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Short report

In vitro evaluation of temozolomide combined with X-irradiation

Stephen R Wedge, Julia K Porteous, Mark G Glaser, Kim Marcus and Edward S Newlands

Department of Medical Oncology, Charing Cross Hospital, Fulham Palace Road, London W6 8RF, UK.
Tel: (+44) 181 846-1410; Fax: (+44) 181 846-1443.

The *in vitro* cytotoxicity of 8-carbamoyl-3-methylimidazo[5,1-*d*]-1,2,3,5-tetrazine-4(3*H*)-one (temozolomide) with concurrent X-irradiation was examined in a human glioblastoma cell line (U373MG) as a potential radio-chemotherapeutic treatment for malignant glioma. The combination was also examined in a human colorectal adenocarcinoma (Mawi) which had 100-fold greater O^6-alkylguanine-DNA alkyltransferase (AGT) activity, a DNA-repair protein which confers resistance to temozolomide. A comparison of IC_{50} values indicated U373MG to be over 32-fold more sensitive to temozolomide than Mawi, but slightly more resistant to X-irradiation ($p < 0.035$; unpaired two-tailed *t*-test). Temozolomide and X-irradiation proved largely additive in U373MG by isobologram analysis (50% iso-effect) and the addition of 10 μM temozolomide to 1–2 Gy of X-irradiation increased cell kill by 2.5- to 3.0-fold. However, the combination was antagonistic in Mawi: an effect attributed to AGT induction by X-irradiation as the antagonism was removed by co-incubation with the AGT inhibitor O^6-benzylguanine (O^6-BG 1 μM; 24 h). O^6-BG did not affect the radiation dose-response curve, but significantly increased temozolomide cytotoxicity ($p < 0.015$). In conclusion, the combination of temozolomide with radiation is at best additive, but could nonetheless be of considerable therapeutic benefit in glioma, particularly if administered for prolonged periods. If AGT induction compromises the efficacy of this therapy, it may be circumvented with an appropriate inhibitor such as O^6-BG.

Key words: Cytotoxicity, glioma, isobologram, O^6-benzylguanine, radiotherapy, temozolomide.

Introduction

Patients with high-grade glioma have a particularly dismal prognosis. Current treatment of this disease involves surgical reduction of the tumor burden, followed by radiotherapy which is at best palliative.[1] Adjuvant chemotherapy is clearly warranted but has proven largely disappointing; the most widely investigated combination involving i.v. administration of 1,3-bis(2-chloroethyl)-nitrosourea (carmustine, BCNU),[2] which is limited by dose-related renal and pulmonary fibrosis.

8-Carbamoyl-3-methylimidazo[5,1-*d*]-1,2,3,5-tetrazine-4(3*H*)-one (temozolomide) is a methylating imidazotetrazinone which is clinically well tolerated, readily bioavailable and has demonstrated promising clinical activity in the treatment of malignant glioma during phase I/II evaluation.[3,4] The antitumor activity of this compound is attributed to the methylation of O^6-guanine in DNA,[5] which is inhibitory to replication when processed by a DNA-mismatch repair pathway.[6,7] Temozolomide exhibits schedule-dependent activity, with repeat dosing yielding the greatest therapeutic effect.[3,8] To capitalize on this phenomenon, a recent phase I study has examined continuous daily administration of the drug for up to 6 or 7 weeks (75–100 mg/m^2/day, p.o., one or two courses)[9] and found an objective response to be produced in 41% of 15 patients with glioma (in preparation). This extended dosing regimen is ideally suited to combination with conventional loco-regional radiotherapy for glioblastoma, which involves daily irradiation with 2 Gy or less, up to a cumulative dose of approximately 60 Gy.[10] An examination of this combination is therefore warranted, given that concurrent administration of two DNA-damaging modalities may potentially prove efficacious.

The primary purpose of this study was to evaluate the *in vitro* cytotoxicity of temozolomide combined with X-irradiation in a human glioblastoma cell line (U373MG). However, clinical responses to temozolomide are also likely to depend upon the activity of

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Correspondence to ES Newlands

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O^6-alkylguanine–DNA alkyltransferase (AGT), a DNA-repair protein which removes O^6-alkylguanine adducts in DNA.[11] This protein is irreversibly inactivated upon adduct removal and so AGT depletion with a pseudosubstrate such as O^6-benzylguanine (O^6-BG) may increase the efficacy of temozolomide treatment.[12,13] The effect of combining O^6-BG with temozolomide and X-irradiation was therefore also examined in a high AGT expressing colorectal carcinoma cell line, as U373MG has little AGT activity.[12]

Materials and methods

Chemicals, drugs and radiation source

Temozolomide was supplied by Dr J Catino (Schering Plough Research Institute, Kenilworth, NJ). O^6-BG was a generous gift from Dr RC Moschel (NCI–Frederick Cancer Research and Development Center, Frederick, MD). All other chemicals were purchased from Sigma (Poole, UK). X-irradiation was provided by a linear accelerator with an output of 318 cGy/min, in the Radiotherapy Department, Charing Cross Hospital.

Cell culture

Two human cell lines were used in this study: U373MG, a glioblastoma astrocytoma obtained from the European Tissue Culture Collection (Porton Down, UK), and Mawi, a colorectal adenocarcinoma established at Charing Cross Hospital.[14] Both cell lines were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM) (ICN Biomedicals, High Wycombe, UK), supplemented with 10% heat-inactivated fetal calf serum (Gibco, Paisley, UK), L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml). Cultures were maintained in exponential growth at 37°C in a humidified 5% CO_2 incubator.

Cytotoxicity assay

Experiments were performed in 96-well microtiter plates, with six wells per plate being used for each drug concentration, or the relevant control. Cytotoxicity was evaluated using the SRB assay for protein,[15] using a pre-determined optimal plating density which enabled logarithmic cell growth for 8 days.[12] Cells were plated and allowed to grow for 24 h before treatment. On day 1 media was removed from all cells and replaced with 0.66% dimethylsulfoxide (DMSO) in DMEM with/without temozolomide (1–1000 μM) for 3 h. After 1 h of drug exposure, plates were briefly removed from the incubator and exposed to X-irradiation under aerobic conditions. Following drug incubation, medium was replenished with fresh drug-free medium and plates reincubated for 7 days before assay. In experiments involving O^6-BG treatment, 1 μM O^6-BG was applied 1 h prior to, during and 20 h following incubation with temozolomide (a treatment known to produce significant AGT inactivation[12]). Relative absorbances were determined using an SLT 340 ATTC plate reader (SLT Instruments, Salzburg, Austria) and Biolise software (Labtech International, East Sussex, UK). IC_{50} values for temozolomide ± X-irradiation were interpolated by cubic spline regression, while values for X-irradiation alone were interpolated by polynomial regression (GraphPad, Prism; GraphPad Software, San Diego, CA). Differences between IC_{50} values were analyzed for statistical significance using a paired two-tailed Student's *t*-test.

Analysis of drug interaction

The interaction between temozolomide and X-irradiation was assessed by the construction of IC_{50} isoeffect curves from the dose–response data, at 5% increments between 100 and 50% cell survival, according to the methods described by Steel and Peckham[16] and Okano *et al.*[17] Briefly, the two outermost lines of the mode I, mode IIa and mode IIb analysis describe an 'additivity envelope', and the isoeffect produced by a combination of therapies is considered synergistic (supra-additive) if displaced to the left of the envelope, additive if within the envelope and antagonistic (sub-additive) if to the right of the envelope.

Results

The two tumor cell lines displayed significantly different sensitivities to temozolomide (Figure 1a): IC_{50} values for a 3 h drug incubation being (mean ± SE) 22 ± 1.5 μM in U373MG and 718 ± 21 μM in Mawi. This effect is undoubtedly related to the relative expression of AGT in these cell lines, as Mawi is known to have an approximately 100-fold greater AGT activity than U373MG.[12] In contrast, the sensitivity of these cell lines to X-irradiation was similar (Figure 1b), but with U373MG being slightly

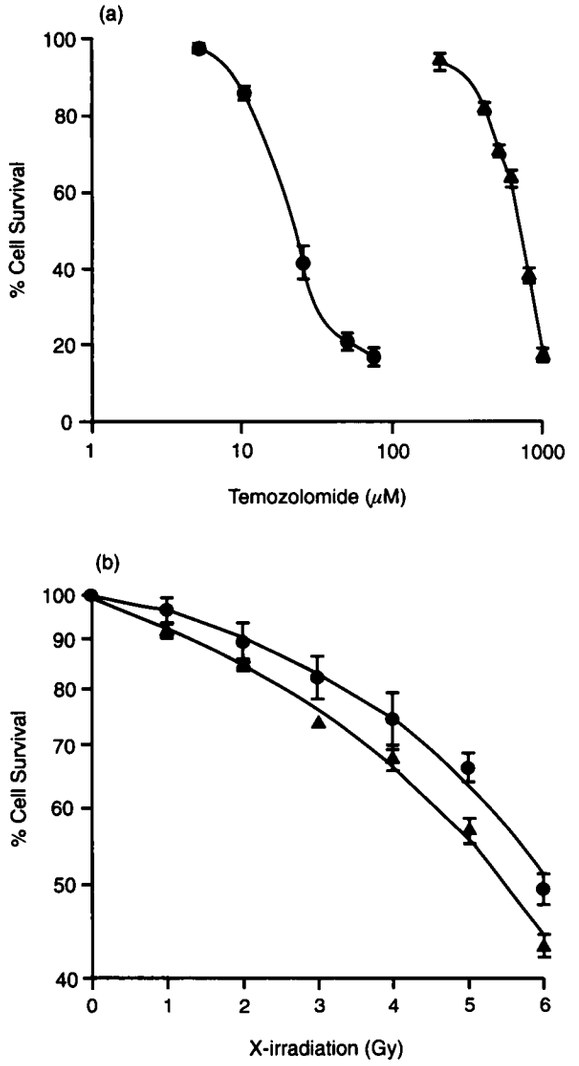

Figure 1. Dose–response curves in U373MG (●) and Mawi (▲) following treatment with (a) temozolomide (3 h exposure) and (b) X-irradiation. Each point represents the mean ± SE as a percentage of control cell growth, from three independent experiments.

more radioresistant ($p < 0.035$; IC_{50} values of 5.98 ± 0.2 and 5.48 ± 0.1 Gy in U373MG and Mawi respectively).

Three independent experiments were performed with each combination. A representative isobologram is shown for illustrative purposes, as the net interaction remained consistent but the exact shape of a given additivity envelope was variable. Isobolograms constructed from IC_{50} data in U373MG indicated that combinations of temozolomide and X-irradiation producing an IC_{50} were largely additive (Figure 2), there being the possibility of a slight

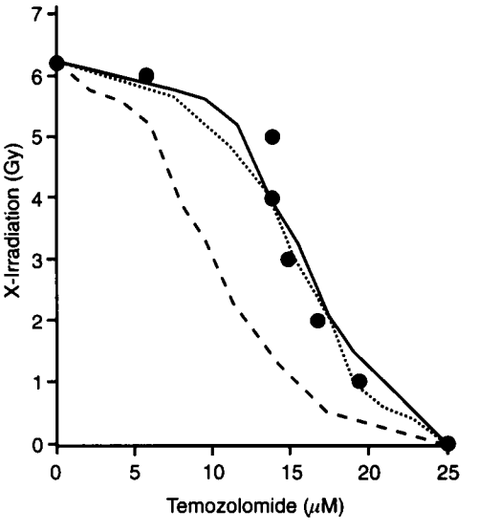

Figure 2. Sample IC_{50} isobologram constructed according to Okano *et al.*[17] from combinations of temozolomide and X-irradiation in U373MG, showing mode I (——), mode IIa (– – –) and mode IIb (·····) iso-effect lines. The majority of combinations producing a 50% inhibition of cell growth are within the envelope of additivity and denote a largely additive interaction.

antagonistic response above 4 Gy. In this cell line, the addition of 5 μM temozolomide to 1 or 2 Gy X-irradiation almost doubled the growth inhibitory effect produced by radiation alone and a combination with 10 μM temozolomide increased cytotoxicity by 2.5- to 3.0-fold (Figure 3). In contrast to these results, combinations of temozolomide and X-irradiation were found to be antagonistic in Mawi (Figure 4a). However, the addition of O^6-BG to the combination resulted in an additive response, indicating that the antagonism was AGT mediated (Figure 4b). O^6-BG had no effect on the radiation survival curve (an identical IC_{50} value of 5.48 ± 0.1 Gy was obtained), but significantly increased the cytotoxicity of temozolomide, reducing the IC_{50} value to 443 ± 18 μM ($p < 0.015$).

Discussion

This study indicates that a combination of temozolomide with X-irradiation in a human glioma cell line is at best additive. Nonetheless, the enhancement of cell kill observed in U373MG suggests that the combination may offer a significant therapeutic advantage. Clinical efficacy will be dependent upon both local control of the tumor and the eradication of disease outside the field of radiation. The addition

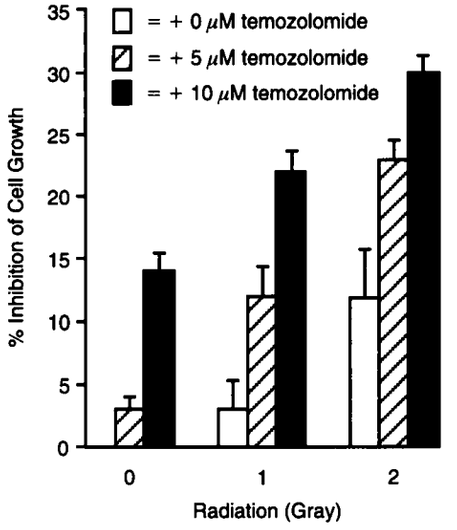

Figure 3. Bar graph showing increase inhibition of U373MG cell growth when temozolomide (5 and 10 μM) is combined with 1 or 2 Gy X-irradiation. Each bar represents the mean + SE of three independent experiments.

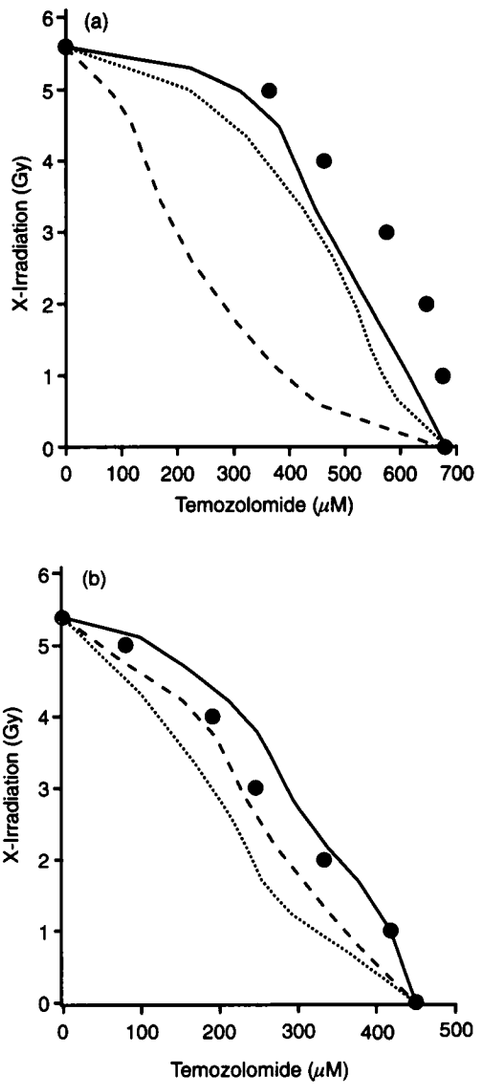

Figure 4. Sample IC_{50} isobolograms (as in Figure 2) from combinations of temozolomide and X-irradiation in Mawi. (a) Combinations of temozolomide with X-irradiation which produced a 50% inhibition of cell growth are to the right of the envelope of additivity and denote an antagonistic interaction. (b) The addition of O^6-BG (1 μM; 1 h pre-, 3 h during and 21 h post-drug incubation) to temozolomide and radiation removes the antagonism and results in an additive response.

of temozolomide to a course of loco-regional irradiation could therefore benefit either process, by providing additional cytotoxic insult to the primary tumor and collateral treatment of life-threatening disseminated disease. Previous attempts to exploit this phenomenon, termed 'spatial co-operation',[18] have led to a moderate therapeutic gain in the treatment of breast and small cell lung cancer.[19,20]

U373MG was particularly sensitive to temozolomide and at concentrations which are clinically achievable. The plasma area under the curve (AUC) produced by oral administration of 75 mg/m^2 temozolomide (0.83 mg/ml·min), which can be given daily for 7 continuous weeks (in preparation), is in excess of the concentration × time ($C \times T$) value required to produce a 50% inhibition of cell growth *in vitro* (0.78 mg/ml·min). This marked sensitivity is likely to be a consequence of low AGT activity, which is evident in many malignant brain tumor cell lines.[21] However, an increase in AGT activity has been shown to accompany tumorigenesis of the brain *in vivo*, suggesting that greater intrinsic resistance to the combination may be encountered in the clinic.[22]

The antagonism observed between temozolomide and radiation in Mawi can be attributed to the induction of AGT following X-irradiation: an effect which is well documented[23–25] and can reduce the antitumor activity of DNA-alkylating chemotherapy.[26,27] A correlation between radiation and AGT may appear surprising, given that the many DNA lesions produced by X-irradiation[28] are not repairable by AGT and that AGT status (and treatment with O^6-BG) has no effect upon radiosensitivity.[29] However, the phenomenon is dependent upon wild-type p53 gene expression, which is thought to up-regulate of a number of cellular DNA-repair activities in response to DNA strand breakage.[25] This effect

may therefore be attenuated or absent in many brain tumors, since although inactivation of the p53 gene is not an obligatory step in glioblastoma genesis,[30] chromosome 17p deletions and p53 gene mutations are frequently found in gliomas of all grades of malignancy.[31] In addition, it is plausible that the loss of chromosome 10 heterozygosity which frequently accompanies the development of high-grade glioma could further constrain AGT regeneration, the gene for AGT being located at 10q26[32] and there being a common deletion region in 10q25.[33] It is encouraging that a number of glioma patients who have received a course of radiotherapy, have subsequently responded to temozolomide treatment.[34]

In conclusion, temozolomide may be a useful adjunct to the radiotherapeutic treatment of malignant glioma, particularly if the combination is administered for a prolonged period. It is uncertain as to whether AGT induction with repeated irradiation will severely reduce the activity of the combination, although it may be possible to circumvent such an effect with an appropriate AGT inhibitor (e.g. O^6-BG). We plan to perform a pilot study, combining 7 weeks of radiotherapy (1.75 Gy/day) with concurrent temozolomide (75 mg/m^2/day) in patients with high-grade glioma, using serial registration magnetic resonance imaging and 2-[^{18}F]fluoro-2-deoxy-D-glucose positron emission tomography to assess tumor response and potential toxicity to normal tissues.

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