_2O to lyse contaminating red blood cells prior to being cultured in DMEM:F12 supplemented with 10% FBS (34).

qPCR analysis of *MGMT* expression

Total RNA was extracted using the RNeasy mini kit (Qiagen, Germany) and 2 µg converted to cDNA following the M-MLV Reverse Transcriptase protocol (Promega, USA) according to manufacturer's instructions. Samples were analysed in triplicate with SYBR Select qPCR master mix (Thermo Fisher Scientific, USA) using 50 ng of cDNA template for MGMT expression on a CFX96 Thermocycler (Bio-Rad, USA) according to the manufacturer instructions (*MGMT* forward primer: 5′-GGGTCTGCACGAAATAAAGC-3′; Reverse primer: 5′-TCCGGACCTCCGAGAAC-3′). Expression data was normalised to the mean Ct value of the reference gene hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) and presented as $2^{-\Delta Ct}$.

***In vitro* cytotoxicity: Sulforhodamine B (SRB) and Cell Counting Kit 8 (CCK8) assays**

Cells were seeded in 96-well plates (Corning, USA) at $2x10^3$ cells per well in their respective culture medium supplemented with 2% FBS. Twenty-four hours post-plating cells were treated with TMZ, Calix, physical mixture of TMZ and Calix or the TMZ@Calix complex in culture medium supplemented with 2% FBS. The concentration of TMZ in the complex was 100ugTMZ/mg complex). To determine the IC50 of native TMZ, cells were treated with 2, 4, 8, 16, 32, 64, 128, 256, 512 uM TMZ and analysed for proliferation using SRB assay 9 days post-treatment as previously described[34]. For experiments comparing the efficacy of the Calixarene complex with native TMZ, doses just below the IC50 of native TMZ was used (5uM and 10uM for U87 and 8MG respectively; 100uM and 200uM for the primary lines) and the equivalent equimolar concentrations of the complex was calculated and used. These experiments were harvested on days 6 and 9 post-treatment and cell proliferation was analysed by SRB for the established GBM cell lines and by the Cell Counting Kit 8 (CCK8, Sigma Aldrich, USA) for the primary lines according to manufacturer's instructions.

***In vivo* pharmacokinetic analysis**

Animal procedures were approved by the Animal Welfare and Ethical Review Body (Imperial College London). C57BL/6 female mice aged 6 weeks (Charles River Laboratories, USA) were separated into two groups (n=3) and injected intraperitoneally either with a single dose of TMZ (30

mg/kg) or a single dose of the TMZ@Calix complex (205.5 mg/kg, equivalent to 30 mg/kg of unbound TMZ). Blood was collected by cardiac puncture prior to treatment (day 0) and then at 0.5, 2 and 4 hrs post treatment in tubes containing heparin. Samples were centrifuged at 2862 g for 10 min to separate the plasma which was acidified (pH<4) with phosphoric acid 85%. All samples were stored at -80 ℃ until further processing. To prepare samples for analysis 200 µL IS solution (theophylline 1 µg/mL in methanol) and 200 µL of 10 mM pH 3.5 ammonium formate buffer were added to 100 µL of acidified mouse plasma and this mixture was then precipitated with a 100 mM 1:1 methanol:zinc sulfate solution. Following a vortex mix for 1 min and centrifugation at 21885 g for 15min, the supernatant was transferred to glass vials and TMZ was quantified by LC-MS/MS as described previously (35)

***In vivo* therapy of U87 intracranial xenografts**

CD1 Swiss nude female mice 6 weeks old (Charles River Laboratories, USA) were stereotactically injected with $3x10^5$ U87-GFP/Luc cells into the right cerebral cortex (n=12). Coordinates for injection were determined from the bregma (Anteroposterior: +0.1; Mediolateral: -0.2; Dorsoventral: -0.3). Mice were first imaged 9 days after cell implantation and randomised into three treatment groups (n=4): p-sulphonatocalix[4]arene only control (175.5 mg/kg), TMZ (30 mg/kg) and TMZ@Calix (205.5 mg/kg), which were carried out by intraperitoneal injections on days 13, 15 and 17 post implantation. Prior to treatment, mice were weighed and the dose adjusted accordingly. Tumour development was followed by bioluminescent imaging on days 16, 20, 24 and 27. Animals were sacrificed on day 28 and perfused with ice cold 4% PFA in PBS before brains were extracted and fixed in 4% PFA in PBS for 1 week. Brains were then transferred to 30% sucrose overnight and then frozen in 2-methylbutane (Sigma, UK). Frozen brains were suspended in OCT compound (VWR, USA), and whole brains were cut into 20 micron thick sections as depicted in Figure 4C using a CM1900 Cryostat (Leica Biosystems, Germany) onto SuperFrost Plus microscope slides (VWR, USA) and stained with hematoxylin and eosin (H&E). Images of H&E stained sections were acquired using a Nikon Eclipse E800 microscope (Nikon, Japan) and the Surveyor imaging software (Objective Imaging, UK).

Statistics

One-way and two-way ANOVA were performed in GraphPad Prism software (version 7.0). Data is presented as mean ± standard error of the mean (s.e.m.).

Results

Encapsulation of TMZ into Calix greatly enhances its stability

TMZ is the only FDA approved drug for the treatment of GBM. However, due to its short half-life (2hrs) it has to be administered repeatedly and at high doses. To address these limitations, we encapsulated TMZ with Calix and tested it's stability and solubility using ^{1}H-NMR spectroscopy, LC-MS/MS and UV-Vis spectroscopy. We detected a significant chemical shift in the NMR spectrum of the TMZ@Calix complex compared to native TMZ indicating that the imidazotetrazine ring of TMZ was incorporated deep inside the hydrophobic cavity (**Supplementary Fig. S1**). We next analysed the 1H-NMR spectrum for the presence of MITC (represented by the 7-H proton) which is highlighted in grey in figure 2A. There was an increase in signal at 7.23 ppm (1H, 7-H) indicating degradation of native TMZ to MTIC. Moreover, the methyl region of the spectrum indicated a decomposition of the singlet peak which is normally representative of intact TMZ (δ 4 ppm, 3H, 12-H). These changes were not detected in the TMZ@Calix ^{1}H-NMR spectrum suggesting no detectable degradation of TMZ in this complex.

To further validate this enhanced stability under physiological conditions, the degradation rate of TMZ and TMZ@Calix was monitored by LC-MS/MS and UV-Vis in aqueous buffer solutions of differing pH **(Fig 2B & C).** These pH values were selected to mimic the conditions present during gastrointestinal (GI) absorption after oral drug administration. Under fasting conditions, the pH range in the GI tract varies from 1.4 to 2.1 in the stomach, 4.9 to 6.4 in the duodenum, 4.4 to 6.6 in the jejunum and 6.5 to 7.4 in the ileum (36). Both analytical techniques show that TMZ remains stable after 24-hour incubation at pH 2.1 and 4.5, whether free or encapsulated. However, at pH 7.1 native TMZ demonstrated a high rate of hydrolysis. Only around 60% of the initial concentration remained

after two hours of incubation. Notably, encapsulation of TMZ in the TMZ@Calix complex profoundly enhanced its stability, delaying hydrolysis by more than 6 hours and increasing the half-life 4-fold under these conditions.

TMZ@Calix is significantly more effective at inhibiting the growth of GBM cells

We next proceeded to test the *in vitro* cytotoxicity of native TMZ and TMZ@Calix against GBM cells. Established (U87/8MG) and patient derived primary GBM cultures (GBM31/GBM59/GBM77) were initially analysed for *MGMT* expression by qPCR, an important determinant of cellular sensitivity to TMZ (**Fig. 3A**). We then treated these cells with varying concentrations of TMZ and analysed them for proliferation (**Fig. 3B**). The *MGMT* expression was near to undetectable in the established cell lines and both were sensitive to TMZ treatment with IC50 doses of 5 µM and 10 µM for U87 and 8MG respectively. The primary lines all expressed *MGMT* and were highly resistant to TMZ with IC50 values of over 300 µM. Having established the sensitivity of GBM cells to native TMZ, the efficacy of the TMZ@Calix complex was tested in these cell lines using equimolar concentrations equivalent to TMZ IC50 doses (**Fig. 3C**). In 8MG and U87, TMZ@Calix reduced the number of cells by 43% ($p < 0.0001$) and 54% ($p < 0.0001$) respectively when compared to equimolar concentrations of native TMZ. Calixarene alone had no significant effect on the growth of either cell line.

Patient derived primary GBM cell cultures were used to validate the enhanced efficacy of TMZ@Calix. GBM31, GBM59 and GBM77 were analysed by the CCK8 assay 9 days post-treatment TMZ@Calix at equimolar concentrations equivalent to 100 and 200uM of native TMZ. While TMZ treatment at high concentrations did not significantly impact the TMZ-resistant primary GBM cultures, the equivalent of 100 µM of TMZ encapsulated in the TMZ@Calix complex caused a highly significant 65% reduction in the proliferation of GBM31 ($p < 0.0001$), a 74% reduction of GBM59 ($p < 0.0001$) and an 81% reduction of GBM77 ($p < 0.0001$), despite these cells expressing high levels of *MGMT* (**Fig. 3D**).

Encapsulation greatly increases the biological half-life of TMZ and improves its therapeutic efficacy *in vivo*

The *in vivo* degradation rates of native TMZ and the TMZ@Calix complex were examined by LC-MS/MS plasma stability assays **(Fig 4A)**. Mice were intraperitoneally injected with 30 mg/kg TMZ and an equimolar dose of the TMZ@Calix complex (205.5 mg/kg) and blood collected by cardiac puncture pre treatment (0) and at 30 minutes, 2 hours and 4 hours post-treatment. As previously reported, we found the half-life of native TMZ to be very short with only 8% of the injected dose remaining 4 hours post-administration. In contrast, 47% of the TMZ administered as the TMZ@Calix complex could be detected at this time point.

To determine if this enhanced stability would lead to greater efficacy, the complex was tested for its therapeutic potential in an intracranial GBM mouse model using U87 GBM cells **(Fig 4B)**. Groups of 4 mice were treated with calixarene only as a control (175.5 mg/kg), native TMZ (30 mg/kg) or the TMZ@Calix complex (205.5 mg/kg). Mice were treated on day 13, 15 and 17 post-implantation and then sacrificed on day 28. Bioluminescence imaging revealed enhanced tumour shrinkage in mice treated with the TMZ@Calix complex compared to those treated with native TMZ or calixarene alone, however, these results appeared not to be significant (Fig 4C). Upon further inspection of the brains by H&E staining of the largest area of the tumour (Fig 4D), there was clear indication of an enhanced therapeutic effect in mice treated with the TMZ@Calix complex where the tumour size was drastically reduced compared to native TMZ **(Fig 4C** lower panel**)**.

Discussion

Parenchymal brain tumours present unique management challenges because of their inherent aggressiveness, cellular heterogeneity and location. While developments in detection methods, surgical techniques and radiology have contributed to a modestly improved clinical outcome, there has been little progress in the development of chemotherapeutics. Since its introduction to the clinic in 2007 TMZ has remained the standard of care systemic therapy for brain tumours, especially for GBM. Notwithstanding promising developments, for example in immunotherapy, TMZ will remain an

essential agent in the management of GBM for the foreseeable future (37). With TMZ now being off patent it is the ideal time to modify this drug to develop a more efficacious and cost-effective treatment.

Here TMZ was encapsulated to form a TMZ@Calix complex in which TMZ is protected from rapid degradation. Native TMZ has a half-life of 1.83 hours at 37℃ in phosphate buffer (0.1M) at pH 7 (38). Evaluation of TMZ@Calix dissociation and stability according to FDA and EMA guidelines confirmed that the hydrolysis of TMZ to its active compound, MITC, is significantly delayed (32). This stabilisation far exceeds what has been achieved by a previous study, where UV-vis spectrometry in a 10 mM buffer solution at pH 7.0 showed an extension of the TMZ half-life by around 2 hours when complexed to cucurbit[7]uril (39). By forming a complex with Calix, we extended the half-life to more than 12 hours. The stability of TMZ is therefore enhanced 4-fold by encapsulating it in a TMZ@Calix complex. Since the therapeutic action of TMZ requires induction of sufficient DNA damage to result in cell death, we determined whether the enhanced stability results in increased chemotherapeutic efficacy and we show that *in vitro* the TMZ@Calix complex is a significantly more potent inhibitor of tumour cell growth than native TMZ.

Particularly striking is the ability of the TMZ@Calix complex to inhibit tumour cell growth even in those primary GBM cells that express high levels of *MGMT* and are normally refractory to therapy. Importantly, we have demonstrated activity of TMZ@Calix in three independent MGMT positive early passage primary GBM cultures. This clearly strengthens the general applicability of our findings, which is in line with a previous publication where platinum-based drugs were stabilised by complexing with *para*-sulfonato-calix[4]arene, resulting in increased anti-cancer activity in a cisplatin-resistant ovarian cancer cell line (30). Overcoming MGMT-mediated innate TMZ resistance would be of major significance in GBM treatment considering that approximately 60% of patients express *MGMT* and therefore do not benefit from TMZ treatment (6) It was observed that the TMZ@Calix complex affects cells earlier than unbound TMZ, which suggests increased cellular uptake. The combination of enhanced cell uptake with increased stability, could explain how the TMZ@Calix complex is able to overcome MGMT-mediated TMZ resistance. Surprisingly, little is known about the cellular uptake of TMZ and the kinetics involved, but Calix has been characterised in

terms of its cellular uptake and has been found to enter cells very efficiently and to accumulate in the cytoplasm (40). In that study, a fluorescently labelled calix[4]arene was used where the fluorescent intensity changes from weak to strong upon conjugation (40). This method could also be employed in future studies to assess the cellular uptake of the TMZ@Calix complex to better understand the kinetics behind its enhanced anti-tumour effects.

Having demonstrated that encapsulation potentiates the *in vitro* stability and anti-tumour activity of TMZ we next tested the *in vivo* pharmacokinetic and therapeutic properties in mice. We demonstrated that encapsulation of TMZ with Calixarene greatly enhanced its stability in plasma and improved its therapeutic efficacy in an orthotopic model of glioblastoma far superior to native TMZ. The increased ability of the TMZ@Calix complex to inhibit intracranial GBM is particularly striking because a previous study reported that unbound para-sulfonato-calix[4]arene do not enter the brain of mice even at high concentrations of 100mg/kg (23). A possible explanation for the higher potency observed with TMZ@Calix is that through enhanced stability provided by para-sulfonato-calix[4]arene higher concentrations of released TMZ will pass through the BBB and enter the tumour site. Moreover, encapsulated TMZ may indeed be able to enter the perturbed BBB increasing the local concentration of TMZ in the brain. Further *in vivo* biodistribution studies will be performed to evaluate the accumulation of TMZ in the brain when administered complexed to Calixarene. Orthotopic models of *MGMT* expressing primary human GBM cells will also be used to further understand the enhanced therapeutic efficacy of TMZ@Calix.

There are several other approaches that could be taken to further enhance the anti-tumour efficacy of the TMZ@Calix complex. For example, co-assembly of amphiphilic p-sulphonatocalix[4]arenes into multifunctional drug carriers that can be functionalised by decorating them with tumour targeting ligands have been explored in recent years and this approach could also be employed to deliver TMZ (41, 42). The TMZ@Calix complex could also be functionalised with a potent MGMT inhibitor to further enhance its efficacy against *MGMT*-expressing TMZ resistant tumours (43). TMZ has also been conjugated directly to compounds where co-administration causes a synergistic anti-tumour effect. For example, a conjugate of TMZ and perillyl alcohol termed NEO212 was found to be 10-fold more cytotoxic than TMZ alone (44). Instead of using conventional TMZ, NEO212 could be loaded

onto calixarene to enhance its stability, efficacy and ease of delivery. Finally, the TMZ@Calix complex could additionally be functionalised by conjugation to angiopep-2, to further enhance active transport across the BBB as has been reported for angiopep-2 functionalised nanoparticles targeting glioma (45-47)**.** This small peptide has been shown to cross the BBB in animal models via transcytosis by binding to low density lipoprotein receptor related protein (LRP) expressed on the surface of the BBB. Moreover, GBM cell lines have been reported to express LRP (48).

These options are currently being explored in our labs because we believe this method of TMZ stabilisation to be a significant enhancement to the current standard of care with potentially broad treatment implications.

To date there has been only one clinical trial listed involving p-sulphonatocalix[4]arenes: OTX-008, is a synthetic p-sulphonatocalix[4]arene molecule that binds directly to galectin-1 and induces a conformational change reducing the binding to carbohydrates. Promising pharmacokinetic data was presented in an abstract at the AACR 104th annual meeting in 2013 (49). With preliminary clinical trial data on the synthetic p-sulphonatocalix[4]arene OTX-008 having been positive and the pre-clinical indications that Calix can function as non-toxic drug carrier (49), we anticipate that the TMZ@Calix complex could quickly move through clinical trials with more robust *in vivo* testing. Due to the lengthy processes involved in translating completely novel effective therapies for GBM treatment, we envisage that the TMZ@Calix complex could be an inexpensive and highly potent treatment option made readily available to GBM patients in the near future.

References

1. Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med*. 2005;352:987-996.

2. Stupp R, Hegi ME, Mason WP, wan den Bent MJ, Taphoorn MJ, Janzer RC, et al. Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. *Lancet Oncol*. 2009;10:459-466.

3. Roos WP, Batista LF, Naumann SC, Wick W, Weller M, Menck CF, et al. Apoptosis in malignant glioma cells triggered by the temozolomide-induced DNA lesion O6-methylguanine. *Oncogene*. 2007;26:186-197.

4. Weller M, Tabatabai G, Kastner B, Felberg J, Steinbach JP, Wick A, et al. MGMT promoter methylation is a strong prognostic biomarker for benefit from dose-intensified temozolomide rechallenge in progressive Glioblastoma: The DIRECTOR Trial. *Clin Cancer Res*. 2015; 21:2057-2064.

5. Happold C, Roth P, Wick W, Schmidt N, Florea AM, Silginer M, et al. Distinct molecular mechanisms of acquired resistance to temozolomide in glioblastoma cells. *J Neurochem*. 2012; 122:444-455.

6. Hegi ME, Diserens A-C, Gorlia T, Hamou MF, de Tribolet N, Weller M, et al. MGMT gene silencing and benefit from temozolomide in glioblastoma. *N Engl J Med*. 2005;352:997-1003.

7. Zhou Q, Guo P, Wang X, Nuthalapati S, Gallo JM. Preclinical pharmacokinetic and pharmacodynamic evaluation of metronomic and conventional temozolomide dosing regimens. *J Pharmacol Exp Ther*. 2007;321:265-275.

8. Portnow J, Badie B, Chen M, Liu A, Blanchard S, Synold TW. The neuropharmacokinetics of

temozolomide in patients with resectable brain tumors: Potential implications for the current approach to chemoradiation. *Clin Cancer Res*. 2009;15:7092-7098.

9. Lopes IC, De Oliveira SCB, Oliveira-Brett AM. Temozolomide chemical degradation to 5-aminoimidazole-4-carboxamide - Electrochemical study. *J Electroanal Chem*. 2013;704:183-189.

10. Meer L, Janzer RC, Kleihues P, Kolar GF. In vivo metabolism and reaction with dna of the cytostatic agent, 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (DTIC). *Biochem Pharmacol*. 1986;35:3243-3247.

11. Ostermann S, Csajka C, Buclin T, Leyvraz S, Lejeune F, Decosterd LA, Stupp R. Plasma and cerebrospinal fluid population pharmacokinetics of temozolomide in malignant glioma patients. *Clin Cancer Res*. 2004;10:3728-3736.

12. Fang C, Wang K, Stephen ZR, Mu Q, Kievit FM, Chiu DT, et al. Temozolomide nanoparticles for targeted glioblastoma therapy. *ACS Appl Mater Interfaces*. 2015;7:6674-6682.

13. Tian XH, Lin XN, Wei F, Feng W, Huang ZC, Wang P, et al. Enhanced brain targeting of temozolomide in polysorbate-80 coated polybutylcyanoacrylate nanoparticles. *Int J Nanomedicine*. 2011;6:445-452.

14. Hu J, Wang J, Wang G, Yao Z, Dang X. Pharmacokinetics and antitumor efficacy of DSPE-PEG2000 polymeric liposomes loaded with quercetin and temozolomide: Analysis of their effectiveness in enhancing the chemosensitization of drug-resistant glioma cells. *Int J Mol Med*. 2016;37:690-702.

15. Irani M, Mir Mohamad Sadeghi G, Haririan I. Gold coated poly (ε-caprolactonediol) based polyurethane nanofibers for controlled release of temozolomide. *Biomed Pharmacother*. 2017; 88:667-676.

16. Irani M, Mir Mohamad Sadeghi G, Haririan I. A novel biocompatible drug delivery system of

chitosan/temozolomide nanoparticles loaded PCL-PU nanofibers for sustained delivery of temozolomide. *Int J Biol Macromol*. 2017; 97:744-751.

17. Di Martino A, Kucharczyk P, Capakova Z, Humpolicek P, Sedlarik V. Enhancement of temozolomide stability by loading in chitosan-carboxylated polylactide-based nanoparticles. *J Nanoparticle Res*. 2017;19:17

18. Clemente N, Ferrara B, Gigliotti CL, Boggio E, Capucchio MT, Biasibetti, et al. Solid Lipid Nanoparticles Carrying Temozolomide for Melanoma Treatment. Preliminary In Vitro and In Vivo Studies. *Int J Mol Sci*. 2018;9:255.

19. Patil R, Portilla-Arias J, Ding H, Inoue S, Konda B, Hu J, et al. Temozolomide delivery to tumor cells by a multifunctional nano vehicle based on poly(β-L-malic acid). *Pharm Res*. 2010;27:2317-2329.

20. Kumari S, Ahsan SM, Kumar JM, Kondapi AK, Rao NM. Overcoming blood brain barrier with a dual purpose Temozolomide loaded Lactoferrin nanoparticles for combating glioma (SERP-17-12433). *Sci Rep*. 2017;7:6602.

21. Blanco E, Shen H, Ferrari M. Principles of nanoparticle design for overcoming biological barriers to drug delivery. *Nat Biotechnol*. 2015;33:941-951.

22. Perret F, Coleman AW. Biochemistry of anionic calix[n]arenes. *Chem Commun*. 2011; 47:7303-7319.

23. Coleman AW, Jebors S, Cecillon S, Perret P, Garin D, Marti-Battle D, et al. Toxicity and biodistribution of para-sulfonato-calix[4]arene in mice. *New J Chem*. 2008;32:780.

24. Da Silva E, Shahgaldian P, Coleman AW. Haemolytic properties of some water-soluble para-sulphonato-calix-[n]- arenes. *Int J Pharm*. 2004;273:57-62.

25. Paclet M-H, Rousseau CF, Yannick C, Morel F, Coleman AW. An Absence of Non-specific Immune Response towards para-Sulphonato-calix[n]arenes. *J Incl Phenom Macrocycl Chem*.

2006;55:353-357.

26. Guo DS, Wang K, Liu Y. Selective binding behaviors of p-sulfonatocalixarenes in aqueous solution. *J Incl Phenom Macrocycl Chem*. 2008;62:1-21.

27. Karakurt S, Kellici TF, Mavromoustakos T, Tzakos AG, Yilmaz M. Calixarenes in lipase biocatalysis and cancer therapy. *Curr Org Chem*. 2016;20:1043-1057.

28. Mo J, Eggers PK, Yuan ZX, Raston CL, Lim LY. Paclitaxel-loaded phosphonated calixarene nanovesicles as a modular drug delivery platform. *Sci Rep*. 2016;6:23489

29. Galindo-Murillo R, Olmedo-Romero A, Cruz-Flores E, Petrar PM, Kunsagi-Mate S, Barroso-Flores J. Calix[n]arene-based drug carriers: A DFT study of their electronic interactions with a chemotherapeutic agent used against leukemia. *Comput Theor Chem*. 2014;1035:84-91.

30. Brown SD, Plumb JA, Johnston BF, Wheate NJ. Folding of dinuclear platinum anticancer complexes within the cavity of para-sulphonatocalix[4]arene. *Inorganica Chim Acta*. 2012; 393:182-186.

31. Yousaf A, Hamid SA, Bunnori NM, Ishola AA. Applications of calixarenes in cancer chemotherapy: Facts and perspectives. *Drug Des Devel Ther*. 2015;9:2831-2838.

32. Ishaq BM, Ahad HA, Muneer S, Parveen S, Fahmida B. Analytical method development and validation for the estimation of temozolomide in phosphate buffer ph 2.0 as a solvent by uv spectroscopy. Int. Res.J.Pharm. 2014;5:7-20.

33. Verbeeck RK, Musuamba FT. The revised 2010 EMA guideline for the Investigation of bioequivalence for immediate release oral formulations with systemic action. *J Pharm Pharm Sci*. 2012;15:376-388.

34. Syed N, Langer J, Janczar K, Janczar K, Singh P, Lo Nigro C, et al. Epigenetic status of argininosuccinate synthetase and argininosuccinate lyase modulates autophagy and cell death in glioblastoma. *Cell Death Dis*. 2013;4:e458.

35. El Mubarak MA, Stylos EK, Chatziathanasiadou M V, Danika C, Alexiou GA, Tsekeris P, et al. Development and validation of simple step protein precipitation UHPLC-MS/MS methods for quantitation of temozolomide in cancer patient plasma samples. *J Pharm Biomed Anal*. 2019;162:164-170.

36. Riethorst D, Mols R, Duchateau G, Tack J, Brouwers J, Augustijns P. Characterization of Human Duodenal Fluids in Fasted and Fed State Conditions. *J Pharm Sci*. 2016;105:673-681.

37. Chamberlain MC, Kim BT. Nivolumab for patients with recurrent glioblastoma progressing on bevacizumab: a retrospective case series. *J Neurooncol*. 2017;133:561-569.

38. Wheelhouse RT, Stevens MFG. Decomposition of the antitumour drug temozolomide in deuteriated phosphate buffer: Methyl group transfer is accompanied by deuterium exchange. *J Chem Soc Chem Commun*. 1993;15:1177-1178.

39. Appel EA, Rowland MJ, Loh XJ, Heywood RM, Watts C, Scherman OA. Enhanced stability and activity of temozolomide in primary glioblastoma multiforme cells with cucurbit[n]uril. *Chem Commun*. 2012;48:9843.

40. Lalor R, Baillie-Johnson H, Redshaw C, Matthews SE, Mueller A. Cellular uptake of a fluorescent calix[4]arene derivative. *J Am Chem Soc*. 2008;130:2892-2893.

41. Eggers PK, Becker T, Melvin MK, Boulos RA, James E, Morellini N, et al. Composite fluorescent vesicles based on ionic and cationic amphiphilic calix[4]arenes. *RSC Adv*. 2012;2:6250.

42. Wang Y-X, Guo D-S, Duan Y-C, Wang Y-J, Liu Y. Amphiphilic p-Sulfonatocalix[4]arene as “Drug Chaperone” for Escorting Anticancer Drugs. *Sci Rep*. 2015;5:9019.

43. Wang C, Abegg D, Hoch DG, Adibekian A. Chemoproteomics-Enabled Discovery of a Potent and Selective Inhibitor of the DNA Repair Protein MGMT. *Angew Chem Int Ed Engl*. 2016; 55:2911-2915.

44. Jhaveri N, Agasse F, Armstrong D, Peng L, Commins D, Wang W, et al. A novel drug conjugate, NEO212, targeting proneural and mesenchymal subtypes of patient-derived glioma cancer stem cells. *Cancer Lett*. 2016;371:240-250.

45. Gao H, Zhang S, Cao S, Yang Z, Pang Z, Jiang X. Angiopep-2 and activatable cell-penetrating peptide dual-functionalized nanoparticles for systemic glioma-targeting delivery. *Mol Pharm*. 2014;11:2755-2763.

46. Wang L. L, Hao Y, Li H, Zhao Y, Meng D, Li D, et al. Co-delivery of doxorubicin and siRNA for glioma therapy by a brain targeting system: angiopep-2-modified poly(lactic-co-glycolic acid) nanoparticles. *J Drug Target*. 2015;23:832-846.

47. Figueiredo P, Balasubramanian V, Shahbazi MA, Correia A, Wu D, Palivan DG, et al. Angiopep2-functionalized polymersomes for targeted doxorubicin delivery to glioblastoma cells. *Int J Pharm*. 2016;511:794-803.

48. Malentinska L, Blakely EA, Bjornstad KA, Deen DF, et al. Human Glioblastoma Cell lines: Levels of Low Density Lipoprotein Receptor and Low Density Lipoprotein Receptor-related Protein. Cancer Research. 2000;60:2300-2303.

49. Rezai K, Durand S, Lachaux N, Raymond E, Herait P, Lokiec F. Abstract 33: OTX008 pharmacokinetics (PK) during the first-in-man phase I study in patients with advanced solid tumors. *Cancer Res*. 2013;73:33.

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Abbreviations and Acronyms

AIC: 5-amino-imidazole-4-carboxamide; BBB: blood brain barrier; Calix: p-sulphonatocalix[4]arene; GBM: glioblastoma multiforme; IS: internal standard; LC-MS/MS: liquid chromatography-tandem mass spectroscopy; NMR: nuclear magnetic resonance; MGMT: O6-methylguanine-DNA methyltransferase; MRM: multiple reaction monitoring; MTIC: 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide; TMZ: temozolomide; UV: ultraviolet

Figure Legends

Fig. 1: Degradation products of native TMZ and structure of the TMZ@calixarene nanocapsule complex

A) Under neutral or alkaline conditions TMZ undergoes rapid hydrolytic ring opening to produce the active metabolite MTIC ($t_{1/2}$=8min at pH = 7.4), the inactive metabolite AIC, and the active electrophile methyl diazonium cation ($t_{1/2}$= 8s at pH = 7.4)[48]. **B)** Schematic representations of the Calixarene (Calix) nanocapsule used in this study and the TMZ@Calix complex.

Fig. 2: **Stability profiles of native TMZ and TMZ@Calix in vitro**

A) ^{1}H NMR spectra of the time-dependent degradation of native TMZ and TMZ@Calix to MTIC at deuterated phosphate buffer in D_2O (10 µm). The highlighted grey peak at 7.23 ppm represents the 7-H proton of the MTIC form. The highlighted peaks at 3.52 ppm represent the 1-H proton of the MTIC form and its adduct. **B)** Stability of TMZ and TMZ@Calix under different pH conditions as monitored by LC-MS/MS. **C)** Stability of TMZ and TMZ@Calix under different pH conditions as monitored by UV-Vis.

Fig. 3: MGMT expression profiling and functional assessment of TMZ and TMZ@Calix on the proliferation of GBM cell lines

A) *MGMT* expression determined by qPCR and represented as $2^{-\Delta ct}$. **B)** Dose-response for TMZ treated GBM cell lines (U87/8MG) and patient derived primary cells (GBM31/GBM59/GBM77) determined by SRB assay 9 days post-treatment. TMZ concentrations used were 2, 4, 8, 16, 32, 64, 128, 256, 512uM. Absorbance was normalised to the untreated control and represented on a log scale. **C)** Cell growth SRB assay on established GBM cell lines 8MG and U87. Cells were treated with IC50 doses of TMZ (U87: 5µM; 8MG: 10µM) and calixarene (U87: 13.5 µM; 8MG: 27 µM) both alone and in combination at equimolar concentrations to those found in the amount of TMZ@Calix complex used. The concentration of TMZ in the complex was 100ugTMZ/mg of complex. Cells were analysed on 6 and 9 days post-treatment. Both 8MG and U87 are *MGMT* negative and sensitive to TMZ. **D)** CCK8 assay to measure the growth of GBM primary cultures GBM31, GBM59 and GBM77, 9 days post-treatment. Cells were treated with 100 µM and 200 µM TMZ and the respective equimolar concentrations of the TMZ@Calix complex. Both primaries are *MGMT* positive and very resistant to TMZ. Two-way ANOVA with Tukeys multiple comparisons test was used to identify significant differences between treatment groups (****$p < 0.0001$).

Fig. 4: Degradation profile of TMZ and TMZ@Calix and assessment of their therapeutic efficacy in an intracranial model of GBM

A) *In vivo* degradation rate in mouse plasma of 30 mg/kg native TMZ and equimolar TMZ encapsulated in the TMZ@Calix complex. Plasma samples were analysed by an optimised LC-MS/MS stability assay. **B)** Therapeutic efficacy of the TMZ@Calix complex was evaluated to determine whether increased stability increases its efficacy compared to unbound TMZ. CD1 Swiss nude mice with intracranial U87 xenograft tumours were randomized into 3 groups on day 10 post-implantation (n=4): p-sulphonatocalix[4]arene only control (175.5 mg/kg), TMZ (30 mg/kg) and TMZ@Calix complex (205.5 mg/kg). Prior to each treatment mice were weighed, and the dose adjusted accordingly. Mice were imaged for luciferase activity by bioluminescence imaging on days 9, 16, 20, 24 and 27 post-implantation. Treatments were administered on days 13, 15 and 17 days post implantation of U87 GBM cells by intraperitoneal injection. The graph shows fold change in tumour size calculated by normalising to the bioluminescent image values of each mouse at day 9 post-

implantation to account for variability in initial tumour formation. **C)** Representative images for bioluminescence at day 9 and day 27 post implantation and representative H&E images from animals in each treatment group. D) Schematic representation of how brains were sectioned. The H&E stains in D represent sections taken from the largest area of the tumour bulk.

Figure 1

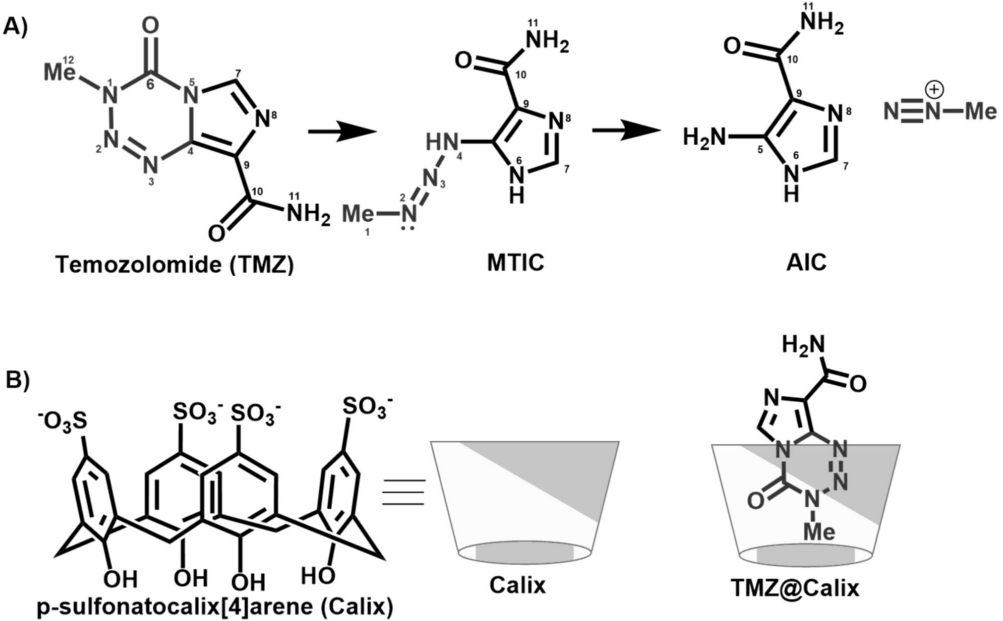


Figure 2

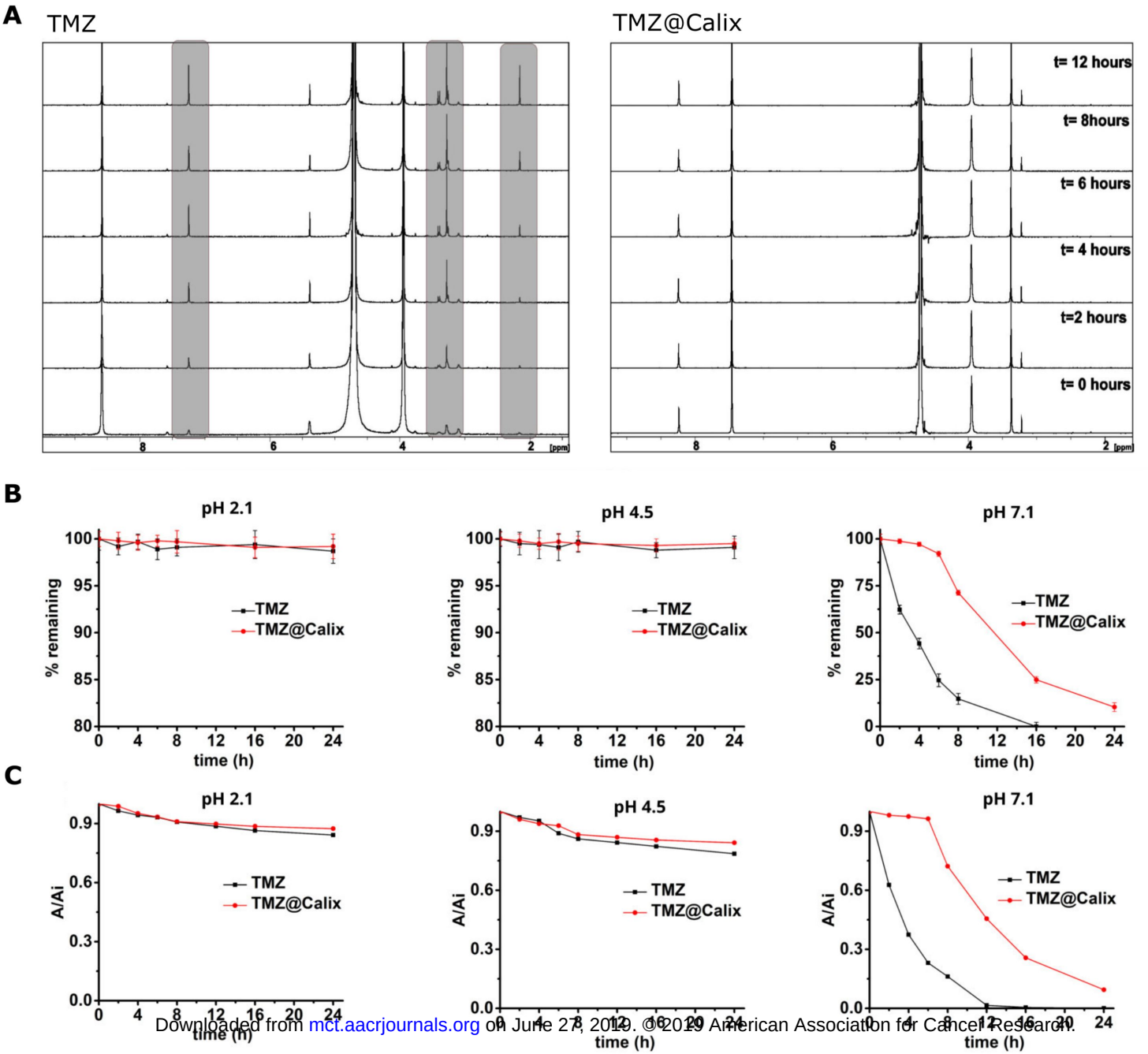


Figure 3

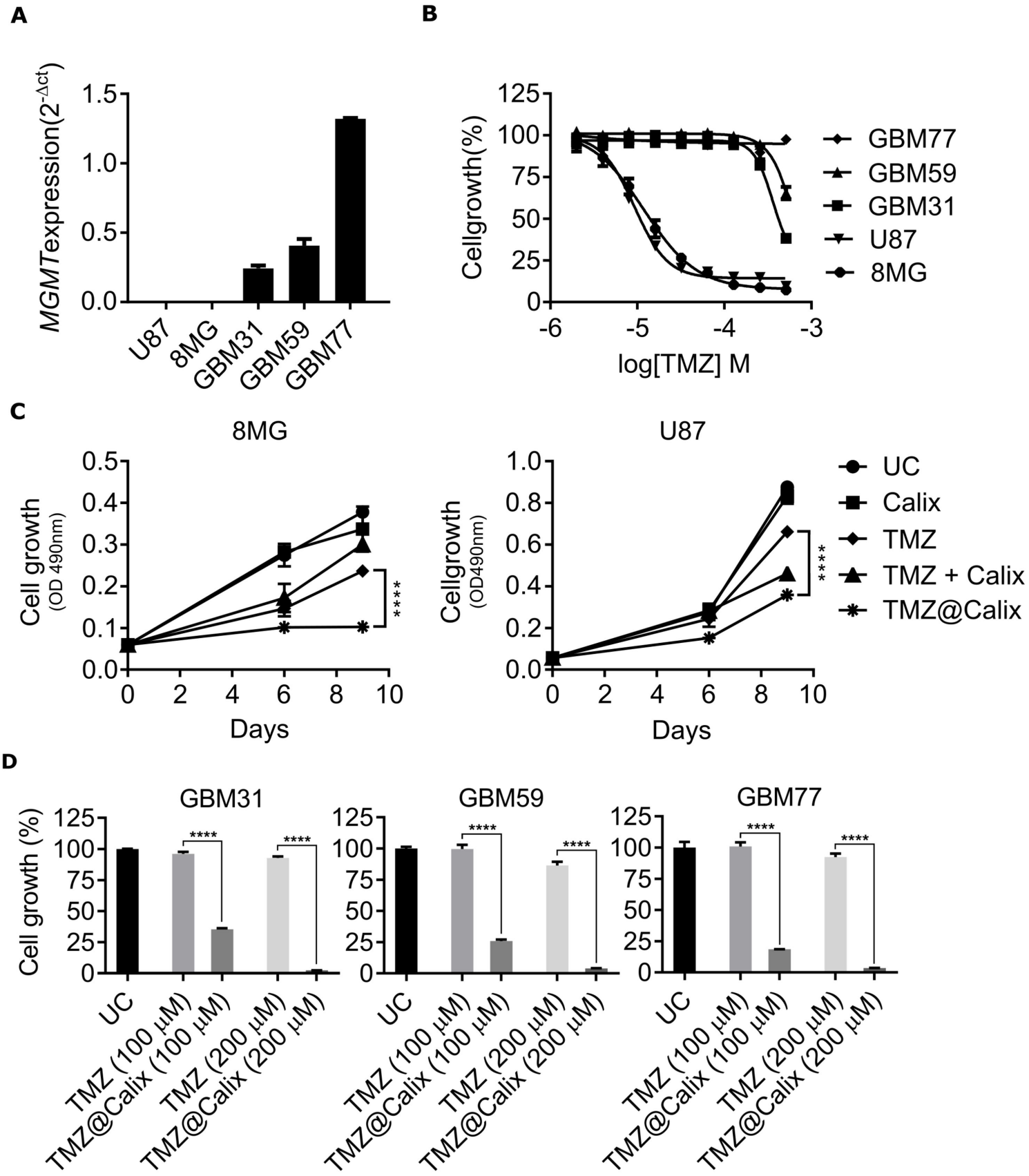
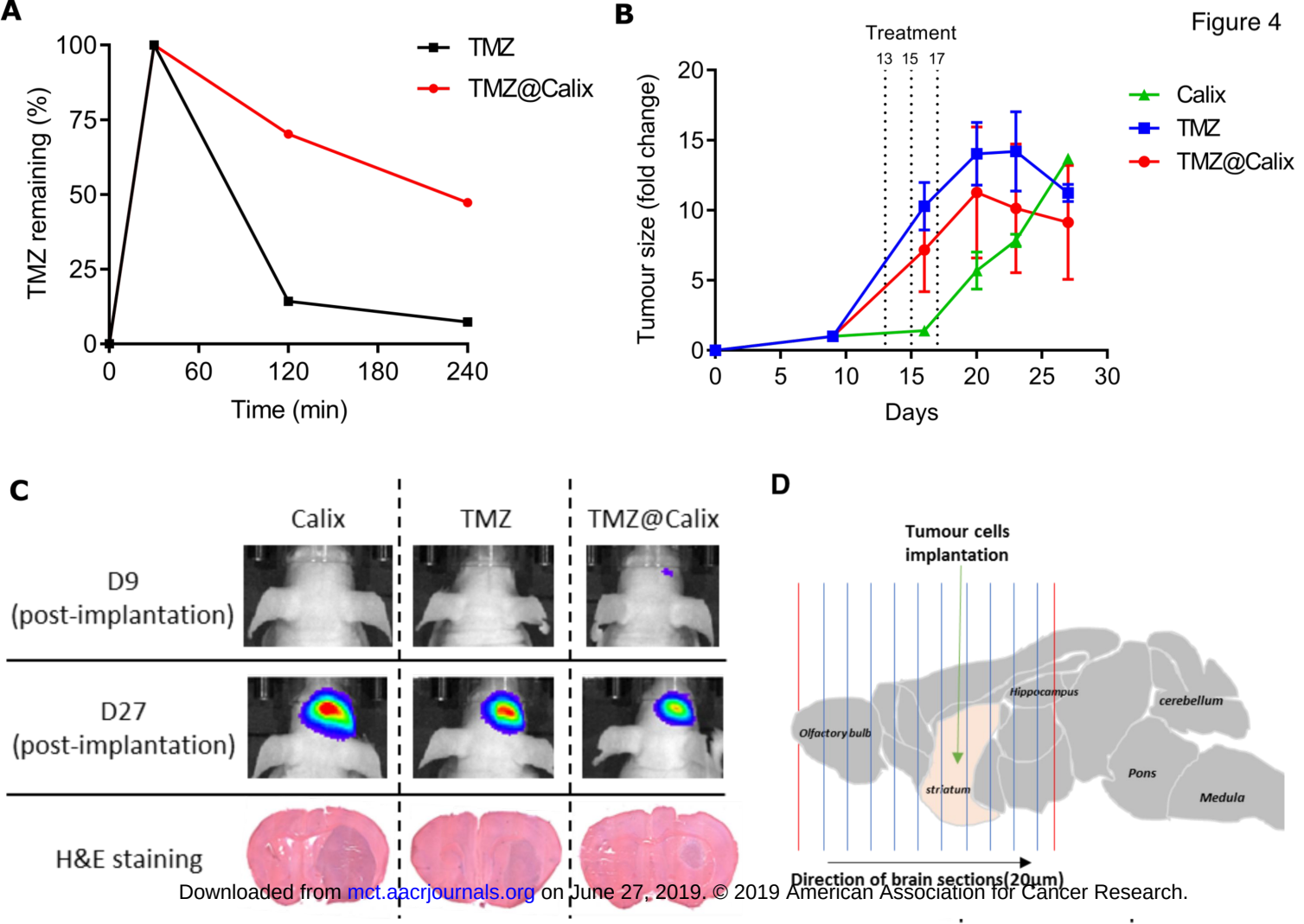


Figure 4



AACR American Association for Cancer Research

Molecular Cancer Therapeutics

Encapsulation of temozolomide in a calixarene nanocapsule improves its stability and enhances its therapeutic efficacy against glioblastoma

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