Int. J. Cancer: **85,** 248–252 (2000)

Publication of the International Union Against Cancer
Publication de l'Union Internationale Contre le Cancer

O^6-(4-BROMOTHENYL)GUANINE IMPROVES THE THERAPEUTIC INDEX OF TEMOZOLOMIDE AGAINST A375M MELANOMA XENOGRAFTS

Mark R. MIDDLETON[1,2], Jane KELLY[1], Nicholas THATCHER[2], Dorothy J. DONNELLY[3], R. Stanley MCELHINNEY[3], T. Brian H. MCMURRY[3], Joan E. MCCORMICK[3] and Geoffrey P. MARGISON[1]*

[1]*Cancer Research Campaign Department of Carcinogenesis, Paterson Institute for Cancer Research, Manchester, UK*
[2]*Cancer Research Campaign Department of Medical Oncology, Christie Hospital NHS Trust, Manchester, UK*
[3]*University Chemical Laboratory, Trinity College, University of Dublin, Dublin, Ireland*

Tumour resistance to methylating agents is linked to expression of the DNA repair protein O^6-alkylguanine-DNA alkyltransferase (ATase). There is considerable interest in improving the efficacy of O^6-alkylating chemotherapy by prior depletion of ATase. We have tested the ability of a modified guanine base, O^6-(4-bromothenyl)guanine (4BTG), to inactivate ATase and to enhance the anti-tumour effect of temozolomide in an animal model system. A375M human melanoma xenografts were established in the flanks of nude mice. ATase depletion after a single dose of 4BTG or O^6-BG (20 mg/kg i.p.) was determined over a 24 hr period. Subsequently, we tested the effect of 4BTG (20 mg/kg i.p. daily) and/or temozolomide (80–175 mg/kg i.p. daily) over a 5-day schedule on tumour growth. 4BTG was an effective inactivator of ATase in tumour, producing complete depletion within 2 hr of dosing. Furthermore, it enhanced the tumour growth delay achieved with temozolomide, increasing the tumour quintupling time by 8.7 days (95% confidence interval 6.1–11.3 days, $p < 0.0001$). Whilst the delay in tumour growth was indistinguishable from that observed with O^6-benzylguanine (O^6-BG) and temozolomide, the 4BTG combination resulted in considerably less toxicity (0/9 *vs.* 2/9 deaths; 6.84% weight loss *vs.* 9.48%, $p = 0.019$). 4BTG is a potent inactivator of ATase and enhances the therapeutic ratio of temozolomide in this model system to a greater extent than O^6-BG. *Int. J. Cancer* 85:248–252, 2000.

Methylating and chloroethylating agents are cytotoxic principally by virtue of their ability to alkylate DNA at the O^6 position of guanine (Domoradzki *et al.,* 1984; Margison and O'Connor, 1990). There is considerable evidence that the DNA repair protein O^6-alkylguanine-DNA alkyltransferase (ATase) plays a key role in determining tumour resistance to these drugs. ATase repairs alkylation at the O^6 position on guanine by accepting the alkyl group onto a cysteine residue in its active site. This reaction is stoichiometric and auto-inactivating, and recovery of ATase activity requires *de novo* protein synthesis (Margison and O'Connor, 1990; D'Incalci *et al.,* 1988). Cellular resistance *in vitro* is correlated with ATase expression and can be engendered in previously susceptible cell lines by transfection with cDNA encoding for the protein (Jelinek *et al.,* 1988; Pegg, 1990). Furthermore, depletion of ATase prior to treatment renders resistant cells sensitive to O^6-alkylating agents (Yarosh *et al.,* 1986; Dolan *et al.,* 1991).

Attempts to exploit this clinically have used methylating agents to deplete ATase, *via* their effect on DNA, prior to the administration of a chloroethylating drug. These have been hampered by the similar toxicities of the 2 agents, and no useful increase in therapeutic index has been demonstrated (Micetich *et al.,* 1992; Lee *et al.,* 1993; Smith *et al.,* 1996). Interest has therefore turned to inactivation of ATase using inherently non-toxic pseudosubstrates for the protein, such as O^6-benzylguanine (O^6-BG) (Dolan and Pegg, 1997).

We previously described a group of novel ATase inactivators, some of which were more potent than O^6-BG (McElhinney *et al.,* 1998). Here, we present results with O^6-(4-bromothenyl)guanine (4BTG), one of the most potent inactivators synthesised to date with an I_{50} for pure recombinant human ATase 10-fold lower than that of O^6-BG, and demonstrate its ability to enhance the anti-tumour efficacy of temozolomide against human melanoma xenografts.

MATERIAL AND METHODS

Drugs

4BTG, O^6-BG and temozolomide were provided by the Cancer Research Campaign Drug Formulation Unit, University of Strathclyde (Glasgow, UK). The inactivators were homogenised and suspended in corn oil at 4 mg/ml immediately prior to i.p. injection. Temozolomide was freshly prepared at 40 mg/ml in DMSO (Sigma, Poole, UK) each time, diluted in 0.9% NaCl solution and injected i.p. within 15 min. Temozolomide was injected 1 hr after the inactivator or appropriate vehicle control.

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 experiments began when tumour volumes reached a suitable size. Animals were cared for in accordance with Home Office guidelines.

ATase depletion was studied in 6 groups of at least 3 mice for each inactivator. Animals received 4BTG or O^6-BG 20 mg/kg i.p. or the vehicle control as a single dose. At varying times after dosing, animals were killed by cervical dislocation and the tissues dissected out and immediately frozen in liquid nitrogen. Tissue was stored at –70°C until assayed according to the method of Lee *et al.* (1994).

The first tumour growth delay experiment, to assess the ability of 4BTG to sensitise human melanoma xenografts to the anti-tumour effects of temozolomide, was started with tumour volumes of between 17 and 58 mm^3. Up to 11 animals were assigned to each group, and mean tumour volume was standardised across the groups. Mice received vehicle controls (corn oil then 20% DMSO in PBS) once daily for 5 days, corn oil then temozolomide i.p. daily for 5 days (at doses of 80, 100, 125 or 175 mg/kg) or 4BTG 20 mg/kg followed by either the DMSO/PBS vehicle or temozolomide (at 80 or 100 mg/kg) i.p. daily for 5 days.

In a second experiment, to compare the relative effects of 4BTG and O^6-BG on temozolomide treatment, drugs were also given over 5 days. Tumour volumes ranged from 20 to 145 mm^3 at the start of the experiment and were standardised across the groups. Mice received daily injections of corn oil, 4BTG 20 mg/kg or O^6-BG 20 mg/kg i.p. followed 1 hr later by either DMSO/PBS or temozolomide 100 mg/kg i.p. daily for 5 days.

In a third experiment, 4BTG or O^6-BG (20 mg/kg i.p.) was administered 1 hr before BCNU (16 mg/kg i.p.) to contrast their

*Correspondence to: Cancer Research Campaign Department of Carcinogenesis, Paterson Institute for Cancer Research, Wilmslow Road, Manchester M20 4BX, UK. Fax: +44 161 4463299.
E-mail: mmiddleton@picr.man.ac.uk

Received 23 March 1999; Revised 12 August 1999

effects on the therapeutic index of BCNU. Tumour volumes ranged from 68 to 184 mm^3 at the start of the experiment and were standardised across the groups.

In all tumour growth delay experiments, animal weights and tumour volumes were measured twice per week. Tumour volumes were calculated using the formula (length $\times$ height $\times$ width) $\times$ $\pi \div 6$, with measurements taken using digital calipers.

Statistical methods

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 this estimate were obtained. These data pairs (estimate, precision) were used in a generalised 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, CA). The maximum relative weight losses observed in each group were compared using the Kruskal-Wallis test.

RESULTS

ATase depletion in tumour and normal tissues

Significant depletion was seen in all tissues analysed after a single 20 mg/kg dose of 4BTG i.p. (Fig. 1*a*), with over 60% reduction in ATase activity in all tissues analysed, except brain. In brain, the ATase nadir was later than in the other tissues and there was only 24% depletion. In normal tissues, substantial activity (over 50%) had returned at 24 hr after dosing. Complete inactivation was seen in tumour, and recovery of ATase levels was delayed. Similar results were achieved in tumour and kidney with 20 mg/kg O^6-BG i.p. (Fig. 1*b*). The same pattern of depletion, with substantial recovery at 24 hr, was seen in liver, lung and bone marrow, though there was greater depletion than with 4BTG. In brain, O^6-BG administration also produced greater depletion, but this was sustained throughout the period studied.

Effect of 4BTG ± temozolomide on A375M tumour xenograft growth

Temozolomide significantly delayed growth of the A375M tumour xenograft in a dose-dependent manner (Fig. 2*a*,*b*, Table I; $p < 0.0001$), with an estimated delay in the time for tumour to quintuple in size of 9.6 days (95% confidence interval 6.3–12.8) for each doubling of dose. Addition of 4BTG to temozolomide significantly enhanced the latter's effect, delaying the quintupling time a further 8.7 days (6.1–11.3, $p < 0.0001$). The tumour growth delay achieved with 4BTG and 100 mg/kg temozolomide daily was greater than that observed with the highest temozolomide dose, though this did not achieve statistical significance (33.0 *vs.* 29.6 days, $p = 0.14$). The inactivator alone had no significant effect on tumour growth. Toxicity, as measured by weight loss, occurred in all temozolomide-treated groups and was log-linearly dose-dependent ($p < 0.0001$). Toxicity was unaffected by addition of 4BTG to treatment regimens (Table I, $p = 0.50$).

Comparison of the effect of 4BTG or O^6-BG on alkylating agent toxicity and efficacy

Both inactivators significantly extended the tumour growth delay observed with temozolomide treatment and to the same extent (quintupling time was extended 12.7 days with 4BTG *vs.* 13.6 with O^6-BG; Table II, Fig. 3). However, 2 of 9 mice died in the O^6-BG/temozolomide group, whereas there were no deaths with the 4BTG/temozolomide combination. Weight loss was more marked in the surviving O^6-BG/temozolomide-treated animals, being a mean of 9.48% of the day 1 value compared to 6.84% with 4BTG/temozolomide ($p = 0.019$). Similar results were seen when the inactivators were combined with BCNU: similar enhancement of efficacy was observed but at the cost of significantly greater toxicity with the O^6-BG/BCNU combination (Table III, Fig. 4).

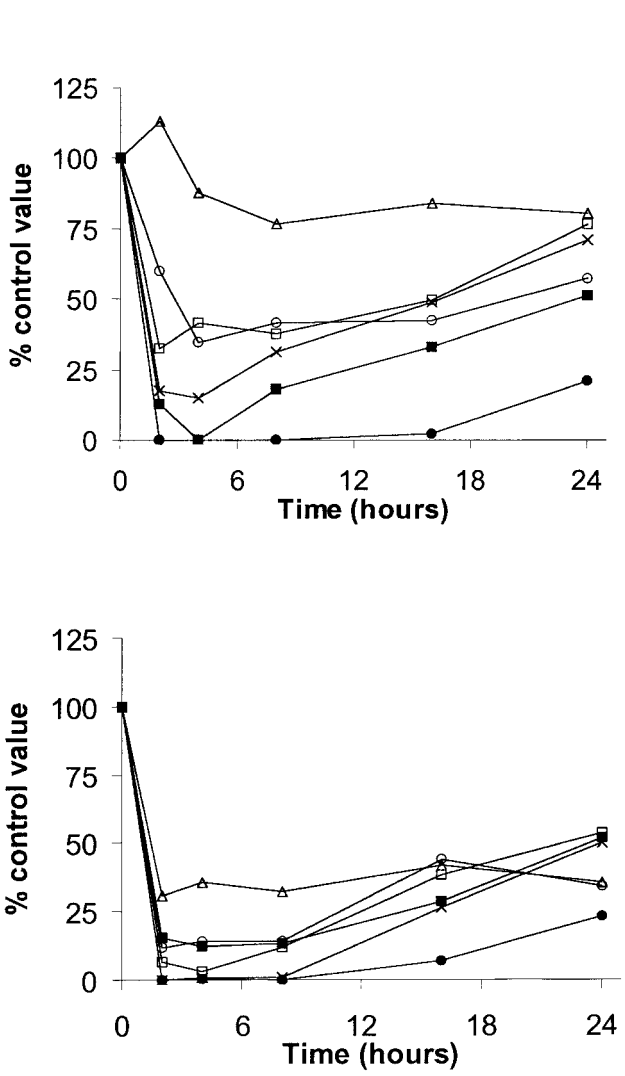

FIGURE 1 – Tumour and normal tissue depletion of ATase after a single i.p. dose of 20 mg/kg 4BTG *(a)* or O^6-BG *(b)*. Determinations were made in A375M tumour xenograft (●), liver (X), kidney (■), lung (○), brain (△) and bone marrow (□). Points are the mean of at least 3 observations (SE not shown for clarity).

DISCUSSION

Temozolomide has been approved for the treatment of malignant glioma and is under consideration with the EAMA for use in melanoma. However, resistance to treatment remains a problem, with only a minority of patients with melanoma responding (Middleton *et al.*, 1999). As discussed above, there is evidence that ATase is the principal mediator of this resistance. Although melanoma expresses relatively low levels of ATase, inter- and intratumour levels can vary considerably for any given patient, with areas of high activity even in tumours with low average levels of expression (Chen *et al.*, 1992; Egyhazi *et al.*, 1995; Lee *et al.*, 1992). Attempts to improve the results of this and other O^6-alkylating agent therapy by prior inactivation of ATase using methylating agents have foundered owing to the similar, and hence cumulative, toxicities of the inactivating and treatment drugs (Lee *et al.*, 1993; Smith *et al.*, 1996). To overcome this, there is a need for non-toxic ATase inactivators, and several potential compounds have been identified. Of these, O^6-BG is by far the most developed and is now being tested in phase II clinical trials (Dolan and Pegg, 1997). It has previously been shown that O^6-BG improves the

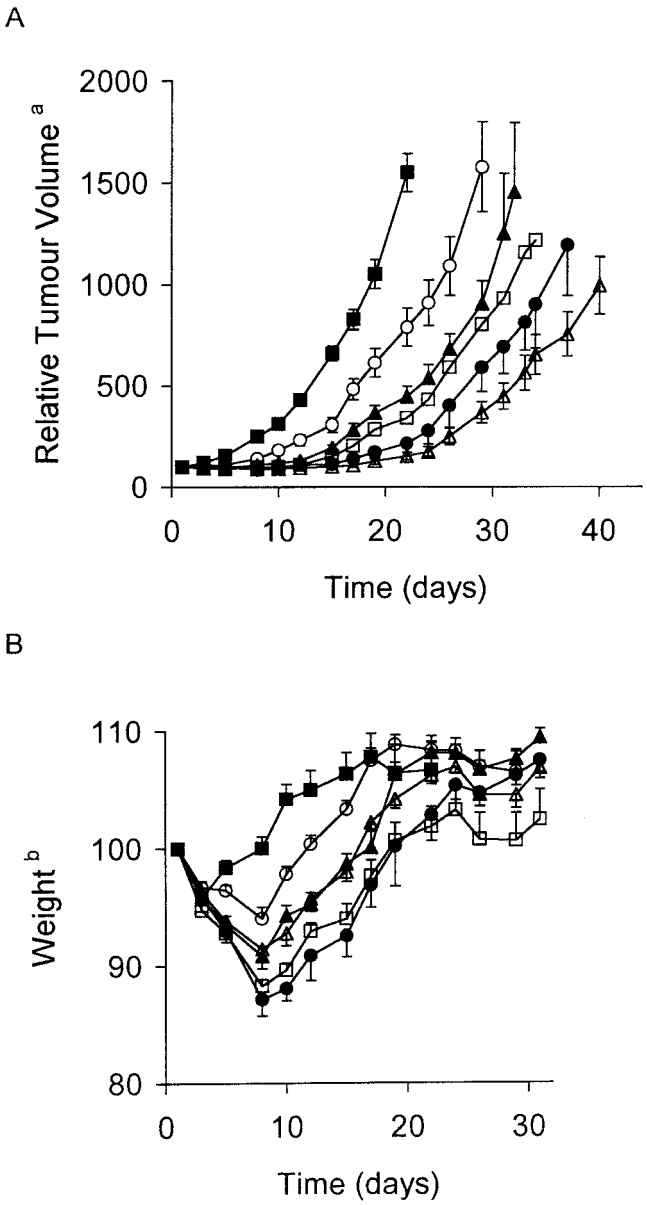

FIGURE 2 – Growth inhibition of A375M xenografts *(a)* and weight change in animals *(b)* treated with DMSO 20% in 0.9% saline and corn oil (■), temozolomide 80 (○), 100 (▲), 125 (□) or 175 (●) mg/kg once daily for 5 days with corn oil or temozolomide 100 mg/kg daily for 5 days preceded by 20 mg/kg 4BTG in corn oil (Δ). All treatments were administered i.p. Points represent the mean for at least 5 mice ± SE, with error bars that overlap being shown in one direction only. *(a)* Mean of the percentage change in tumour volume for each group (compared with the day 1 volume). *(b)* Mean of the percentage change in animal weight for each group (compared with the day 1 value).

TABLE I – A375M HUMAN TUMOUR XENOGRAFT QUINTUPLING TIME AND ANIMAL WEIGHT LOSS AFTER TREATMENT WITH TEMOZOLOMIDE ± 4BTG

Temozolomide dose (mg/kg/day)[1]	4BTG dose (mg/kg/day)[1]	Quintupling time (days)	Weight loss (% of day 1 value)	Deaths
0	0	13.8 ± 1.21	4.2 ± 1.34	0/11
0	20	14.2 ± 1.22	4.0 ± 0.84	0/11
80	0	19.2 ± 1.35	6.3 ± 0.84	0/9
100	0	24.0 ± 1.28	9.4 ± 0.71	0/10
125	0	26.0 ± 1.43	11.9 ± 1.85	0/8
150	0	27.9 ± 1.49	11.0 ± 1.71	0/8
175	0	29.6 ± 1.81	13.9 ± 1.25	0/5
80	20	27.2 ± 1.28	3.9 ± 1.16	0/10
100	20	33.0 ± 1.39	9.0 ± 1.46	0/9

[1]Administered i.p. on days 1–5.

TABLE II – A375M HUMAN TUMOUR XENOGRAFT QUINTUPLING TIME AND ANIMAL WEIGHT LOSS AFTER TREATMENT WITH TEMOZOLOMIDE ± 4BTG OR O^6-BG

Temozolomide dose (mg/kg/day)[1]	4BTG dose (mg/kg/day)[1]	O^6-BG dose (mg/kg/day)[1]	Quintupling time (days)	Weight loss (% of day 1 value)	Deaths
0	0	0	17.4 ± 1.44	3.7 ± 0.95	0/9
0	20	0	15.8 ± 1.45	0.1 ± 0.14	0/9
0	0	20	17.3 ± 1.44	0.3 ± 0.17	0/9
100	0	0	22.1 ± 1.44	7.9 ± 1.00	0/9
100	20	0	30.1 ± 1.50	6.8 ± 0.62[2]	0/9
100	0	20	31.0 ± 1.53	9.5 ± 0.80[2]	2/9

[1]Administered i.p. on days 1–5.–[2]$p = 0.019$ (4BTG/temozolomide *vs.* O^6-BG/temozolomide).

therapeutic effect of alkylating agents in a number of tumour models (Dolan *et al.,* 1993; Wedge *et al.,* 1996), but clinical studies have yet to demonstrate that this yields an increase in therapeutic index, *i.e.,* that the improvement in efficacy outweighs any extra toxicity (Schilsky *et al.,* 1998). This is because O^6-BG depletes ATase in normal tissues, such as bone marrow, potentiating adverse as well as anti-tumour effects (Fairbairn *et al.,* 1995).

Our results show that 4BTG is also a potent inactivator of ATase *in vivo,* producing significant inactivation in all normal tissues measured and 100% tumour depletion at a dose that is itself not toxic. Furthermore, 4BTG produces improvement in the anti-tumour efficacy of temozolomide above and beyond that which can be achieved by escalating the dose of temozolomide. In our experience, mice do not survive dosing at higher levels (*e.g.,* 200 mg/kg daily i.p. for 5 days) than those used in this experiment. Despite this, there is no increased toxicity, as measured by weight loss or toxic death. Thus, the therapeutic index of temozolomide is increased by 4BTG in this animal model. It remains to be seen if this increase will be evident in clinical practice, for 4BTG will undoubtedly deplete ATase in normal tissues too. The dose-limiting toxicity in our model is gastro-intestinal but likely to be haematological in humans, making extrapolation from data on animals difficult.

Temozolomide is already licensed for the treatment of primary brain tumours, and it may be possible to use this approach in the treatment of patients with these and other CNS tumours. Although we have no direct evidence of 4BTG entering the CNS, we have observed ATase depletion in brain after dosing with the inactivator. However, much greater depletion was observed here with O^6-BG, and it is known that the active 8-hydroxy metabolite penetrates the CNS.

The key to successful clinical application of an ATase inactivator lies in providing the greatest possible improvement in the therapeutic index of the co-administered O^6-alkylating agent. We compared the efficacy and toxicity of 4BTG and temozolomide with O^6-BG, the best-developed ATase inactivator to date, in the same combination. We ascertained beforehand that the pattern and extent of ATase depletion and recovery in tumour were similar for the 2 drugs. A confounding factor in comparing toxicity with the 2 inactivators is the relative sensitivity of murine and human ATase to 4BTG and O^6-BG. *In vitro,* human ATase is more sensitive to inactivation by these drugs than is the murine form. Human ATase is 14-fold more sensitive to 4BTG than the murine form, but the difference is only 5-fold with O^6-BG. *In vivo,* much of the activity of O^6-BG is conferred by metabolites such as 8-hydroxybenzylguanine, which has activity similar to that of the parent drug. This is not the case with 4BTG, whose 8-hydroxy metabolite has

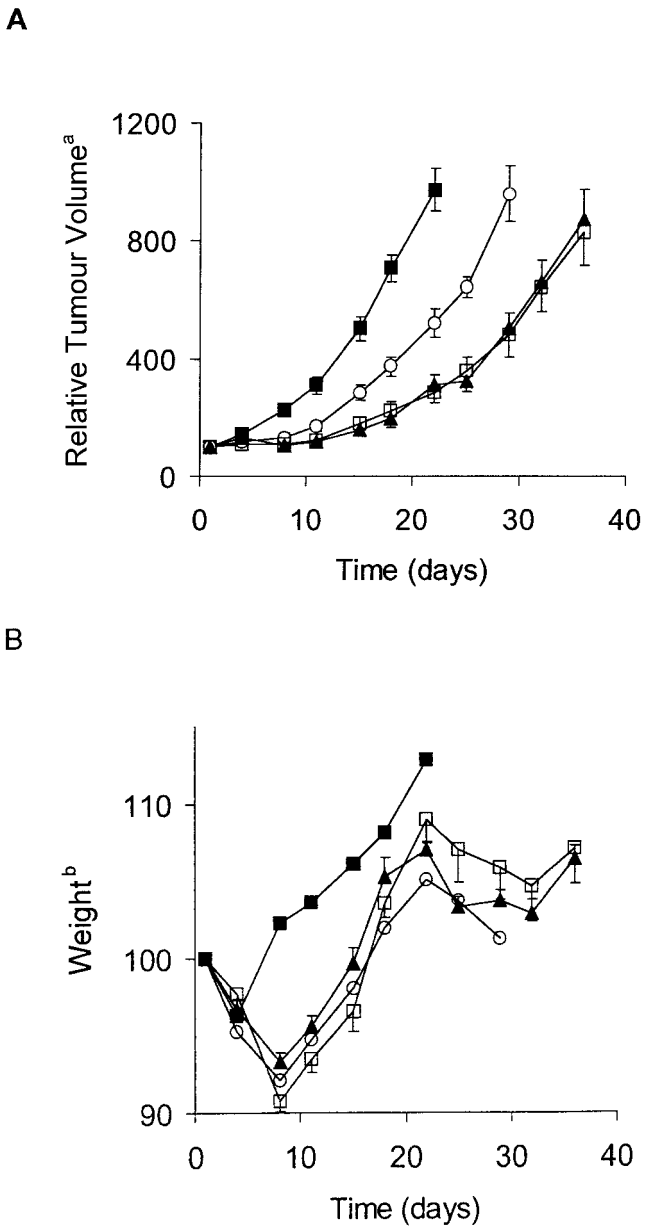

FIGURE 3 – Growth inhibition of A375M xenografts *(a)* and weight change in animals *(b)* treated with DMSO 20% in 0.9% saline and corn oil (■), temozolomide 100 mg/kg once daily for 5 days with corn oil (○), temozolomide 100 mg/kg once daily for 5 days preceded by 20 mg/kg 4BTG in corn oil (▲) or temozolomide 100 mg/kg once daily for 5 days preceded by 20 mg/kg O6-BG in corn oil (□). All treatments were administered i.p. Points represent the mean for 9 mice ± SE, with error bars that overlap being shown in one direction only. *(a)* Mean of the percentage change in tumour volume for each group (compared with the day 1 volume). *(b)* Mean of the percentage change in animal weight for each group (compared with the day 1 value). SE shown only for combination treatments for the sake of clarity.

TABLE III – A375M HUMAN TUMOUR XENOGRAFT QUINTUPLING TIME AND ANIMAL WEIGHT LOSS AFTER TREATMENT WITH BCNU ± 4BTG OR O^6-BG

BCNU dose (mg/kg)[1]	4BTG dose (mg/kg)[1]	O^6-BG dose (mg/kg)[1]	Quintupling time (days)	Weight loss (% of day 1 value)	Deaths
0	0	0	14.2 ± 1.35	2.43 ± 0.52	0/8
16	0	0	14.4 ± 1.13	3.49 ± 0.75	0/8
16	20	0	20.9 ± 1.32[2]	6.11 ± 1.25[3]	0/8
16	0	20	22.5 ± 0.87[2]	18.6 ± 2.51[3]	1/8

[1]Administered i.p. on day 1.–[2]No significant difference.–[3]$p = 0.019$ (4BTG/BCNU *vs.* O^6-BG/BCNU).

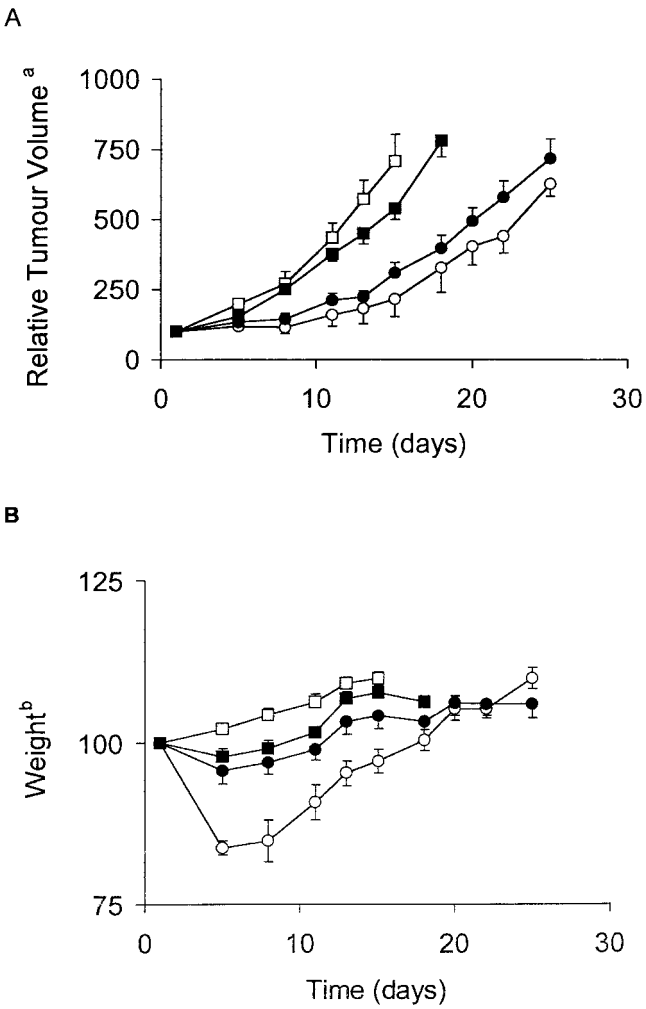

FIGURE 4 – Growth inhibition of A375M xenografts (*a*) and weight change in animals (*b*) treated with DMSO 20% in 0.9% saline and corn oil (□), BCNU 16 mg/kg preceded by corn oil (■), 20 mg/kg 4BTG in corn oil (●) or 20 mg/kg O^6-BG in corn oil (○). All treatments were administered i.p. Points represent the mean for 8 mice ± SE, with error bars that overlap being shown in one direction only. *(a)* Mean of the percentage change in tumour volume for each group (compared with the day 1 volume). *(b)* Mean of the percentage change in animal weight for each group (compared with the day 1 value).

only one-fifteenth the activity of the parent compound. Thus, by selecting inactivator doses with similar effects on tumour ATase, it might be expected that greater depletion would be seen in normal tissues with O^6-BG, despite the much lower I_{50} for recombinant human ATase of 4BTG. Although the pattern of depletion in normal tissues was similar, greater depletion was seen in most tissues after O^6-BG administration. The increased normal tissue ATase depletion may account for the additional toxicity of the O^6-BG combination regimens. However, the difference in normal tissue depletion is likely to be less significant with repeated administration over 5 days in combination with temozolomide. Since the drug also

inactivates ATase (Lee *et al.,* 1994), it is likely that no measurable activity remains in normal tissues with either inactivator.

Our results demonstrate that 4BTG and O^6-BG bring about a similar improvement in the efficacy of temozolomide or BCNU but that this is at the expense of greater toxicity with O^6-BG. If these observations are borne out in clinical practice, then, of the 2 ATase inactivators, 4BTG is the one most likely to enhance O^6-alkylating agent treatment.

REFERENCES

CHEN, J., ZHANG, Y., WANG, C., SUN, Y., FUJIMOTO, J. and IKENAGA, M., O^6-methylguanine-DNA methyltransferase activity in human tumors. *Carcinogenesis,* **13,** 1503–1507 (1992).

D'INCALCI, M., CITTI, L., TAVERNA, P. and CATAPANO, C.V., Importance of the DNA repair enzyme O^6-alkylguanine alkyltransferase (AT) in cancer chemotherapy. *Cancer Treat. Rev.,* **15,** 279–292 (1988).

DOLAN, M.E., MITCHELL, R.B., MUMMERT, C., MOSCHEL, R.C. and PEGG, A.E., Effect of O^6-benzylguanine analogues on sensitivity of human tumor cells to the cytotoxic effects of alkylating agents. *Cancer Res.,* **51,** 3367–3372 (1991).

DOLAN, M.E. and PEGG, A.E., O^6-benzylguanine and its role in chemotherapy. *Clin. Cancer Res.,* **3,** 837–847 (1997).

DOLAN, M.E., PEGG, A.E., MOSCHEL, R.C. and GRINDEY G.B., Effect of O^6-benzylguanine on the sensitivity of human colon tumor xenografts to 1,3-*bis*(2-chloroethyl)-1-nitrosourea (BCNU). *Biochem. Pharmacol.,* **46,** 285–290 (1993).

DOMORADZKI, J., PEGG, A.E., DOLAN, M.E., MAHER, V.M. and MCCORMICK, J.J., Correlation between O^6-methylguanine-DNA methyltransferase activity and resistance of human cells to the cytotoxic and mutagenic effect of *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine. *Carcinogenesis,* **5,** 1641–1647 (1984).

EGYHAZI, S., HANSSON, J. and RINGBORG, U., O^6-methylguanine-DNA methyltransferase activities in biopsies of human melanoma tumours. *Brit. J. Cancer,* **71,** 37–39 (1995).

FAIRBAIRN, L.J., WATSON, A.J., RAFFERTY, J.A., ELDER, R.H. and MARGISON, G.P., O^6-benzylguanine increases the sensitivity of human primary bone marrow cells to the cytotoxic effects of temozolomide. *Exp. Haematol.,* **23,** 112–116 (1995).

JELINEK, J., KLEIBL, K., DEXTER, T.M. and MARGISON, G.P., Transfection of murine multi-potent haemopoietic stem cells with an *E. coli* DNA alkyltransferase gene confers resistance to the toxic effects of alkylating agents. *Carcinogenesis,* **9,** 81–87 (1988).

LEE, S.M., MARGISON, G.P., WOODCOCK, A. and THATCHER, N., Sequential administration of varying doses of dacarbazine and fotemustine in advanced malignant melanoma. *Brit. J. Cancer,* **67,** 1356–1360 (1993).

LEE, S.M. and 12 OTHERS, Immunohistological examination of the inter- and intracellular distribution of O^6-alkylguanine-DNA alkyltransferase in human liver and melanoma. *Brit. J. Cancer,* **66,** 355–360 (1992).

LEE, S.M., THATCHER, N., CROWTHER, D.C. and MARGISON, G.P., Inactivation of O^6-alkylguanine-DNA alkyltransferase in human peripheral blood mononuclear cells by temozolomide. *Brit. J. Cancer,* **69,** 452–456 (1994).

MARGISON, G.P. and O'CONNOR, P.J., Biological consequences of reactions with DNA: role of specific lesions. *In:* C. Cooper and P. Grover (eds.), *Handbook of Experimental Pharmacology,* pp. 547–571, Springer-Verlag, Berlin (1990).

MCELHINNEY, R.S., DONNELLY, D.J., MCCORMICK, J.E., KELLY, J., WATSON, A.J., RAFFERTY, J.A., ELDER, R.H., MIDDLETON, M.R., WILLINGTON, M.A., MCMURRY, B.H. and MARGISON, G.P., Inactivation of O^6-alkylguanine-DNA alkyltransferase. Novel O^6-(hetarylmethyl)guanines having basic rings in the side-chain. *J. med. Chem.,* **41,** 5265–5271 (1998).

MICETICH, K.C., FUTSCHER, B.W., KOCH, D., FISHER, R.I. and ERICKSON, L.C., Phase I study of streptozocin- and carmustine-sequenced administration in patients with advanced cancer. *J. nat. Cancer Inst.,* **84,** 256–260 (1992).

MIDDLETON, M.R., GORE, M., TILGEN, W., FIERLBECK, G., GROB, J.J., THATCHER, N. and FRAASS, U., A randomized, phase III study of temozolomide (TMZ) versus dacarbazine (DTIC) in the treatment of patients with advanced, metastatic malignant melanoma. *Proc. Amer. Soc. clin. Oncol.,* **19,** (1999). Abstr. 2069.

PEGG, A.E., Mammalian O^6-alkylguanine-DNA alkyltransferase: regulation and importance in response to alkylating carcinogenic and therapeutic agents. *Cancer Res.,* **50,** 6119–6129 (1990).

SCHILSKY, R.L., DOLAN, M.E., VOGELZANG, N.J., MANI, S., PERTUCCI, D., COOPER, N., ROY, S. and RATAIN, M.J., Phase I clinical and pharmacologic study of O^6-benzylguanine (BG) plus carmustine (BCNU) in patients with advanced cancer. *Proc. Amer. Soc. clin. Oncol.,* **17,** 198a (1998).

SMITH, D., GERSON, S.L., LIU, L., DONELLY, S., DAY, R., TRUMP, D. and KIRKWOOD, J., Carmustine and streptozotocin in refractory melanoma: an attempt at modulation of O^6-alkylguanine-DNA alkyltransferase. *Clin. Cancer Res.,* **2,** 1129–1134 (1996).

WEDGE, S., PORTEOUS, J. and NEWLANDS, E.S., 3-Aminobenzamide and/or O^6-benzylguanine evaluated as an adjuvant to temozolomide or BCNU treatment in cell lines of variable mismatch repair status and O^6-alkylguanine-DNA alkyltransferase. *Brit. J. Cancer,* **74,** 1030–1036 (1996).

YAROSH, D.B., HURST-CALDERONE, S., BABICH, M.A. and DAY, R.S. III, Inactivation of O^6-methylguanine-DNA methyltransferase and sensitization of human tumour cells to killing by chloroethylnitrosourea by O^6-methylguanine as a free base. *Cancer Res.,* **46,** 1663–1668 (1986).