ChemComm

Cite this: *Chem. Commun.*, 2012, **48**, 9843–9845

www.rsc.org/chemcomm

COMMUNICATION

Enhanced stability and activity of temozolomide in primary glioblastoma multiforme cells with cucurbit[*n*]uril†

Eric A. Appel,‡[a] Matthew J. Rowland,‡[a] Xian Jun Loh,[a] Richard M. Heywood,[b] Colin Watts[bc] and Oren A. Scherman*[a]

Received 17th July 2012, Accepted 15th August 2012
DOI: 10.1039/c2cc35131e

Temozolomide (TMZ) is the primary chemotherapeutic agent for treatment of glioblastoma multiforme (GBM) yet it has a fast rate of degradation under physiological conditions to the 'active' MTIC, which has poor penetration of the blood–brain barrier and cellular absorption. Herein we have demonstrated binding of TMZ within the cavity of nano-container cucurbit[7]uril, resulting in a decreased rate of drug degradation. Prolonging the lifetime of the TMZ under physiological conditions through encapsulation dramatically improved the drug's activity against primary GBM cell lines as more TMZ could be absorbed by the cells before degradation. This work can potentially lead to increases in the drug's propensity for crossing the blood–brain barrier and absorption into the GBM cells, thereby increasing the efficacy of this chemotherapy.

Approximately 10% of diagnosed primary tumors are brain cancers, the most common of which is glioblastoma multiforme (GBM).[1] Despite this seemingly low occurrence rate, targeting GBM is of major importance on account of the high average number of years of life lost (AYLL > 20 years), survival rates (only 1.16 years) and disease recurrence (often within one year) being some of the most dismal statistics across all cancer types.[2–4] Once a patient has been diagnosed with GBM, prognosis is unfortunately extremely poor and life expectancy can often be as short as a few months.[5]

One of the major chemotherapeutic agents used to treat GBM, alongside surgical resection and radiotherapy, is temozolomide (TMZ), which is orally administered as it is soluble and stable under acidic conditions.[6,7] TMZ readily crosses the blood–brain barrier and is a prodrug to a DNA alkylating agent, 5-(3-methyl-triazen-1-yl)imidazole-4-carboxamide (MTIC), *via* hydrolysis under physiological conditions. Following uptake by the GBM cells in the brain, TMZ degrades to MTIC (Fig. 1),

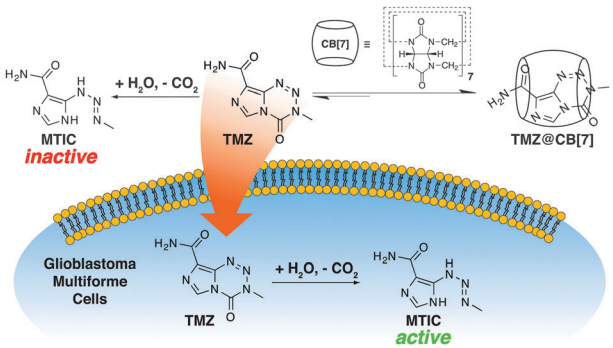

Fig. 1 Schematic representation of the degradation of temozolomide (TMZ) to 5-(3-methyl-triazen-1-yl)imidazole-4-carboxamide (MTIC) outside of the cell and the mechanism of stabilisation of TMZ through complexation with the nanocontainer cucurbit[7]uril.

which methylates the O6 position of the guanine residues of DNA, causing cytotoxic DNA damage.[8] However, TMZ can also degrade to MTIC in the blood stream, which is problematic for treatment as MTIC does not cross the blood–brain barrier and is not absorbed well by cells.[9] Therefore, despite the use of TMZ as an adjuvant in surgical procedures and radiotherapy, patient survival rates remain poor.

It was hypothesised that improving the half-life of TMZ under physiological conditions would promote a greater accumulation of TMZ accessing the GBM site, prior to degradation. The drug would therefore be more effective and lower doses could be utilised to maintain the current therapeutic window. This would make chemotherapy less expensive, more efficient, and would reduce side-effects and toxicity, improving patient life expectancy.

In the recent literature it has been shown that the family of macrocyclic host molecules, cucurbit[*n*]urils (CB[*n*]), can complex drug compounds, increasing their solubility, stability and cellular uptake as often the complexes can cross the cell membrane.[10–14] The CB[*n*] family are cyclic, methylene-linked oligomers of glycoluril that have a symmetric 'barrel' shape with two identical portal regions laced by ureido-carbonyl oxygens. The number of glycoluril units determines the size of the CB[*n*] cavity without affecting the height of the molecular container (approximately 0.9 nm). CB[7][15–17] is a particularly attractive host on account of its large cavity volume (210 Å^3)

[a] *Melville Laboratory for Polymer Synthesis, Department of Chemistry, University of Cambridge, Cambridge, CB2 1EW, UK. E-mail: oas23@cam.ac.uk; Fax: +44 (0)1223 334866; Tel: +44 (0)1223 331508*
[b] *Cambridge Centre for Brain Repair, E.D. Adrian Building, Robinson Way, Cambridge CB2 0PY, UK*
[c] *Division of Neurosurgery, Department of Clinical Neurosciences, University of Cambridge, UK*

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c2cc35131e

‡ These two authors contributed equally to this work.

and extremely high equilibrium binding constants (K_{eq} up to 10^{15} M^{-1} for some CB[7] complexes).[18] Not only does this macrocyclic host enhance drug properties, it is non-toxic, making it useful for biomedical applications.[19,20] Thus, the CB[*n*] family represents an attractive form of nano-container for the stabilisation of TMZ.

With these advantages, studies on binding of TMZ into the cavity of both CB[7] and CB[8] were performed along with investigation into the ability of the nano-container to facilitate extension of the TMZ half-life under physiological conditions. The effect of enhanced TMZ half-life on primary GBM cell lines was also of interest in order to investigate the effects of the nano-container on cellular uptake and activity of the drug.

Isothermal titration calorimetry (ITC) is a powerful physical technique for measuring solution binding thermodynamics and stoichiometry and has previously been utilised to measure K_{eq} values in CB[*n*] host–guest systems.[21–25] Investigations of TMZ binding to CB[7] were performed with this technique. TMZ binds to CB[7] in a 1 : 1 fashion ($n = 1.0$) with a high equilibrium binding constant (see ESI†) determined to be $K_{eq} = (2.00 \pm 0.09) \times 10^3$ M^{-1} when fitted with a 'single site' binding model. From this information the binding energy was calculated to be $\Delta G = -19 \pm 1$ kJ mol^{-1}. The strong binding was determined to be almost entirely enthalpically driven as $\Delta H = -39 \pm 2$ kJ mol^{-1} while the entropic contributions were $\Delta S = -67 \pm 7$ J mol^{-1}.

The binding of TMZ to CB[7] was also observable by ^{1}H NMR by performing a titration experiment of CB[7] into a solution of TMZ. Whilst at the concentrations measured it was not expected that 100% of the TMZ would be bound to CB[7] (on account of the equilibrium binding constant), a slight upfield shift was observed for the aromatic proton on TMZ with increasing addition of CB[7] (see ESI†). This upfield shift clearly identifies that the bulk of the TMZ structure binds inside of the CB[7] cavity. Furthermore, the presence of only one peak indicates that dynamics of exchange between 'bound' and 'unbound' states is rapid as they are faster than the NMR timescale.

With binding of TMZ to the CB[7] cavity clearly evident, investigation into the ability of the complex formation to stabilise the drug from degradation under physiological conditions was undertaken. A 1 : 1 solution of TMZ and CB[7] (30 mM) was prepared in pH 7.0 buffer, along with a control solution of TMZ alone, and the two were incubated at 37 °C. At this concentration it was calculated that approximately 89% of the TMZ present would be bound to CB[7] according to the K_{eq} determined previously. We therefore postulated that this concentration would provide an observable change in half-life upon stabilisation. The change in concentration of TMZ upon degradation was then quantified by UV-Vis spectroscopy at 329 nm, the results of which are shown in Fig. 2. The first order degradation rate constant of TMZ decreased tremendously from 7.9 ± 0.6 s^{-1} to 3.6 ± 0.1 s^{-1} upon binding with CB[7], corresponding to an increase in the half-life of TMZ from 3 to 5 hours.

With a system in hand, which clearly stabilises TMZ, the effect of the complex on primary GBM cell lines was investigated. The use of primary GBM cell lines can provide insight into utility of real clinical targets and these were obtained by surgical resection from GBM patients (see ESI†). There were two possible outcomes from enhanced stabilisation we believed could occur: (1) stabilisation slows degradation to the active MTIC form, thereby reducing cytotoxic activity and increasing the IC_{50}, or (2) TMZ exists longer in solution thereby allowing more to be absorbed by cells, decreasing the IC_{50}. GBM cells were seeded in 96 well plates and exposed to varying concentrations of TMZ and TMZ@CB[7] (Fig. 3). The IC_{50} observed for TMZ alone was relatively high (8.0 ± 0.6 mM), while that for the TMZ@CB[7] complex was much lower (2.9 ± 0.6 mM). Therefore, a four-fold increase in the activity of the TMZ towards the GBM cells is observed upon complexation to the CB[7] host. Two possible mechanisms can be envisaged from this: (1) CB[7] acts as a chaperone whereby the complex is better absorbed by the cells than TMZ alone, or (2) enhanced stability of the TMZ in the TMZ@CB[7] complex allows for a greater proportion of the TMZ to be absorbed by the cells. By either mechanism more TMZ is absorbed in the cell before degradation and thus

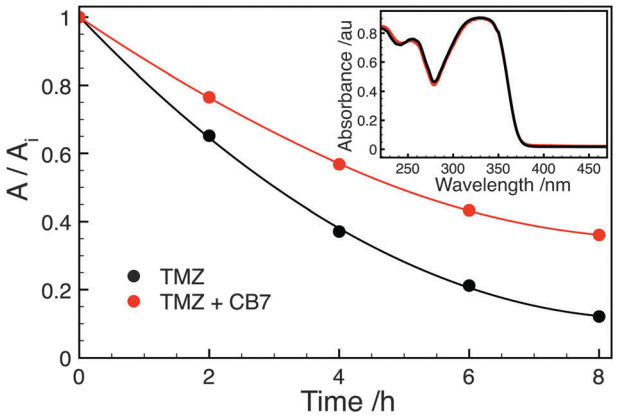

Fig. 2 Stability of TMZ alone and bound in CB[7] (1 eq.) monitored with UV-vis spectrometry in a 10 mM buffer solution at pH 7.0. The inset displays the UV-vis absorbance traces of TMZ alone and when complexed with CB[7], demonstrating that the absorbance of the drug does not change upon complexation within the cavity of the CB[7] host molecule.

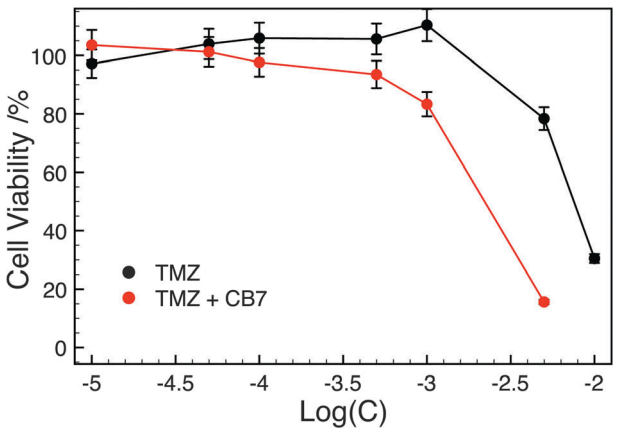

Fig. 3 Cell viability studies, black circles show the response of GBM cell lines to TMZ alone, red circles show the response of the GBM cells to the TMZ/CB[7] combination. Activity of the complex represents a four-fold improvement on TMZ treatment.

a lower IC_{50} is achieved. Again, as MTIC has an extremely low propensity for crossing the blood–brain barrier and cellular uptake, maintaining TMZ concentration is of high value.

Intuitively one might expect that if CB[7] were acting as a chaperone, a decrease in cell viability would be observed even at very low concentrations of the complex, as a greater proportion of the bound species would be transported into the cells. However, as this is not the case, it is believed that the latter mechanism is the major contributor to the observed decrease in IC_{50}. The TMZ@CB[7] complex is dynamic, as determined previously, whereby a 'bound' species leads to stabilisation of the TMZ, while the 'unbound' species is free to enter the cells, as demonstrated in Fig. 1. Further studies are currently underway to elucidate the exact mechanism, the results of which will appear in a forthcoming publication.

In summary, the encapsulation of the important anti-cancer drug TMZ in the cavity of the macrocyclic host, CB[7], has been demonstrated and thermodynamic parameters for binding have been characterised by ITC. ^{1}H NMR and UV-Vis spectroscopy studies clearly corroborate the ITC data and a stabilising effect of the TMZ drug by encapsulation has been achieved. This enhanced stabilisation has led to a dramatic increase in the activity of the drug on primary GBM cell lines. We envisage that this effect may improve the bioavailability of TMZ in patients, allowing for administration of TMZ@CB[7] *via* intravenous injection and perhaps in combination with other chemotherapeutic agents that cannot be orally administered.[26] Furthermore, the stabilisation of the drug in the TMZ@CB[7] complex can potentially increase the drug's propensity for crossing the blood–brain barrier and absorption into the GBM cells, thereby increasing the efficacy of treatment for this extremely vicious disease.

E.A. thanks Schlumberger for financial support. M.J.R. thanks the University of Cambridge Chemical Biology and Molecular Medicine program for funding. X.J.L. thanks A*STAR for a postdoctoral fellowship. R.M.H. thanks the Sims Scholarship at the University of Cambridge for funding. C.W. is supported by the NIHR Cambridge Biomedical Research Centre. This work was also supported by an ERC Starting Investigator Grant (ASPiRe) and a Next Generation Fellowship provided by the Walters-Kundert Foundation.

References

1 *Cancer incidence and mortality in the UK, 2007–2009*, Office for National Statistics, United Kingdom, 2010.
2 N. D. Burnet, S. J. Jefferies, R. J. Benson, D. P. Hunt and F. P. Tresure, *Br. J. Cancer*, 2005, **92**, 241–245.
3 D. Krex, B. Klink, C. Hartmann, A. von Deimling, T. Pietsch, M. Simon, M. Sabel, J. P. Steinbach, O. Heese, G. Reifenberger, M. Weller and G. Schackert, *Brain*, 2007, **130**, 2596–2606.
4 E. L. Chang, S. Akyurek, T. Avalos, N. Rebueno, C. Spicer, J. Garcia, R. Famiglietti, P. K. Allen, K. C. Chao, A. Mahajan, S. Y. Woo and M. H. Maor, *Int. J. Radiat. Oncol., Biol., Phys.*, 2007, **68**, 144–150.
5 E. G. Van Meir, C. G. Hadjipanayis, A. D. Norden, H.-K. Shu, P. Y. Wen and J. J. Olson, *Ca–Cancer J. Clin.*, 2010, **60**, 166–193.
6 G. Dresemann, *OncoTargets Ther.*, 2010, **3**, 139–146.
7 R. Stupp, W. Mason, M. J. van den Bent, M. Weller, B. Fisher, M. Taphoorn, K. Belanger, A. Brandes, C. Marosi, U. Bogdahn, J. Curschmann, R. janzer, S. Ludwin, T. Gorlia, A. Allgeier, D. Lacombe, G. Cairncross, E. Eisenhauer and R. Mirimanoff, *N. Engl. J. Med.*, 2005, **352**, 987–996.
8 B. J. Denny, R. T. Wheelhouse, M. F. G. Stevens, L. L. H. Tsang and J. A. Slack, *Biochemistry*, 1994, **33**, 9045–9051.
9 L. Meer, R. C. Janzer, P. Kleihues and G. F. Kolar, *Biochem. Pharmacol.*, 1986, **35**, 3243–3247.
10 N. J. Wheate, D. P. Buck, A. I. Day and J. G. Collins, *Dalton Trans.*, 2006, 451–458.
11 P. Montes-Navajas, M. Gonzalez-Bejar, J. C. Scaiano and H. Garcia, *Photochem. Photobiol. Sci.*, 2009, **8**, 1743–1747.
12 N. J. Wheate, *J. Inorg. Biochem.*, 2008, **102**, 2060–2066.
13 I. Ghosh and W. M. Nau, *Adv. Drug Delivery Rev.*, 2012, **64**, 764–783.
14 D. Ma, G. Hettiarachchi, D. Nguyen, B. Zhang, J. B. Wittenberg, P. Y. Zavalij, V. Briken and L. Isaacs, *Nat. Chem.*, 2012, **4**, 503–510.
15 C. Marquez, R. R. Hudgins and W. M. Nau, *J. Am. Chem. Soc.*, 2004, **126**, 5806–5816.
16 J. Lagona, P. Mukhopadhyay, S. Chakrabarti and L. Isaacs, *Angew. Chem., Int. Ed.*, 2005, **44**, 4844–4870.
17 S. Liu, C. Ruspic, P. Mukhopadhyay, S. Chakrabarti, P. Zavalij and L. Isaacs, *J. Am. Chem. Soc.*, 2005, **127**, 15959–15967.
18 M. V. Rekharsky, T. Mori, C. Yang, Y. H. Ko, N. Selvapalam, H. Kim, D. Sobransingh, A. E. Kaifer, S. Liu, L. Isaacs, W. Chen, S. Moghaddam, M. K. Gilson, K. Kim and Y. Inoue, *Proc. Natl. Acad. Sci. U. S. A.*, 2007, **104**, 20737–20742.
19 V. D. Uzunova, C. Cullinane, K. Brix, W. M. Nau and A. I. Day, *Org. Biomol. Chem.*, 2010, **8**, 2037–2042.
20 G. Hettiarachchi, D. Nguyen, J. Wu, D. Lucas, D. Ma, L. Isaacs and V. Briken, *PLoS One*, 2010, **5**, e10514.
21 M. Bush, N. Bouley and A. Urbach, *J. Am. Chem. Soc.*, 2005, **127**, 14511–14517.
22 L. M. Heitmann, A. B. Taylor, P. J. Hart and A. R. Urbach, *J. Am. Chem. Soc.*, 2006, **128**, 12574–12581.
23 P. Rajgariah and A. R. Urbach, *J. Inclusion Phenom. Macrocyclic Chem.*, 2008, **62**, 251–254.
24 J. J. Reczek, A. A. Kennedy, B. T. Halbert and A. R. Urbach, *J. Am. Chem. Soc.*, 2009, **131**, 2408–2415.
25 U. Rauwald, F. Biedermann, S. Deroo, C. V. Robinson and O. A. Scherman, *J. Phys. Chem. B*, 2010, **114**, 8606–8615.
26 E. A. Appel, X. J. Loh, S. T. Jones, C. A. Dreiss and O. A. Scherman, *Biomaterials*, 2012, **33**, 4646–4652.