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ORIGINAL ARTICLE

Mark R. Middleton · Jane Kelly · Sarah Goodger
Nicholas Thatcher · Geoffrey P. Margison

Four-hourly scheduling of temozolomide improves tumour growth delay but not therapeutic index in A375M melanoma xenografts

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Abstract *Purpose*: To establish whether temozolomide is more effective against A375M human melanoma xenografts if given every 4 h rather than every 24 h, in order to exploit depletion of the DNA repair protein O^6-alkylguanine-DNA alkyltransferase (ATase) by prior doses of the drug. *Methods*: ATase depletion in A375M human melanoma xenografts was determined over 24 h after a single dose of temozolomide. The effect of different drug schedules (all of total dose 500 mg/kg) in delaying the growth of the xenografts was tested, and ATase depletion and DNA methylation damage assessed in tumour and normal tissue. *Results*: Maximal depletion of ATase in tumour, to 2.52 ± 0.23% of pretreatment levels, occurred 4–8 h after a single 100 mg/kg i.p. dose of temozolomide, with 23.0% recovery of protein levels at 24 h. Scheduling of temozolomide every 4 h increased tumour growth delay (33.6 ± 1.39 days with temozolomide 100 mg/kg 4-hourly ×5 versus 23.2 ± 1.43 days with temozolomide 100 mg/kg once daily ×5; $P < 0.0001$) at the expense of increased toxicity (17.4 ± 1.55% animal weight loss versus 10.6 ± 1.27%, respectively). Temozolomide every 4 h did not increase ATase depletion compared with the 5-day schedule, but resulted in greater DNA O^6-guanine methylation (29.0% more in tumour, 20.8% in liver and 56.0% in brain, comparing areas under the methylation-time curve). *Conclusions*: The 4-hourly schedule of temozolomide delayed tumour growth significantly more than the once-daily and 12-hourly schedules, probably as a result of greater DNA damage inflicted, but also increased toxicity. It remains to be seen if this regimen confers a net benefit over the standard schedule.

Keywords Temozolomide · Alkyltransferase · Melanoma · Schedule · Xenograft

M.R. Middleton (✉) · N. Thatcher
Cancer Research Campaign Department of Medical Oncology, Christie Hospital NHS Trust, Manchester, M20 9BX, UK
e-mail: mmiddleton@picr.man.ac.uk
Tel.: +44-161-446-3745; Fax: +44-161-446-3299

M.R. Middleton · J. Kelly · S. Goodger
N. Thatcher · G.P. Margison
Cancer Research Campaign Section of Genome Damage and Repair, Paterson Institute for Cancer Research, Wilmslow Road, Manchester M20 4BX, UK

Introduction

The antitumour agent temozolomide has activity in malignant melanoma similar to that of dacarbazine, the most effective single agent to date [1]. The activity of the drug is schedule-dependent [2], and it is currently given once daily by mouth for five consecutive days at the start of each 28-day cycle. Temozolomide causes a number of DNA lesions, of which O^6-methylguanine is the most important determinant of cytotoxicity.

There is considerable evidence that the principal mechanism of resistance to temozolomide is through the DNA repair protein O^6-alkylguanine-DNA alkyltransferase (ATase). Cell lines and tumour xenografts with low levels of ATase expression are sensitive to the drug. Resistant cell lines can be sensitized by prior depletion of the protein and sensitive cell lines made resistant by the transfection of cDNA coding for the protein [3–7]. ATase repairs the cytotoxic DNA adduct O^6-methylguanine (O^6-MeG) in a stoichiometric reaction, and is itself inactivated in the process. *De novo* synthesis is then required to restore protein levels. Concentrations of the protein, therefore, fall in peripheral blood mononuclear cells (PBMCs) and in tumour after administration of temozolomide, but there is partial recovery of levels within PBMCs at 24 h, the time of subsequent dosing [8]. In order to maximize the tumour cell cytotoxicity of the drug it would seem logical to administer subsequent doses at the ATase nadir. In human PBMCs this occurs 4 h after treatment [8], but there is little published information about the kinetics of ATase depletion in tumours in clinical practice. Therefore, we determined this in a tumour model, before designing an appropriate

schedule to test the hypothesis that repeat dosing at the ATase nadir enhances antitumour efficacy, in comparison with the standard once-daily schedule.

We have examined ATase depletion in human melanoma xenografts after a single dose of temozolomide (as used daily in our standard 5-day schedule) to determine the nadir and the rate at which ATase levels recover. This confirmed that in the tumour model, as in PBMCs in clinical practice, maximal ATase depletion occurs at 4 h with evidence of some recovery by 24 h. Taking these results into account, we investigated in the animal model whether altering the schedule of temozolomide – but not the total dose – improves the antitumour activity of the drug, with a view to conducting a similar trial in the clinic. We compared the standard, once-daily, schedule with a twice-daily regimen, as used in a recent phase I trial [9], and with one in which temozolomide was administered every 4 h for five doses. A single dose of temozolomide at 500 mg/kg was not tested, as this schedule had proved ineffective in early clinical trials [10]. We then determined the ability of the most effective schedule (100 mg/kg 4-hourly for five doses) and the standard regimen (100 mg/kg per day for 5 days) to deplete ATase and to methylate DNA in tumour and normal tissue, in order to seek to explain the differences we had observed in tumour growth delay.

Materials and methods

Drugs

Temozolomide was provided by the Cancer Research Campaign Drug Formulation Unit, University of Strathclyde, Glasgow, UK. Temozolomide was freshly prepared at 40 mg/ml in dimethyl sulphoxide (DMSO; Sigma, Poole, UK) each time, diluted in 0.9% NaCl solution, and injected intraperitoneally (i.p.) within 15 min.

Animal studies

Male nude mice (O/Nu: outbred ALPK Nu/Nu) were purchased from Zeneca (Macclesfield, UK). Animals were housed in a sterile environment and allowed free access to food and water. A375M human melanoma xenograft samples (1 to 2 mm^3) were implanted in the right flank and the experiments begun when tumour volumes had reached a suitable value. ATase depletion after a single dose of temozolomide was studied in six groups of five mice. The animals received temozolomide 100 mg/kg i.p. (12.5 μl/g injection volume), or the vehicle control, as a single dose. At various times after dosing animals were killed by cervical dislocation, and the tumour excised and immediately frozen in liquid nitrogen. The tumours were stored at −70 °C until assayed according to the method of Lee et al. [8].

The tumour growth delay experiment was started with tumour volumes of between 16 and 219 mm^3. Eight animals were assigned to each of four groups, and tumour volume was standardized across the groups. The nude mice received (a) vehicle control once-daily for 5 days, (b) temozolomide 100 mg/kg i.p. daily for 5 days, (c) 100 mg/kg on the morning of the first day followed by 45 mg/kg 12-hourly for nine doses, or (d) 100 mg/kg 4-hourly for five doses (Table 1). Animal weights and tumour volumes were measured twice per week. Tumour volumes (in cubic millimetres) were calculated from the dimensions in millimetres using the formula [length × height × width × $\pi \div 6$], with measurements taken using digital calipers.

ATase depletion and DNA methylation were studied in six groups of four mice. The animals received temozolomide 100 mg/kg i.p. every 4 or 24 h for five doses. At various times after dosing animals were killed by cervical dislocation, and the tumour, liver and brain excised and immediately frozen in liquid nitrogen. These were stored at −70 °C until assayed according to the method of Lee et al. for ATase [8] and by a competition assay for O^6-methylguanine content in DNA [11].

Statistical methods

The relative tumour volumes were plotted for each animal. In the growth/regrowth phase these profiles were log-linear. Separate log-linear regressions were fitted to each animal's data, and estimates of the tumour quintupling time and a measure of the precision of these estimates obtained. These data pairs (estimate, precision) were used in a generalized regression model for the group mean tumour quintupling time structure, which was fitted by maximum likelihood using program LE of the BMDP statistical package (Version 7.0; University Press of California, Berkeley). The maximum weight losses observed in each group were compared using the Kruskal-Wallis test.

Results

ATase depletion after a single dose of temozolomide

The A375M xenograft was found to express ATase levels of 290 (± 50.7) fmol/mg protein (± SEM). Maximal depletion, of over 97% of starting levels, was evident 4 and 8 h after dosing with temozolomide. By 16 h after dosing a recovery in levels to 13.5% (± 4.65%) of pretreatment values was evident, and at 24 h 23.0% (± 6.41%) of the initial ATase activity could be detected (Fig. 1).

Tumour growth delay

Temozolomide given once daily for 5 days significantly delayed tumour regrowth (quintupling time of 23.2 days vs 16.7 days with vehicle controls; $P = 0.003$). No

Table 1 Treatment regimens used (▮ temozolomide 100 mg/kg; ▮ temozolomide 45 mg/kg; *v* vehicle, 20% DMSO in 0.9% saline; all injections given i.p.)

Regimen	Day					Total dose (mg/kg)
	1	2	3	4	5	
A: control	v	v	v	v	v	Nil
B: once-daily	▮	▮	▮	▮	▮	500
C: 12-hourly	▮ ▮	▮ ▮	▮ ▮	▮ ▮	▮ ▮	500
D: 4-hourly	▮ ▮ ▮ ▮ ▮					500

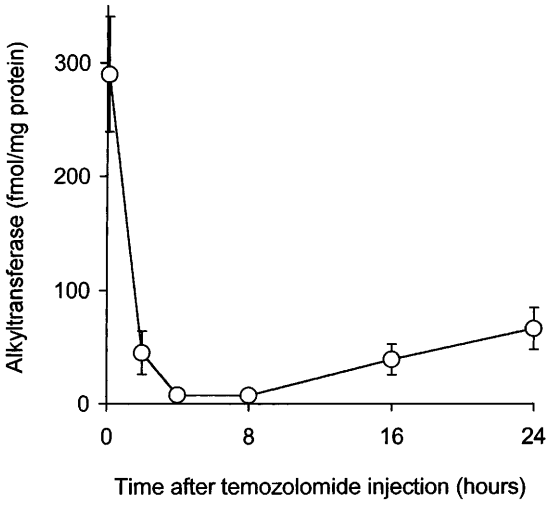

Fig. 1 Depletion of ATase in A375M xenografts after a single 100 mg/kg i.p. injection of temozolomide. Points are the means (±SE) of five observations

difference in delay was seen when the schedule was altered to allow for dosing every 12 h ($P = 0.57$), but there was significantly greater delay when the drug was given every 4 h for five doses ($P < 0.0001$). The time to tumour quintupling was 33.6 days with the 4-hourly schedule (Table 2, Fig. 2a).

Toxicity, as measured by weight loss in the animals, was similar in the two 5-day regimens, but more marked with the 4-hourly schedule (Table 2; Fig. 2b). The volume of DMSO given over 16 h in the latter group may have accentuated this effect, which was significant ($P = 0.046$). The difference in weight loss was temporary, being evident only in the first 2 weeks after treatment started. There were no deaths in any of the four groups during the 6 weeks of the experiment.

ATase depletion and O^6-MeG formation with temozolomide schedule

Both schedules resulted in complete depletion of ATase in tumour, but only the 4-hourly regimen achieved this in liver, and neither schedule did so in brain (Fig. 3a–c). Recovery in ATase activity was quickest in liver, starting soon after cessation of treatment, and was complete at 1 week. The process was slower in tumour, where pretreatment levels of ATase were only achieved by day 16 of the experiment. No recovery was evident in brain over this time. There was no significant difference between the two schedules in terms of area under the concentration-time curve for ATase. The protein was detectable in tumour 24 h after a single dose of temozolomide (Fig. 1) but could not be found at this time after starting the 4-hourly schedule, suggesting that greater depletion was initially achieved with the latter regimen.

The administration of temozolomide every 4 h resulted in greater DNA O^6-guanine methylation in all three tissues studied (Fig. 4a–c). Peak O^6-MeG levels were 69.4% higher in tumour (9.83 vs 5.80 μmol O^6-methylguanine/mol guanine) and 85.7% higher in liver (10.4 vs 5.58) with the 4-hourly regimen. However, in brain the peak O^6-MeG levels was only 25.6% greater with 4-hourly administration (35.9 vs 28.6). The areas under the O^6-MeG/time curve were also greater with the compressed schedule: by 29.0% in tumour, 20.8% in liver and 56.0% in brain.

Discussion

To date, attempts to improve the efficacy of O^6-alkylating agent chemotherapy in the clinic have concentrated on the depletion of ATase, either with methylating agents or the direct inactivator O^6-benzylguanine, prior to the administration of a chloroethylating agent [12, 13].

Although the sequential combination of methylating and chloroethylating agents has proved synergistic in vivo [14, 15] both classes of drug have myelosuppression as their dose-limiting toxicity. In clinical practice this has limited the dose of chloroethylating agent used, when given after a methylating drug, to around half of that given as a single agent [12], and response rates have been little improved in comparison with conventional therapy [13].

Direct inactivators such as O^6-benzylguanine, that are not inherently myelotoxic, hold more promise. Depletion of ATase by such agents does enhance the myelotoxicity of O^6-alkylating agents [16] but animal studies have demonstrated a useful increase in therapeutic index [17, 18]. Human ATase is more sensitive to O^6-benzylguanine than murine and this may have affected the outcome of such studies, in that antitumour activity is a function of the former and toxicity of the latter protein. Nonetheless, the combination of O^6-benzylguanine and carmustine has recently been tested in a

Table 2 Effect of temozolomide schedules on A375M tumour growth and on animal weights

Regimen	Tumour quintupling time (days)[a]	Tumour growth delay (days)	Weight loss (% day 1)[b]
A: control	16.7 ± 1.42	0	4.44 ± 0.43
B: once-daily	23.2 ± 1.43	6.48	10.6 ± 1.27
C: 12-hourly	22.1 ± 1.39	5.34	8.79 ± 0.46
D: 4-hourly	33.6 ± 1.39	16.9	17.4 ± 1.55

[a] Mean time taken for tumours to reach five times the volume on day 1 (±SE)
[b] Mean body weight nadir as a percentage of day 1 value (±SE)

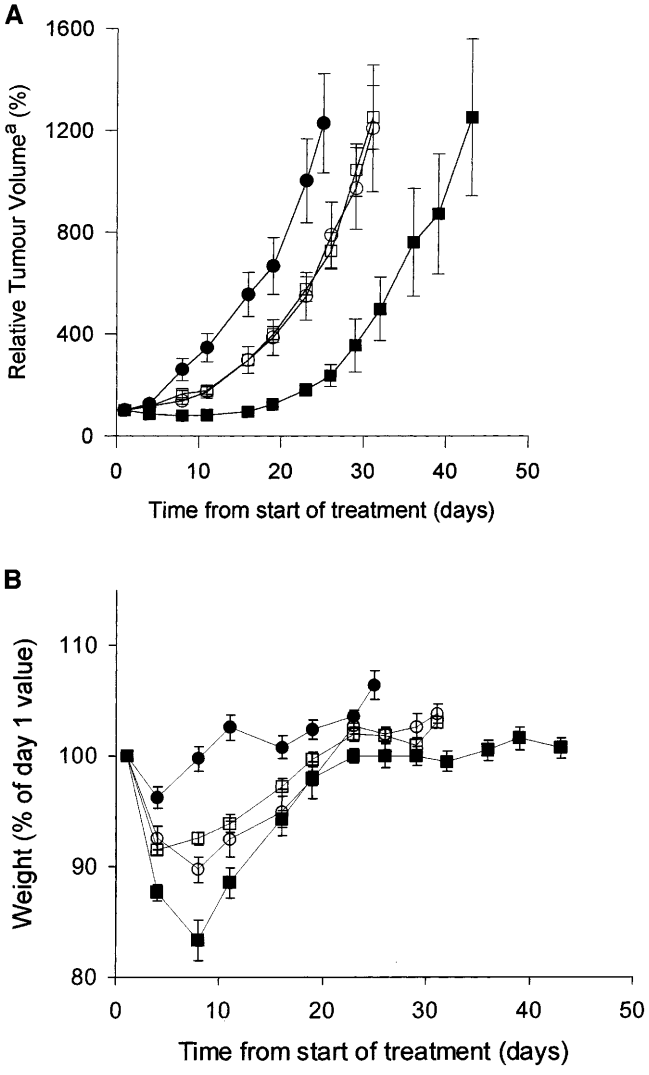

Fig. 2A,B Growth inhibition of A375M xenografts (**A**) and animal weight loss (**B**) in mice treated with DMSO 20% in 0.9% saline (●), temozolomide 100 mg/kg once daily ×5 (○), temozolomide 100 mg/kg ×1 then 45 mg/kg 12-hourly ×9 (□) or temozolomide 100 mg/kg 4-hourly ×5 (■). Points represent the means (±SE) from eight mice, with error bars that overlap being shown in one direction only. All treatments were administered intraperitoneally. [a]Mean of the percentage change in tumour volume for each group (compared with the day-1 volume)

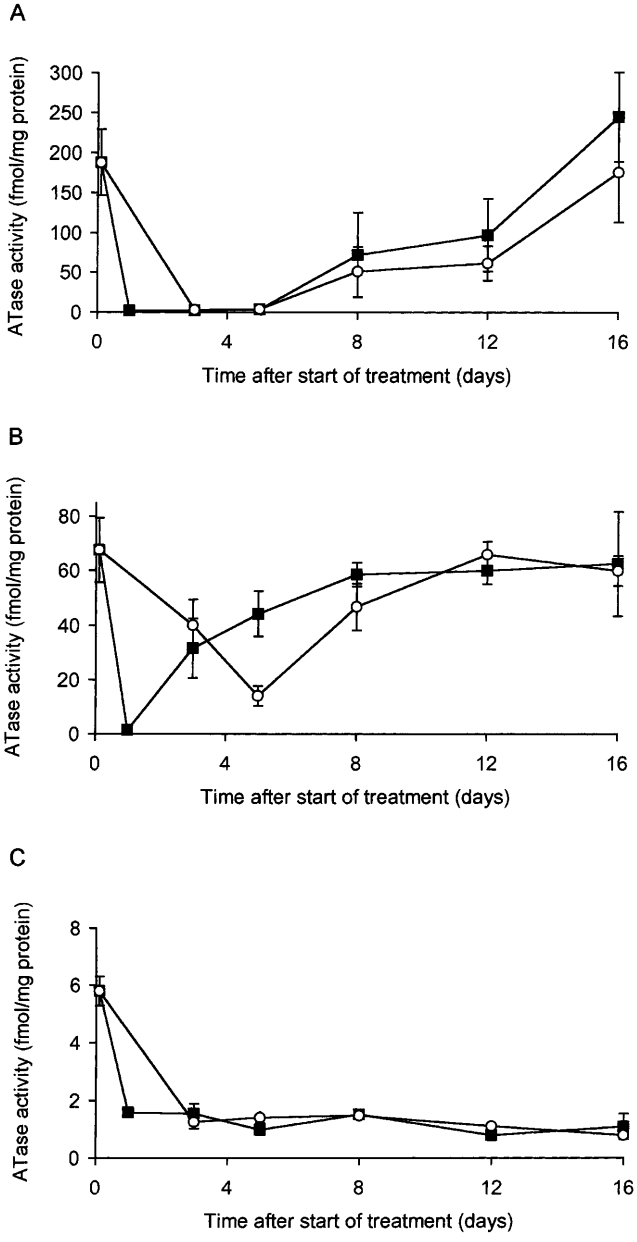

Fig. 3A–C O^6-alkylguanine-DNA alkyltransferase (ATase) activity in A375M xenografts (**A**), liver (**B**) and brain (**C**) during and after treatment with temozolomide 100 mg/kg 4-hourly ×5 (■) or once daily ×5 (○) intraperitoneally. Points shown are the means (±SE) from at least three mice

phase I clinical trial [19], and phase II studies are now in progress. Even if these are successful it is likely to be some time before such agents are widely available.

By altering the schedule of temozolomide to take into account our knowledge of ATase depletion we have significantly enhanced its antitumour activity in this melanoma xenograft model. The basis for this is an increase in tumour methylation damage, presumably as a result of exhausting the DNA repair mechanism. The extent to which the effect observed here would occur in clinical practice remains uncertain, for ATase depletion in PBMCs and in this xenograft model may not accurately reflect events in human tumours. However, it seems likely that 4-hourly scheduling of temozolomide will result in increased tumour methylation damage.

There was a significant increase in weight loss in the 4-hourly treatment group, which gives cause for caution in interpreting the increased tumour growth delay. Although the dose of DMSO given to the mice over a 16-h period may have played a part, it cannot account for the observed increase in weight loss. In a separate experiment, mice dosed 4-hourly with equivalent amounts of DMSO exhibited on average 5% weight loss (data not shown), which is similar to that seen in the control group

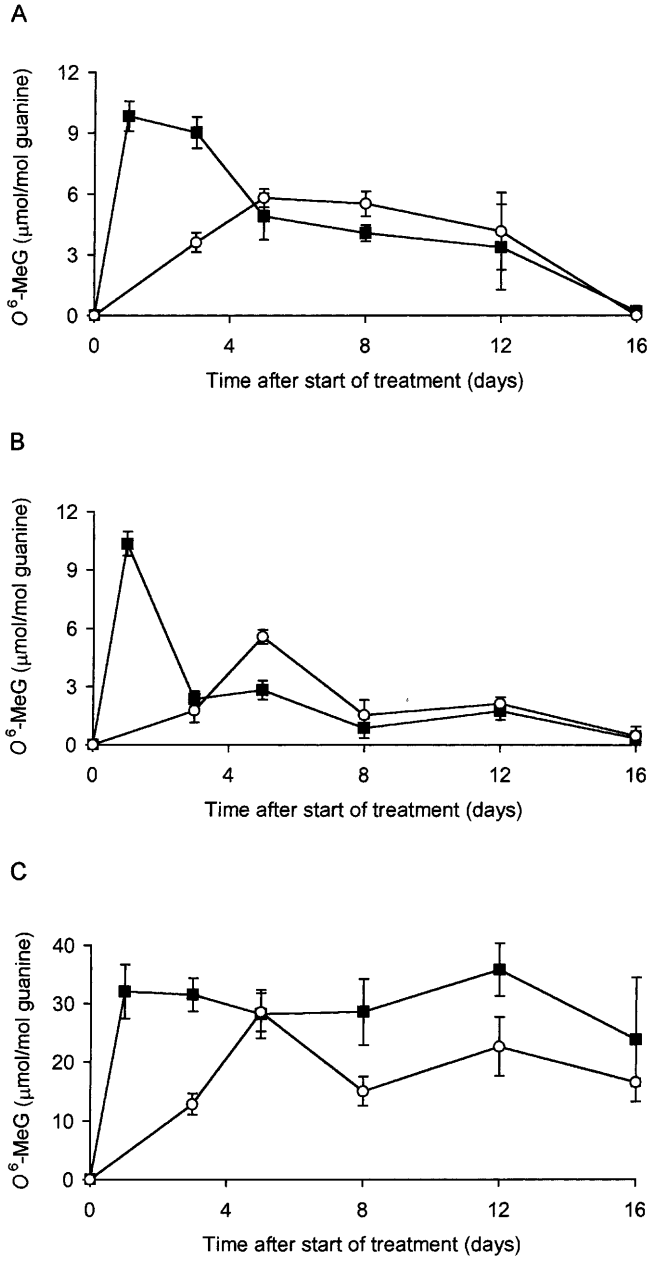

Fig. 4A–C O^6-methylguanine-DNA (O^6-MeG) formation activity in A375M xenografts (**A**), liver (**B**) and brain (**C**) during and after treatment with temozolomide 100 mg/kg 4-hourly ×5 (■) or once daily ×5 (○) intraperitoneally. Points shown are the means (± SE) from at least three mice

in this experiment. We cannot therefore claim an increase in the therapeutic index of temozolomide for this schedule on the basis of these data. We have shown that methylation damage is increased in normal tissues too, so an increase in toxicity is expected with 4-hourly scheduling. The clinical utility of this regimen rests with the balance between increased efficacy and increased toxicity, but it is difficult to predict what this will be in humans from this model: the principal toxicity observed was gastrointestinal, but is likely to be haematological in humans.

There is evidence that the area under the DNA methylation-time curve (AUC) is important in determining cytotoxicity, rather than the O^6-MeG peak [20]. With the 4-hourly regimen, the AUC was increased to a greater extent in tumour than in liver tissue in this experiment, although the greatest increase was seen in brain. Given the relative levels of ATase expression in the tissues this is to be expected, and raises the concern that the compressed schedule will greatly enhance toxicity in bone marrow – another tissue expressing low levels of ATase. The maximum tolerated dose of temozolomide is constant over a variety of schedules, being 1000 mg/m^2 for a single intravenous infusion, a twice-daily 5-day regimen and the standard once-daily 5-day schedule [2, 9, 10]. Thus, we anticipate that any excess toxicity in translating the 4-hourly regimen to the clinic, which is likely to be haematological, will be manageable. If the observed increase in antitumour activity can be replicated in the clinic, then the scope for the use of temozolomide may widen to include tumours that express levels of ATase higher than are found in melanoma or glioma.

In conclusion, we have demonstrated that altering the schedule of temozolomide to take into account our knowledge of ATase depletion in tumour xenografts increases tumour growth delay and DNA O^6-guanine methylation. This is at the expense of increased toxicity as measured by animal weight loss. It therefore remains to be seen whether 4-hourly scheduling of temozolomide will produce a net clinical benefit. A phase II study in advanced malignant melanoma is now under way to address this question.

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