O6-(4-bromothenyl)guanine reverses temozolomide resistance in human breast tumour MCF-7 cells and xenografts

M Clemons1,2,4, J Kelly1, AJ Watson1, A Howell3, RS McElhinney3, TBH McMurry3 and GP Margison*,1

1Cancer Research UK Carcinogenesis Group, Paterson Institute for Cancer Research, Wilmslow Road, Manchester M20 9BX, UK; 2Cancer Research UK Department of Medical Oncology, Christie Hospital NHS Trust, Wilmslow Road, Manchester M20 4BX, UK; 3University Chemical Laboratory, Trinity College, University of Dublin, Dublin 2, Ireland

Tumour resistance to chemotherapy involving methylating agents such as DTIC (dacarbazine) and temozolomide is linked to expression of the DNA repair protein O6-alkylguanine-DNA alkyltransferase (MGMT). There is considerable interest in improving the efficacy of such O6-alkylating chemotherapy by the prior inactivation of MGMT. We have examined the effect of the modified guanine base, O6-(4-bromothenyl)guanine (PaTrin-2, Patrin™, Lomeguatrib) on MGMT activity and cell or xenograft tumour growth inhibition by temozolomide in the human breast carcinosarcoma cell line, MCF-7. PaTrin-2 effectively inactivated MGMT in MCF-7 cells (IC50 ~ 6 h) and in xenografts there was complete inactivation of MGMT within 2 h of dosing (20 mg kg−1 i.p.) and only slight recovery by 24 h. MGMT inactivation in a range of murine host tissues varied between complete and ~60%, with extensive recovery by 24 h. PaTrin-2 (10 μM) substantially increased the growth inhibitory effects of temozolomide in MCF-7 cells (D50 = 10 μM with PaTrin-2 vs 400 μM without). In MCF-7 xenografts, neither temozolomide (100 mg kg−1 day−1 for 5 days) nor PaTrin-2 (20 mg kg−1 day−1 for 5 days) had any significant effect on tumour growth. In contrast, the PaTrin-2–temozolomide combination produced a substantial tumour growth delay: median tumour quintupling time was increased by 22 days (P < 0.005) without any significant increase in toxicity as assessed from animal weight. A PaTrin-2–temozolomide combination may therefore be beneficial in the treatment of human breast cancers.

British Journal of Cancer (2005) 93, 1152–1156. doi:10.1038/sj.bjc.6602833 www.bjcancer.com

Keywords: MGMT; temozolomide; breast cancer; MCF-7; breast tumour xenografts

Standard first-line chemotherapy for advanced breast cancer results in disease regression in the majority of patients, but these responses are rarely complete or sustained (Clemons et al, 1997). There is, therefore, a continuing need to develop new treatments and one potential strategy is the biochemical modulation of tumour drug resistance.

The chemotherapeutic methylating (e.g. dimethyltriazenomida- zole-4-carboxamide (DTIC)) and chloroethylating (e.g. bischloroethylniminosourea (BCNU)) agents are well established in oncology and are a component of high-dose therapy regimens used in the treatment of breast cancer (Peters et al, 1994; Antman, 2001; Bengala et al, 2001). These agents are cytotoxic principally by virtue of their ability to alkylate DNA at the O6 position of guanine, and there is now considerable evidence that the DNA repair protein O6-alkylguanine-DNA alkyltransferase (MGMT) plays a key role in determining tumour resistance to these drugs (Margison and Santibáñez Koref, 2002; Gerson, 2002, 2004). MGMT repairs alkylation at the O6 position on guanine by accepting the alkyl group onto a cysteine residue in its active site in a stoichiometric and autoinactivating reaction. Cellular resistance in vitro and in vivo is correlated with elevated MGMT expression, and can be achieved in previously susceptible cell lines and organisms by transfer and expression of cDNAs or genes encoding MGMT. Furthermore, inactivation of MGMT, or gene deletion in mice, renders resistant cells and tissues sensitive to O6-alkylating agents (Pegg, 1990; Margison et al, 2002, 2003; Margison and Santibáñez Koref, 2002; Gerson, 2002, 2004).

Attempts to exploit this clinically have used methylating agents to deplete MGMT, via the formation of O6-methylguanine (O6-meG) in DNA, prior to the administration of a chloroethylating drug (e.g. Clemons et al, 2003). These have been hampered by the similar toxicities of the two agents, and no useful increase in therapeutic index has been demonstrated (Mietech et al, 1992; Lee et al, 1993; Smith et al, 1996; Hammond et al, 2004). Because of this, interest has turned to inactivation of MGMT using inherently nontoxic pseudosubstrates for the protein, such as O6-benzylguanine (O6-BeG; reviewed in Pegg et al, 1995; Dolan and Pegg, 1997) and O6-(4-bromothenyl)guanine (PaTrin-2, Lomeguatrib; McElhinney et al, 1998; Middleton et al, 2002; Middleton and Margison, 2003).

Temozolomide has recently been tested in breast cancer patients using a 5-day regimen and was found to be ineffective (NCIC, 2001). In order to establish if this resistance can be reversed in model systems, we have used the human breast adenocarcinoma cell line, MCF-7, that, like many of the breast tumours we have examined, expresses very high levels of MGMT. We show that
PaTrin-2 inactivates MGMT in cells and xenografts and that this results in substantial increases in their sensitivity to growth inhibition by temozolomide.

MATERIALS AND METHODS

Drugs
PaTrin-2 and temozolomide were provided by the Cancer Research Campaign Drug Formulation Unit, University of Strathclyde, Glasgow, UK. For cell culture studies, a stock solution of PaTrin-2 (20 mM) was prepared in dimethylsulfoxide (DMSO; Sigma, Poole, Dorset, UK) and diluted into culture medium just prior to use. For animal studies, PaTrin-2 was ground to a fine powder and suspended in corn oil at 4 mg ml⁻¹ immediately prior to intraperitoneal (i.p.) injection. Temozolomide was freshly prepared at 40 mg ml⁻¹ in 20% DMSO/80% phosphate-buffered saline (PBS) and diluted in 0.9% NaCl solution.

Cell culture studies
MCF-7 cells (a human breast adenocarcinoma cell line) were grown as a monolayer in RPMI medium containing 10% foetal bovine serum (Gibco BRL, Paisley, Scotland), at 37°C in a humidified atmosphere of 5% CO₂/95% air.

To determine MGMT inactivation, cells (5 x 10⁶) were incubated in the presence of increasing concentrations of PaTrin-2 at 37°C, 5% CO₂. After 2h, cells were pelleted and resuspended in 10 ml PBS. This was repeated three times in order to remove any residual PaTrin-2. Finally, cells were pelleted and assayed for MGMT activity as previously described (Watson and Margison, 2000). Activity remaining, based on at least three points on the linear part of the protein-dependence curve, was calculated as a percentage of the activity in untreated cells.

To determine toxicity, the MTT [3’-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] growth inhibition assay, based on the method of Carmichael et al (1987) was employed. Cells (1000 per well) were plated into a 96-well plate and followed a 24 h attachment period. PaTrin-2 was added to the cells. After 2 h incubation with PaTrin-2 (10 μM) at 37°C, 5% CO₂, increasing doses of temozolomide or vehicle were added and the cells were incubated for a further 4–5 days. At the end of the exposure period 150 μM MTT was added to each well and plates were incubated for 3 h at 37°C, 5% CO₂. The media were removed and the formazan crystals formed in the viable cells were solubilised in 200 μM DMSO. The absorbances at 540 and 690 nm were determined using a Titertek Multiscan ELISA plate reader and growth inhibition calculated as a percentage of the A₅₄₀/A₆₉₀ of untreated wells.

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. 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.

MCF-7 cells expressed high levels of MGMT (~1540 fmols mg⁻¹ total protein). Exposure to PaTrin-2 for 2h resulted in extensive inactivation of MGMT in MCF-7 cells: the concentration required to inactivate 50% of the MGMT was around 6 nM (Figure 1). The sensitivity of the MCF-7 cells to the growth inhibitory effects of temozolomide was substantially increased by PaTrin-2. Growth amounting to 60% of control was seen after 400 μM temozolomide alone but following preincubation with 10 μM PaTrin-2, 60% growth occurred at 10 μM temozolomide (Figure 2), indicating a 40-fold increase in sensitivity. PaTrin-2 itself had no growth inhibitory effect.

RESULTS

Effect of PaTrin-2 on MGMT activity and temozolomide sensitivity in MCF-7 cells

Extensive depletion of MGMT activity was seen in all host tissues measured after a single i.p. dose of 20 mg kg⁻¹ PaTrin-2 (Figure 3). Depletion to below the limits of detection occurred in the kidney, while it was to ~20, ~35 and ~40% of pretreatment values in
liver, lung and bone marrow, respectively. The nadir was generally between 2 and 8 h and substantial activity (to over 50% of pretreatment levels) had returned by 24 h after dosing. In the MCF-7 xenografts, complete MGMT inactivation was seen between 2 and 8 h, and recovery of levels was only to ~20% of pretreatment levels by 24 h after dosing. Slower recovery in the xenograft might reflect the relative strength of the human MGMT promoter or that the human protein is more extensively inactivated by PaTrin-2 or its putative metabolites.

Effect of PaTrin-2 and temozolomide on MCF-7 tumour growth

The median MCF-7 tqt in the vehicle control and PaTrin-2 only groups were ~21 and ~17 days, respectively. Neither temozolomide (tqt ~17 days) nor PaTrin-2 alone had any significant effect on xenograft growth. However, the combination of PaTrin-2 and temozolomide resulted in a median tumour quintupling time of ~43 days representing an increase of ~22 days (P<0.005; Figure 4, Table 1). Toxicity, as measured by weight loss, was essentially unaffected by the addition of PaTrin-2 to the temozolomide treatment regimen. At the end of the treatment period, weight loss was ~5% in both the temozolomide alone and combination groups (Figure 5, Table 1).

DISCUSSION

The O6-alkylating agent, temozolomide has been approved for the treatment of malignant glioma and is under consideration for use in melanoma, for which it is extensively prescribed, off license (e.g. Kiebert et al., 2003). It has also been tested in patients with metastatic breast cancer (NCIC, 2001) and prostate cancer (van Brussel et al., 2000) but inherent drug resistance has resulted in no clinical benefit. Several groups have measured MGMT expression in human breast tumours and report activity that is low (Cao et al., 2003). The O6-(4-bromothenyl)guanine that we have now synthesized is a potential MGMT inhibitor, which may reverse the resistance to temozolomide seen in patients with high-level MGMT expression. We have shown that PaTrin-2 inhibits MGMT activity in vitro and in vivo and is able to reverse resistance to temozolomide in the MCF-7 xenograft model.
samples are dramatically sensitised to the toxic effects of temozolomide by pre-exposure to $\text{O}^\alpha\text{-BeG}$ (Fairbairn et al, 1995; Clemons et al, 2000). So far, phase II clinical trials have yet to demonstrate that $\text{O}^\alpha\text{-BeG}$ provides an increase in therapeutic index, that is, that the improvement in efficacy outweighs any additional toxicity (Quinn et al, 2002).

PaTrin-2 is a more potent MGMT inactivator in vitro than $\text{O}^\alpha\text{-BeG}$, and we considered it worthwhile to examine the extent to which PaTrin-2 could inactivate MGMT and increase sensitivity to temozolomide in a human breast tumour model as a prerequisite for any potential clinical trial in breast cancer. Our results show that PaTrin-2 is also a potent inactivator of MGMT in MCF-7 cells both in culture and in xenografts in vivo. Tumour MGMT depletion by PaTrin-2 was as extensive as was reported with $\text{O}^\alpha\text{-BeG}$ in other tumour types (see Dolan and Pegg, 1997). We also showed that MGMT was inactivated in all host tissues with complete inactivation in kidney and extensive inactivation in other tissues. This collateral depletion again raises the concern about the potentiality of toxicity in healthy tissues following PaTrin-2/alkylating agent combinations.

The MGMT inactivation by PaTrin-2 in MCF-7 cells resulted in marked sensitisation to temozolomide growth inhibition. Following implantation into immune deficient mice, the resulting xenografts are completely resistant to growth inhibition by a 5-day treatment regimen using temozolomide alone. This was probably a result of the resistance conferred by high levels of expression of MGMT. PaTrin-2 alone had, as anticipated, no effect on tumour growth rates. However, PaTrin-2 overcame the resistance to temozolomide producing highly significant tumour growth delays, but without increasing toxicity as judged by animal weights. Thus, the therapeutic index of temozolomide is increased by PaTrin-2 in this animal model. We have previously shown that human melanoma xenografts expressing moderate levels of MGMT do respond to growth inhibition by temozolomide, but this is considerably enhanced by pretreatment with PaTrin-2 (Middleton et al, 2000, 2002). Current phase I studies are investigating the dose of PaTrin-2 that is necessary for complete inactivation of MGMT in patients with a variety of cancer types, prior to phase II studies. Given the results of the xenograft studies with melanoma, and now breast cancer, it seems reasonable to speculate that the greatest benefit from PaTrin-2-mediated inactivation of MGMT might be seen in tumours with the highest levels of MGMT expression and inherent resistance to temozolomide. This might best be assessed in a phase II clinical trial in breast cancer, particularly since temozolomide alone has been shown to be ineffective in the MCIC trial. However, given the variable levels of expression of MGMT in this tumour type, it would be beneficial to assess MGMT levels in tumour biopsies from all patients in such a study so that the hypothesis that MGMT inactivation will be beneficial can be effectively tested.

ACKNOWLEDGEMENTS

MC was supported by a Leukemia Research Clinical Fellowship. We thank Cancer Research-UK for support.

REFERENCES

Antman KH (2001) Randomized trials of high dose chemotherapy for breast cancer. Biochim Biophys Acta 1471: 89 – 98
Bengala C, Pazzagli I, Innocenti F, Donati S, Favre C, Menconi MC, Greco F, Dunesi R, Orlandini C, Guarneri V, Del Taccia M, Conte PF (2001) High-dose thiopeta and melphalan with hemopoietic progenitor support following induction therapy with epirubicin-paclitaxel-containing regimens in metastatic breast cancer (MBC). Ann Oncol 12: 69 – 74
Cao E-H, Fan XJ, Yuan XH, Xin SM, Liu YY, Yu HT (1991) Levels of $\text{O}^\alpha$-methylguanine acceptor protein in extracts of human breast tumour tissues. Cancer Biochem Biophys 121: 55 – 58
Carmichael J, De Giagg WG, Gazdav AF, Mina JD, Mitchell JB (1987) Evaluation of a tetrazolium-based semiautomated colorimetric assay; assessment of chemosensitivity testing. Cancer Res 47: 936 – 942
Chen JM, Zhang YP, Wang C, Sun Y, Fujimoto J, Ikenega M (1992) O6-methylguanine-DNA methyltransferase activity in human tumours. Carcinogenesis 13(9): 1503 – 1507

Chinnasamy N, Rafferty JA, Hickson I, Ashby J, Tinwell H, Margison GP, Dexter TM, Fairbairn LJ (1997) O6-benzylguanine potentiates the in vivo toxicity and clastogenicity of temozolomide and BCNU in mouse bone marrow. Blood 89: 1566 – 1573

Citrin M, Schoenhaus M, Rothenberg H, Kostroff K, Wasserman P, Kahn L, White A, Burns G, Held D, Yarosh D (1994) O6-methylguanine-DNA methyltransferase in normal and malignant-tissue of the breast. Cancer Invest 12: 605 – 610

Clemons MJ, Bibby MC, El Teraifi H, Forster G, Kelly J, Banerjee S, Cadman B, Ryder WD, Howell A, Margison GP (2002) Heterogeneity of O6-alkylguanine DNA-alkyltransferase expression in human breast tumours. Br J Cancer 86: 1797 – 1802

Clemons MJ, Leahy MG, Valle J, Jayson G, Ranson M, Hayes S, Howell A (1997) Review of recent trials for advanced breast cancer. Part I: Studies excluding taxanes. Eur J Cancer 33: 2171 – 2182

Clemons M, Watson A, Howell A, Chang J, Heyworth C, Lord B, Testa N, french J, Chabner B, Ryder WD, Howell A, Margison GP (2002) Heterogeneity of 6-alkylguanine-DNA alkyltransferase in human bone marrow granulocyte–macrophage colony-forming cells. Clin Cancer Res 6: 966 – 970

Dolan ME, Pegg AE (1997) O6-Benzyguanine and its role in chemotherapy. Clin Cancer Res 16: 837 – 847

Fairbairn L, Watson AJ, Rafferty J, Elder RH, Margison GP (1995) O6-benzylguanine increases the sensitivity of human primary bone marrow cells to the cytotoxic effects of temozolomide. Exp Haematol 23: 112 – 116

Friedman HS, Pluda J, Quinn JA, Ewesuedo RB, Long L, Friedman AH, Martin JN, Sampson J, Pegg AE, Moschel RC, McLeod RE, Provenzale JM, Stewart ES, Tourtoulis S, Garcia-Turner AM, Hendron II JE, Bigner DD, Dolan ME (2000) Phase I trial of carmustine plus O6-benzylguanine for patients with recurrent or progressive malignant glioma. J Clin Oncol 18: 3522 – 3528

Gerson SL (2002) Clinical relevance of MGMT in the treatment of cancer. J Clin Oncol 20: 2388 – 2399

Gerson SL (2004) MGMT: its role in carcinogenesis and therapeutic agents. Cancer Res 50: 6119 – 6129

Pegg AE, Dolan ME, Moschel RC (1995) Structure, function and inhibition of O6-alkylguanine-DNA alkyltransferase. Prog Nucleic Acid Res Mol Biol 51: 167 – 223

Peters WP, Ross M, Vredenburgh JJ, Hussein A, Rubin P, Dukelow K, Cavanaugh C, Beauros S, Kaspurz S (1994) The use of intensive clinical support to permit outpatient autologous bone marrow transplantation for breast cancer. Semin Oncol 21(4 Suppl 7): 25 – 31

Quinn JA, Pluda J, Dolan ME, Delaney S, Kaplan R, Rich JN, Friedman AH, Reardon DA, Sampson JH, Kolvin OM, Haglund MM, Pegg AE, Moschel RC, McLeod RE, Provenzale JM, Gururangan S, Tourtoulis S, Hendron II JE, Bigner DD, Friedman HS (2002) Phase II trial of carmustine plus O(6)-benzylguanine for patients with nitrosourea-resistant recurrent or progressive malignant glioma. J Clin Oncol 20: 2277 – 2283

Schilske RL, Dolan ME, Bertucci D, Ewesuedo RB, Vogelzang NJ, Mani S, Wilson LR, Ratain MJ (2000) Phase I clinical and pharmacological study of O6-benzylguanine followed by carmustine in patients with advanced cancer. Clin Cancer Res 6: 3025 – 3031

Smith DC, Gerson SL, Liu L, Donnelly S, Day R, Trump DL, Kirkwood JM (1996) Carmustine and streptozocin in refractory melanoma: an attempt at modulation of O6-alkylguanine-DNA alkyltransferase. Clin Cancer Res 2: 1129 – 1134

Spiro TP, Gerson SL, Liu L, Majia S, Haaga J, Hoppcl CL, Ingalls ST, Pluda JM, Wilson JK (1999) O6-benzylguanine: a clinical trial establishing the biochemical modulatory action in tumor tissue for alkyltransferase-directed DNA repair. Cancer Res 59: 2402 – 2410

van Brussel JP, Bussela MB, Lang MS, Cartsburg T, Schroder FH, Mickisch GH (2000) Phase II study of temozolomide in hormone-refractory prostate cancer. Cancer Chemother Pharmacol 45: 509 – 512

Watson AJ, Margison GP (2000) O6-alkylguanine-DNA alkyltransferase assay. Methods Mol Biol 152: 49 – 61

Wedge SR, Porteous JK, Newlands ES (1997) Effect of single and multiple administration of an O6-benzylguanine/temozolomide combination: an evaluation in a human melanoma xenograft model. Cancer Chemother Pharmacol 40: 266 – 272