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Effect of single and multiple administration of an O^6-benzylguanine/temozolomide combination: an evaluation in a human melanoma xenograft model

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Abstract The purpose of the present study was to examine the effect of O^6-benzylguanine (O^6-BG) on the antitumour activity and toxicity of 8-carbamoyl-3-methylimidazo [5, 1-*d*]-1,2,3,5-tetrazine-4(3*H*)-one (temozolomide) in a human malignant melanoma xenograft model following single and multiple administration of the combination. O^6-BG irreversibly inactivates the DNA-repair protein O^6-alkylguanine-DNA alkyltransferase (AGT), which confers resistance to temozolomide. Preadministration of O^6-BG (35 mg/kg, i.p.) 1 h prior to temozolomide (i.p.) was examined using single and daily × 5 dosing regimens in athymic mice bearing subcutaneous A375P xenografts. The AGT activity of A375P tumors was 95 ± 8 fmol/mg protein (mean ± SE, $n = 4$). O^6-BG alone completely suppressed xenograft AGT activity within 1 h of administration but had no effect upon tumor growth. O^6-BG did not significantly increase the tumor growth delay induced by a single 200-mg/kg dose of temozolomide ($P > 0.05$, two-tailed Mann-Whitney test) but did increase the associated mean body weight loss ($P < 0.025$). In contrast, when the same dose of temozolomide was divided into five equal fractions (40 mg/kg) and given with O^6-BG on 5 consecutive days, a comparable increase in toxicity was accompanied by a very significant increase in tumor growth delay ($P < 0.0025$), equivalent to that produced by a 3-fold greater dose of temozolomide alone. O^6-BG with temozolomide also produced a greater antitumour effect than an equitoxic dose of temozolomide alone on this schedule ($P < 0.005$). These data indicate that the enhancement of temozolomide antitumour activity by O^6-BG preadministration is dependent upon the schedule of drug administration, with multiple dosing of O^6-BG + temozolomide producing the greatest effect. The results also suggest that prolonged administration of the combination can lead to an increase in the therapeutic index of temozolomide.

Key words Temozolomide · O^6- Benzylguanine · Schedule-dependent activity · Therapeutic index · Melanoma xenograft

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Introduction

Temozolomide {8-carbamoyl-3-methylimidazo[5, 1-*d*]-1,2,3,5-tetrazine-4(3*H*)-one} is an imidazotetrazinone derivative that demonstrates activity in metastatic melanoma equivalent to that of the most successful single agent, dacarbazine (5-[3, 3-dimethyl-triazen-1-yl]imidazole-4-carboxamide, DTIC) [1, 22]. Nonetheless, malignant melanoma remains relatively unresponsive to chemotherapy, and patients with disseminated disease have a median survival of less than 1 year [27]. Additional therapeutic strategies are therefore required to increase the effectiveness of treatment.

The antitumour activity of temozolomide is dependent upon its chemical decomposition under mildly alkaline conditions to form the unstable linear triazene 3-methyl-(triazen-1-yl)imidazole-4-carboxamide (MTIC) [4] and subsequent unimolecular fragmentation to produce a reactive methyldiazonium ion. Temozolomide cytotoxicity correlates with the methylation of O^6-guanine in DNA [21, 31], a lesion that induces futile cycling of a mismatch repair pathway, leading to DNA strand breakage and cell death [15, 17]. Intrinsic or acquired resistance to this class of methylating agent may therefore involve the DNA-repair protein O^6-alkylguanine-DNA alkyltransferase (EC 2.1.1.63, AGT), which removes O^6-guanine adducts in a stoichiometric autoinactivating reaction [3, 26, 30]. Since AGT activity may be regenerated only by de novo protein synthesis, its inactivation with a pseudosubstrate such as O^6-benzylguanine (O^6-BG) may circumvent resistance to temozolomide. Indeed, O^6-BG pretreatment has recently been shown to increase both the cytotoxicity of temozolomide in vitro

[34] and its antitumour activity in malignant glioma xenograft models in vivo [11, 33]. However, only single administration of an O^6-BG/temozolomide combination has been examined, although temozolomide is known to exhibit schedule-dependent activity [29]. In addition, there is no available evidence to suggest that O^6-BG can increase the therapeutic index of temozolomide. The purpose of this study, therefore, was to evaluate the effect of O^6-BG on both the antitumour activity and the toxicity of temozolomide using both single and multiple (daily × 5) administration schedules in a human melanoma xenograft model.

Materials and methods

Chemicals and drugs

Temozolomide was supplied by Dr. J. Catino (Schering-Plough Research Institute, Kenilworth, N.J., USA), and the [^{3}H]-methyl-labeled DNA substrate for the assay of AGT, by Dr. G.P. Margison (Paterson Institute for Cancer Research, Christie Hospital NHS Trust, Manchester, UK). O^6-BG was a generous gift from Dr. R.C. Moschel (NCI-Frederick Cancer Research & Development Center, Frederick, Md., USA). Polyethylene glycol (PEG) 400 was obtained from Brenntag (Kingston-upon-Thames, Surrey, UK). All other chemicals were purchased from Sigma Chemical Co. (Poole, UK).

Tumor and mouse model

The human malignant amelanotic melanoma tumour cell line A375P was kindly supplied by Prof. I. Hart (ICRF/R. Dimbleby Department of Cancer Research, St. Thomas's Hospital, Lambeth Palace Rd., London, UK). A375P was grown as a monolayer in Dulbecco's modified Eagle's medium (ICN Biomedicals, High Wycombe, UK) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Gibco, Paisley, UK), L-glutamine (2 m*M*), penicillin (100 U/ml) and streptomycin (100 μg/ml).

Athymic MF-1 (*nu*/*nu* genotype) mice were bred and maintained as previously described [33]. All procedures were performed with Home Office approval on mice that were at least 8 weeks of age. A375P was found to be negative for Sendai virus, mouse hepatitis virus, and pneumonia and minute virus of mice by a mouse antibody-production test [enzyme-linked immunosorbent assays (ELISAs) were performed by the Microbiology Laboratories, North Harrow, Middlesex, UK]. Xenografts were initially established in the hind flank of mice by s.c. injection of a cell suspension in phosphate-buffered saline (PBS; 5×10^7 cells/ml, 200 μl) and were maintained thereafter in vivo by serial passage of 1 to 2-mm^3 tumour sections.

Treatment

Tumour length and width were measured in situ with digital calipers (Cole-Palmer Instrument Co., Illinois, USA) and tumour volumes were calculated using the formula for a prolate ellipsoid [12]. Mice were randomised (seven per group in the single-dose study and six per group in multiple-dosing studies) and treated (day 1) when tumours had reached a volume of between 70 and 360 mm^3. Compounds were prepared immediately prior to use and given at a volume of 100 μl/10 g of body weight as a single i.p. injection. O^6-BG (in a 40% solution of PEG 400 in PBS) was given 1 h before treatment with temozolomide (in a 10% solution of dimethyl sulfoxide in PBS). Control mice, or mice receiving O^6-BG or temozolomide alone, also received the corresponding vehicle(s). Treatments were repeated at 24-h intervals for a total of five doses in repeat dosing experiments.

Evaluation of response

Tumour volume and body weight were recorded at two daily intervals. The growth (or regrowth) of each individual tumour was fitted to an unweighted exponential equation using a computational non-linear regression program (GraphPad Prism, GraphPad Software, San Diego, Calif., USA). The median coefficient of determination (r^2) from 107 analyses was 0.98. The time taken for each tumour to grow to 5 times its untreated volume on day 1 (the tumour-quintupling time) was interpolated from the relevant curve, and the mean value calculated for each group was used to assess tumour response by comparison with the control value. The statistical significance between treated and control tumours was evaluated by a one-tailed Mann-Whitney test. Intra-animal weight loss was expressed as a percentage of the relevant untreated weight (day 1), and the mean nadir was calculated for each group. Differences in tumour growth delay or weight loss between treatments with/without O^6-BG were examined for statistical significance ($P < 0.05$) using a two-tailed Mann-Whitney test. The effect of O^6-BG on the therapeutic index of temozolomide was determined by a relative comparison of weight loss and tumour growth delay.

AGT assay

Mice bearing A375P tumours of 250- to 400-mm^3 volume were randomised, divided into groups of four, and treated as described with a single dose of O^6-BG (35 mg/kg) or the corresponding vehicle as a control. Tumour xenograft and liver samples were harvested from control mice after 1 h and from O^6-BG-treated mice after 1, 6, 24 and 48 h. Samples were snap-frozen in liquid nitrogen until the determination of AGT activity by the removal of [^{3}H]-methylguanine from a [^{3}H]-methylated DNA substrate [18]. Protein concentrations were determined using the method of Bradford [2]. All AGT activities are expressed as mean values ± SE.

Results

Suppression of AGT activity in the A375P tumour xenograft and the liver

The AGT activities in the A375P tumour and the liver of control mice were determined to be 95 ± 8 and 67 ± 2 fmol/mg protein, respectively. The AGT activity in both the tumour and the liver tissue was undetectable (i.e. < 2 fmol/mg protein) within 1 h of O^6-BG administration and remained so for at least 6 h. Tumor and liver AGT activities were found to have regenerated to 16% and 54% of their respective control values by 24 h and to 88% and 140% of their control values by 48 h (Fig. 1).

Single-dose temozolomide ± O^6-BG

Single-dose studies were conducted in mice bearing tumours of 75- to 250-mm^3 volume. Temozolomide alone (100–300 mg/kg) produced significant tumour growth delay as compared with control values, whereas O^6-BG

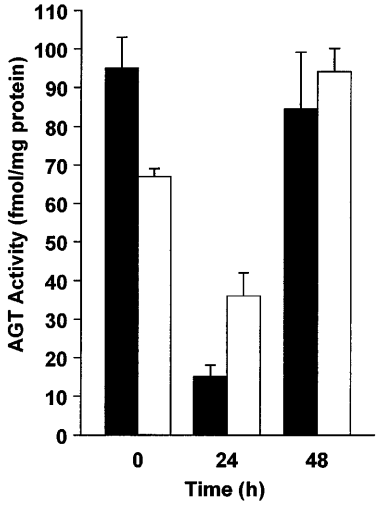

Fig. 1 Depletion and regeneration of AGT activity in (■) A375P tumour xenografts and (□) mouse liver following a single i.p. injection of O^6-BG (35 mg/kg). The AGT activity in both the liver and the tumour tissue was undetectable at 1 and 6 h after O^6-BG administration. Each *bar* represents the mean value + SE for 4 separate determinations

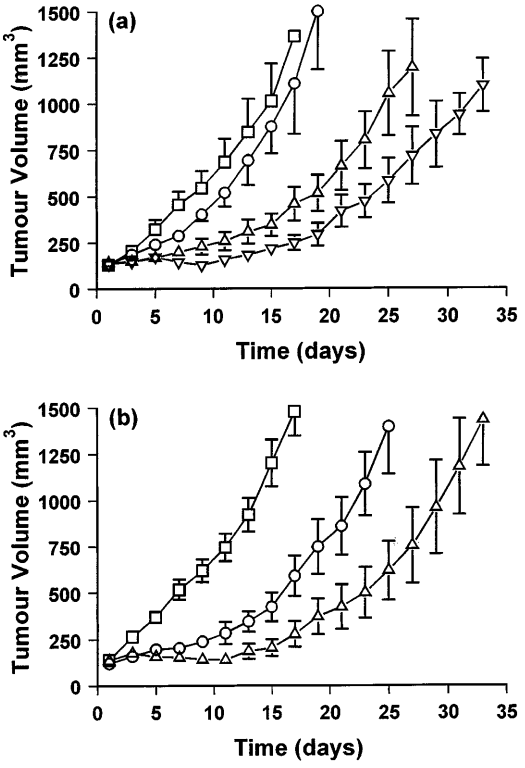

Fig. 2a,b Growth inhibition of A375P xenografts treated with a single dose of O^6-BG ± temozolomide. Nude mice bearing A375P xenografts were given i.p. injections of **a** O^6-BG vehicle (40% PEG400 in PBS) 1 h before administration of temozolomide vehicle (10% DMSO in PBS, □), 100 mg/kg temozolomide (○), 200 mg/kg temozolomide (△) or 300 mg/kg temozolomide (▽) and **b** 35 mg/kg O^6-BG 1 h before temozolomide vehicle (□), 100 mg/kg temozolomide (○) or 200 mg/kg temozolomide (△). *Points* represent mean values ± SE for 7 mice, with errors smaller than the data symbol being omitted and those that overlap being illustrated in one direction only

alone had no effect (Fig. 2, Table 1). O^6-BG preadministration marginally enhanced the tumour growth delay induced by a single dose of temozolomide (Fig. 2), although even the most pronounced effect (100 mg/kg temozolomide ± O^6-BG) did not quite reach statistical significance ($P = 0.053$). The body weight loss induced by treatment with 200 mg/kg temozolomide alone (9.5 ± 1.6%, mean value ± SE) was significantly increased by O^6-BG (16.3 ± 0.9%, $P < 0.025$), although a comparable increase was not observed when O^6-BG was combined with 100 mg/kg temozolomide (Table 1). The mean tumour-quintupling time, mean weight loss, and number of toxicity-related deaths produced by 200 mg/kg temozolomide plus O^6-BG were equivalent to those produced by a 1.5-fold greater dose of temozolomide alone (Table 1), indicating that the therapeutic index of temozolomide was unaffected by O^6-BG on this schedule.

Table 1 Effect of a single dose of temozolomide ± O^6-BG on A375P human melanoma xenografts grown in athymic mice

O^6-BG[a] (mg/kg)	Temozolomide (mg/kg)	Tumour-quintupling time[b] (days)	Tumour growth delay[c] (days)	Weight loss[d] (%)	Toxicity-related deaths
0	0	11.2 ± 0.8	0.0	3.0 ± 0.7	0/7
0	100	14.1 ± 1.3	2.9*	9.1 ± 1.9	0/7
0	200	22.9 ± 1.8	11.7**	9.5 ± 1.6	0/7
0	300	25.5 ± 1.6	14.3**	16.7 ± 1.7	1/7
35	0	10.5 ± 0.2	−0.7	2.2 ± 0.3	0/7
35	100	18.2 ± 1.1	7.0**	12.1 ± 2.3	0/7
35	200	26.9 ± 1.6	15.7**	16.3 ± 0.9	1/7

[a] O^6-BG (35 mg/kg) was given 1 h before temozolomide. The relevant vehicle was given when O^6-BG or temozolomide was not required
[b] Time taken for individual tumours to reach a volume of 5 times their untreated volume on day 1 (mean ± SE)
[c] The difference between the mean time required for tumours in treated and control mice to reach a volume of 5 times their untreated volume on day 1
[d] Mean body weight nadir (±SE) as a percentage of the day-1 untreated weight
* $P < 0.05$; ** $P < 0.001$ vs control (Mann-Whitney test)

Multiple administration of temozolomide ± O^6-BG

Two separate daily × 5 experiments were performed (experiments A and B). In both experiments, repeat administration of temozolomide (40–120 mg/kg per day) or of O^6-BG (35 mg/kg per day) plus temozolomide (20–40 mg/kg per day) produced a statistically significant growth delay as compared with the control value, whereas O^6-BG alone had no effect (Table 2).

Experiment A

Treatment commenced when tumours had reached a volume of 130–360 mm^3. O^6-BG + temozolomide produced a mean tumour-quintupling time equivalent to that resulting from a 3-fold greater dose of temozolomide alone (Fig. 3, Table 2) and usually with less weight loss, although differences in mean weight loss did not quite reach statistical significance ($P = 0.065$). In addition, whereas 120 mg/kg per day of temozolomide alone or 40 mg/kg per day temozolomide plus O^6-BG produced comparable delays in tumour growth, these treatments resulted in 3/6 and 1/6 toxicity-related deaths, respectively. Taken together, these data may suggest that an O^6-BG/temozolomide combination is less toxic than temozolomide alone for a given antitumour effect.

Experiment B

Treatment commenced when tumours had reached a volume of 70–210 mm^3. O^6-BG in combination with 40 mg/kg temozolomide produced a very significant increase in tumour-quintupling time ($P < 0.0025$), whereas the corresponding increase in weight loss remained comparable with that observed in the single-dose study (with 200 mg/kg temozolomide ± O^6-BG; Fig. 4, Table 2). In addition, although the mean weight loss induced by 13.3 mg/kg per day of temozolomide + O^6-BG was equivalent to that produced by a 3-fold greater dose of temozolomide alone (40 mg/kg per day), the tumour-quintupling time in animals treated with 13.3 mg/kg per day of temozolomide + O^6-BG was significantly greater ($P < 0.005$). This would suggest that an O^6-BG/temozolomide combination produces a greater antitumour effect than an equitoxic dose of temozolomide alone.

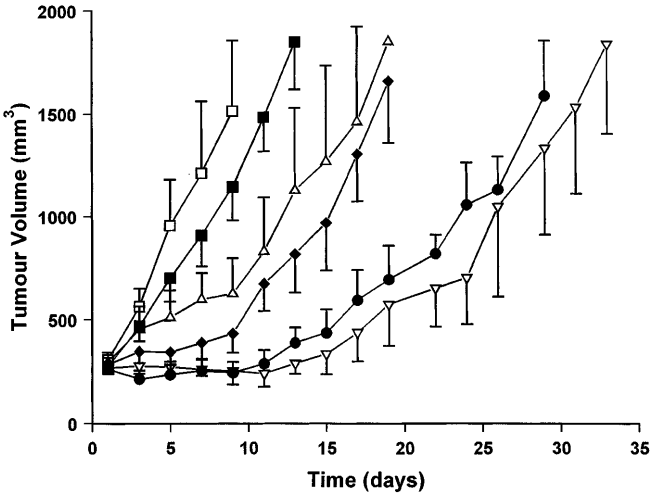

Fig. 3 Experiment A: growth inhibition of A375P xenografts treated with O^6-BG ± temozolomide on 5 consecutive days (days 1–5). Nude mice bearing A375P xenografts received i.p. injections of either (1) O^6-BG vehicle (40% PEG400 in PBS) 1 h before temozolomide vehicle (10% DMSO in PBS, □), 60 mg/kg temozolomide (△) or 120 mg/kg temozolomide (▽) or (2) 35 mg/kg O^6-BG 1 h before temozolomide vehicle (■), 20 mg/kg temozolomide (◆) or 40 mg/kg temozolomide (●). *Points* represent mean values ± SE for 6 mice

Table 2 Effect of a repeat daily × 5 dose of temozolomide ± O^6-BG on A375P human melanoma xenografts grown in athymic mice

Experiment[a]	O^6-BG[b] (mg/kg per day)	Temozolomide (mg/kg per day)	(mg/kg total)	Tumour-quintupling Time[c] (days)	Tumour growth delay[d] (days)	Weight loss[e] (%)	Toxicity-related deaths
A	0	0	0	9.7 ± 1.0	0.0	4.6 ± 0.4	0/6
	0	60	300	19.8 ± 5.1	10.1[*1]	19.7 ± 1.3	1/6
	0	120	600	30.7 ± 2.0	21.0[*2]	20.8 ± 2.0	3/6
	35	0	0	10.7 ± 0.5	1.0	5.1 ± 1.0	0/6
	35	20	100	20.8 ± 2.3	11.1[*4]	15.4 ± 1.4	1/6
	35	40	200	27.7 ± 0.5	18.0[*4]	15.5 ± 2.5	1/6
B	0	0	0	5.9 ± 0.7	0.0	3.1 ± 1.2	0/6
	0	40	200	9.7 ± 1.0	3.8[*3]	11.4 ± 1.4	0/6
	35	0	0	5.8 ± 0.4	−0.1	4.7 ± 0.5	0/6
	35	13.3	66.5	19.0 ± 1.7	13.1[*5]	14.0 ± 1.8	0/6
	35	40	200	23.4 ± 1.6	17.5[*5]	16.7 ± 1.5	0/6

[a]Tumour volumes (mean ± SD) at the start of treatment were 283 ± 69 and 107 ± 30 mm^3 in experiments A and B, respectively
[b]O^6-BG (35 mg/kg) was given 1 h before temozolomide. The relevant vehicle was given when O^6-BG or temozolomide was not required
[c]Time taken for tumours to reach a volume of 5 times their untreated volume on day 1 (mean ± SE)
[d]The difference between the mean time required for tumours in treated and control mice to reach a volume of 5 times their untreated volume on day 1
[e]Mean body weight nadir (± SE) as a percentage of the day-1 untreated weight
[*1]$P < 0.05$; [*2]$P < 0.015$; [*3]$P < 0.005$; [*4]$P < 0.0025$; [*5]$P < 0.0015$ vs control (Mann-Whitney test)

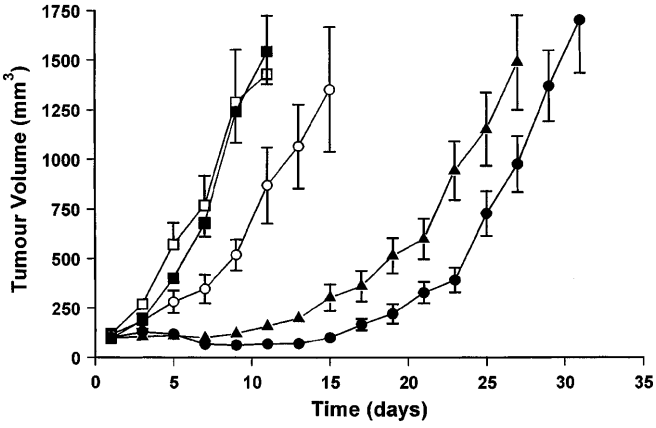

Fig. 4 Experiment B: growth inhibition of A375P xenografts treated with O^6-BG ± temozolomide on 5 consecutive days (days 1–5). Nude mice bearing A375P xenografts received i.p. injections of either (1) O^6-BG vehicle (40% PEG400 in PBS) 1 h before temozolomide vehicle (10% DMSO in PBS, □) or 40 mg/kg temozolomide (○) or (2) 35 mg/kg O^6-BG 1 h before temozolomide vehicle (■), 13.3 mg/kg temozolomide (▲) or 40 mg/kg temozolomide (●). *Points* represent mean values ± SE for 6 mice, with errors smaller than the data symbol being omitted and those that overlap being illustrated in one direction only

Discussion

Previous attempts to increase the antitumour efficacy of DNA-alkylating chemotherapy by manipulating AGT activity have primarily focused upon a combination of O^6-BG with the bifunctional chloroethylating agent carmustine (BCNU) since AGT removes O^6-guanine-linked chloroethyl adducts, which are a precursor to the formation of a highly cytotoxic DNA cross-link [14, 32]. In the many experiments examining an O^6-BG/BCNU combination, only single administration has been studied since the chloroethylnitrosoureas are profoundly myelosuppressive. Nevertheless, significant increases in the therapeutic index of BCNU have been observed following O^6-BG administration in a number of human glioma and colon cancer xenografts [10, 13, 23]. These results have recently prompted phase I clinical examination of O^6-BG in combination with BCNU [6, 28].

The present study indicates that O^6-BG administration has little effect on the antitumour activity of a single 200-mg/kg dose of temozolomide in a human melanoma tumour model (A375P). Similar results have been obtained by Friedman et al. [11], who examined a medulloblastoma tumour model displaying AGT activity comparable with that of the A375P xenograft; O^6-BG (30 mg/kg) pretreatment afforded a small increase in the antitumour activity of a single 250-mg/kg dose of temozolomide but also enhanced its toxicity. The antitumour activity of a single dose of temozolomide has been enhanced by up to 3.5-fold following O^6-BG (40 mg/kg) administration, but only in a human glioblastoma xenograft with very little AGT activity (approximately 5 fmol/mg protein) [33].

In contrast, when 200 mg/kg temozolomide was divided into five equal fractions (40 mg/kg) and given with O^6-BG on 5 consecutive days, a very significant increase in the mean time taken for A372P tumours to grow to 5 times their untreated volume was apparent ($P < 0.0025$). These results are supported by in vitro data, which indicate that the enhancement of temozolomide cytotoxicity by O^6-BG can increase linearly with each subsequent dose of a repeat dosing schedule [34], and in vivo data that indicate that the antitumour activity of temozolomide is schedule-dependent [29]. Multiple administration of temozolomide with an AGT inhibitor would be expected to result in a potentiating effect greater than that produced by an equivalent single dose, as it would be accompanied by a more extensive depletion of AGT and an increased retention of O^6-methyguanine in DNA [19].

Collectively, the results of the multiple-dosing studies also suggest that O^6-BG can increase the therapeutic index of temozolomide. However, these experiments, and all similar investigations performed in mice, may be favourably biased, since the inactivation of AGT has been found to be species-selective, human AGT being approximately 5- to 10-fold more sensitive to inhibition by O^6-BG than murine AGT [8, 20]. This suggests that the toxicity of an O^6-BG/temozolomide combination in normal human tissues may be greater than anticipated. Nevertheless, the potential for a clinical therapeutic gain remains as there are data to suggest that O^6-BG produces a small increase in the therapeutic index of BCNU in a rat prostatic tumour model [5], and rat and human AGT exhibit comparable sensitivity to O^6-BG [8].

Differences in the sensitivity of murine and human AGT to inhibition by O^6-BG may partly explain the greater suppression of AGT activity observed in the A375P tumour xenograft in comparison with mouse liver, but this does not exclude the possibility that AGT regeneration rates may be variable in different syngeneic tissues and influence the efficacy of an O^6-BG/temozolomide therapy. These data are consistent with the results of a previous study in a human colon xenograft model, in which tumour and liver AGT activity were approximately 17% and 42% of their respective control values at 24 h after the administration of 30 mg/kg O^6-BG [13].

Although clinical administration of O^6-BG is likely to exacerbate any myelosuppression produced by temozolomide [9, 24], it may be possible to manage an increase in this toxicity with appropriate haematopoietic support [16]. Of greater concern is the possibility that mutagenesis will be enhanced in tissues with low-level AGT activity [35, 36], although this would not prevent clinical use of the combination if it were found to improve patient survival.

It is likely that any enhancement of temozolomide activity by O^6-BG will be significantly greater if the administration schedule is further prolonged. Extended treatment is clinically feasible as temozolomide is well tolerated and can be safely given at 75 mg/m^2 per day

for a continuous 7-week period [25]. Clinical examination of an O^6-BG/temozolomide combination may therefore be warranted, especially in the treatment of disseminated melanoma, which is particularly chemoresistant and is known to involve variable AGT expression among different metastases of the same patient [7].

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