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Research paper

Sensitivity of short-term cultures derived from human malignant glioma to the anti-cancer drug temozolomide

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The activity of temozolomide, which has shown clinical activity against malignant glioma, has been assessed *in vitro* against short-term cultures derived from these tumors using an intermediate duration microtitration assay with MTT reduction as the end-point This assay has previously been shown to correlate closely with a monolayer clonogenic assay. Sensitivity was assessed in 15 short-term cultures (passage levels 3–9) derived from WHO grade III and IV astrocytomas. These cultures had a median ID_{50} value of 258 μM for temozolomide and 16.13 μM for CCNU. Maximum serum concentrations of temozolomide are of the order of 75 μM but only three of 15 (20%) cultures had ID_{50}s below this value. Fourteen of 15 (93%) cultures displayed cross-resistance between temozolomide and CCNU, although one line which was extremely resistant to CCNU retained sensitivity to temozolomide. Comparative studies of published clonogenic survival curves indicate that the short-term glioma cell lines used in this study have similar sensitivities to established glioma cell lines, whilst colon carcinoma cell lines and bladder carcinoma are often more resistant to these drugs. Cell lines from testicular teratoma cell lines may show exquisite sensitivity to temozolomide and this level of sensitivity is seen only occasionally in short-term cultures derived from malignant glioma. [© **1999 Lippincott Williams & Wilkins.**]

Key words: CCNU, chemosensitivity, glioma, O^6-alkylguanine-DNA alkyltransferase, temozolomide.

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Introduction

Patients with high-grade astrocytomas have a poor prognosis with less than 20% expected to survive for more than 2 years after diagnosis. Surgery and radiotherapy play an established role in the treatment of these tumors and chemotherapy has been shown to be of benefit for up to 50% of patients.[1,2] At present, chemotherapy is limited to a small number of drugs which can be used either individually or in combination. The nitrosoureas are the most effective of these drugs, with BCNU and CCNU producing radiological responses in between 30 and 50% of patients at either diagnosis or relapse.[3] However, response is often short lived and neutropenia is usually dose limiting. There is a clear need for the development of new, relatively non-toxic drugs with activity against CNS tumors.

Temozolomide is a small molecular weight (M_r 194) imidazotetrazine derivative which passes the blood-brain barrier in mice and presumably humans by passive diffusion,[4] and has been shown to have activity against xenografted human gliomas[5,6] and clinically in single center clinical trials.[4,7,8] Temozolomide at a concentration of less than 1000 mg/m^2 has been shown to produce minimal myelosuppresion and side effects, with little accumulative toxicity. A recent multicenter phase II trial organized by the Cancer Research Campaign in the UK examined the efficacy of temozolomide at doses of 150–200 mg/m^2/day orally for 5 days repeated every 4 weeks in patients with recurrent malignant glioma.[9] Of the 103 evaluable patients, 11 (11%) achieved an objective radiological response and a further 48 (47%) had stable disease, and the median time to progression for patients achieving stable disease was 4.2 months.

As short-term cultures derived from malignant glioma have been shown to retain both glial-like and malignancy-related characteristics *in vitro*,[10,11,12] these are useful model systems for examining drug sensitivity either on an individual basis[13,14] or for screening drugs for activity against malignant glioma.[14] It has been suggested that panels of these cultures might be used for screening drugs in the manner of an *in vitro* phase II trial.

The aim of this study therefore was to examine the spectrum of sensitivity of a panel of short-term cell cultures derived from malignant glioma to temozolomide at doses which could be achieved clinically and to compare this with sensitivity to CCNU, a drug used clinically in the treatment of malignant glioma and resistance to which is known to be modulated by O^6-alkylguanine-DNA alkyltransferase (ATase) levels.

Materials and methods

Cell culture

Short-term cell lines were derived from 14 WHO grade III or IV adult astrocytomas and one grade III oligodendroglioma as previously described.[11,12] Briefly, the specimens were prepared by mechanical mincing, with crossed-scalpel blades and digested in Sigma (grade IA) for 1 h at 37°C, before being plated in a 25 cm^2 culture flask overnight to allow the living cells to attach. The cultures were routinely fed with Hams F-10 medium buffered with 20 mM HEPES and supplemented with 10% selected fetal calf serum (complete growth medium). Antibiotics were not used during routine cell culture or in chemosensitivity assays. All cultures were routinely screened (and found negative) for mycoplasma infection using Hoechst 33258 staining as previously described.[12] Cell counts were routinely carried out using a ZM Coulter Counter calibrated for use with human glioma cells. Cell cultures between passage levels 4 and 12 were used for chemosensitivity experiments.

Chemosensitivity assay

Chemosensitivity was assessed using the MTT [3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-tetrazolium bromide] reduction assay as described earlier,[15,16] which involves the mitochondrial reduction of the soluble tetrazolium salt into the insoluble formazan product. Cells were trypsinized from culture flasks and plated onto 96-well microtitration plates at a concentration of 1500 cells/well. These were incubated at 37°C for between 24 and 72 h to allow the cells to reach exponential growth, and the medium replaced with 100 μl of drug solution. Temozolomide was dissolved in dimethylsulfoxide (DMSO) at a concentration of 5 mM and further diluted in complete growth medium to produce a concentration range from 3.1 to 1000 μM. CCNU was dissolved in ethanol at a concentration of 17.2 mM and further diluted in complete growth medium to produce a concentration range of 1.4–430 μM. Previous experiments had shown that the concentration of DMSO (0.5% v/v) and ethanol (0.1% v/v) present in the highest drug concentrations produced no cytotoxicity (data not shown). All stock solutions were stored at −20°C and drug dilutions were prepared freshly for each experiment. Drug exposure was for three consecutive days, with daily renewal of the drug. After a total drug exposure time of 72 h, wells were rinsed with Hanks balanced salt solution and the cells were allowed a recovery period of 72 h in 100 μl of fresh growth medium per well. On the final day, all media was aspirated and replaced with 100 μl MTT (Sigma, St Louis, MO) solution at a concentration of 1 mg/ml in Hams F-10. Cells were incubated for 4 h at 37°C to allow formation of the formazan product. The supernatant was gently removed and 100 μl of DMSO added to each of the wells. Plates were then gently agitated on a gyratory shaker to solubulize the formazan crystals and the optical density (OD) was determined, at a wavelength of 570 nm, *in situ* using a Dynatech MR600 microtitration plate reader. The ID_{50} (dose of drug which inhibits formazan product formation by 50%) was determined for each cell line. To ensure that the cells remained in exponential growth phase throughout the assay, a separate plate was prepared for each cell line. This was re-fed each day with fresh medium and six wells were trypsinized each day, and the contents pooled and counted.

Results

There was considerable heterogeneity in the responses of cultures to both temozolomide and CCNU (Figure 1 and Table 1). The range of sensitivities for temozolomide was approximately 30-fold, as compared to approximately 10-fold for CCNU. For temozolomide, the median ID_{50} was 257.7 μM (mean 14.6 ± 2.3 SEM) and ID_{50}s ranged from 22.7 μM for IN949, the most sensitive cell culture, to temozolomide, to 541.1 μM for IN336, the most resistant cell culture. For CCNU, the median ID_{50} was 16.1 μM (mean 245.8 ± 40.5 SEM) and ID_{50}s ranged from 2.8 μM for IN35, the most sensitive cell culture, to

CCNU, to 30.84 μM for IN336, the most resistant cell culture. There did not appear to be a relationship between sensitivity to either temozolomide or CCNU and culture doubling time (data not shown) or with histological grade within the astrocytomas (Table 1). The culture derived from the anaplastic oligodendroglioma appeared to be moderately resistant to both CCNU and temozolomide (Table 1). For 14 of 15 cultures, there was marked cross-resistance between the cell lines (Figure 2). In general, cell cultures resistant to temozolomide were also resistant to CCNU. For example, IN336 proved to be the most resistant cell culture to both drugs, whilst IN35 was the most sensitive cell culture towards CCNU and the second most sensitive culture to temozolomide. However, there was one exception, cell line IN949, which displayed sensitivity towards temozolomide, but resistance to CCNU. Regression analysis of the ID_{50}s for all 15 cell lines produced a Spearman correlation coefficient of 0.57 (p=0.028). Re-analysis, excluding IN949, produced a correlation coefficient of 0.82 (p=0.0004).

Discussion

Temozolomide is thought to have considerable potential as a new drug in the treatment melanoma, mycosis fungoides and, in particular, the treatment of brain tumors.[4] The drug has excellent bioavailability when

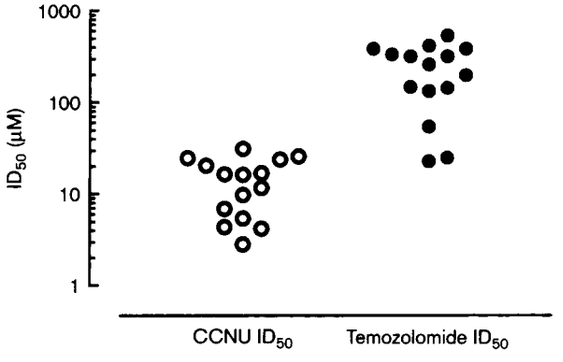

Figure 1. Sensitivity of short-term cultures derived from malignant glioma to CCNU (●) and temozolomide (○). Each symbol represents the ID_{50} (dose of drug which inhibits formazan production by 50%) for a single short-term culture.

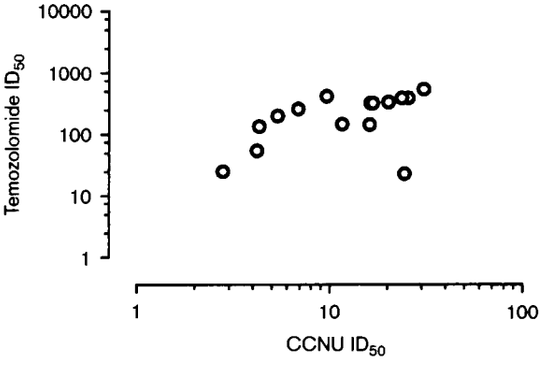

Figure 2. Pattern of cross-resistance between temozolomide and CCNU in short-term cultures derived from malignant glioma.

Table 1. Cell cultures used in the study and their *in vitro* sensitivity

Cell line	Tumor type	CCNU ID_{50} (μM)	Temozolomide ID_{50} (μM)
IN1078	glioblastoma multiforme	4.30	134.00
IN1257	grade III astrocytoma	25.59	386.60
IN336	glioblastoma multiforme	30.84	541.24
IN1752	glioblastoma multiforme	9.67	414.00
IN949	glioblastoma multiforme	24.51	22.68
IN1077	glioblastoma multiforme	16.34	319.58
IN1056	glioblastoma multiforme	16.75	319.58
IN46	glioblastoma multiforme	4.19	54.12
IN1240	grade III oligodendroglioma	16.13	144.32
IN34	glioblastoma multiforme	11.61	146.91
IN97	glioblastoma multiforme	20.22	335.60
IN1303	glioblastoma multiforme	23.66	386.60
IN35	glioblastoma multiforme	2.80	25.00
IN1125	grade III astrocytoma	6.88	257.73
IN2340	grade III astrocytoma	5.38	198.45

administered either orally or i.v., and phase I clinical trials have demonstrated minimal and predictable systemic toxicity at effective doses.[7] In BALB/c mice implanted with PC66 plasmocytoma, temozolomide appears to be widely distributed in tissues, including the brain, although levels here were consistently lower than plasma levels by about 4-fold and lower than liver or lung by about 2-fold. The results of the present study indicate, however, that CCNU is considerably more toxic at equivalent molar concentrations than temozolomide to glioma cells by a factor of approximately 20-fold. Similar results have been reported in two established glioma cells lines, U87 and U373, where a 6- and 9-fold greater sensitivity to CCNU was observed[17] and in colorectal carcinoma cell lines which were approximately 30-fold more sensitive to nitrosoureas than temozolomide at equivalent molar concentrations.[18]

In *in vitro* studies it is important to be sure that the doses of drug which are tested *in vitro* are comparable to those which can be achieved clinically. In most published clinical trials, temozolomide is given either i.v. at dose levels between 50 and 200 mg/m^2 or orally at dose levels upto 1200 mg/m^2, over a period of 5 days. With either route of administration temozolomide plasma levels reached a peak of approximately 60 μM, 2 h after administration, then sharply declined to base levels within 12 h.[7] Although there is no data about the levels which are attained in human brain tumors following treatment with therapeutic doses of temozolomide, drug levels have been determined in the interstitial fluid of experimental tumors in rats bearing the C_6 glioma following a dose of 40 mg/kg, a dose which is rather higher than has been routinely administered in clinical trials. This produced concentrations of around 80 μM which were maintained for upto 2 h after administration.[8] From the data in the present study it is clear that only a small proportion of short-term cultures derived from malignant glioma have ID_{50}s which are below the levels of temozolomide attainable clinically. Only three of 15 cultures (20%) have ID_{50}s below 60 μM, which suggests that only a small proportion of patients treated with this drug are likely to achieve a clinical response. The response rate *in vitro* to temozolomide appears to be consistent with the objective response rate of 11% using radiological criteria. However, the criteria for determining radiological response in patients with recurrent malignant glioma are somewhat controversial and it has been proposed that the progression-free survival of patients at 6 months be employed as an alternative end-point in phase II trials of patients with malignant glioma.[9] Using this criteria 22% of patients in the phase II trial responded to temozolomide. Of course it is possible that there is some degree of accumulation of temozolomide within the glioma tissue, but data from studies of temozolomide pharmacokinetics using experimental brain tumor models[19] and preliminary results from PET studies of glioma patients administered [^{11}C]temozolomide[20] would seem to indicate that although temozolomide is retained for some hours within the tumor, there is little evidence of long-term accumulation.

The levels of CCNU which can be attained within glioma tissue have been suggested to be only of the order of 22 μM.[21] However, even at these levels, around 70% (11 of 15) of cultures had ID_{50}s below this concentration. The objective response rate of patients with malignant glioma treated with single-agent nitrosourea at recurrence are higher than those seen for temozolomide with 30–50% of patients with glioblastoma and 40–60% of patients with anaplastic astrocytomas responding, and the mean time to progression for such patients treated with BCNU is 22 weeks.[3]

There is plainly marked cross-resistance between temozolomide and CCNU (Figure 2). Whilst it is well established that the DNA adduct at the O^6-methyl position of guanine produced by CCNU which ultimately yields interstrand cross-links can be removed by ATase, with levels of this enzyme correlating both *in vitro*[22,23] and *in situ*[24] with resistance to chloroethylnitrosoureas. Temozolomide degrades at physiological pH to 5-(3-methyl-triazeno) imidazole-4-carboxamide (MTIC) and ultimately to a reactive methyldiazonium species which methylates DNA. The adduct most frequently produced by temozolomide appears to be N^7-guanine, which accounts for more than 70% of total DNA methylation with a further 10% of total DNA methylation at N^3-adenine. Temozolomide does methylate the O^6-position of guanine, but this lesion constitutes only about 5% of total DNA methylation. Clearly, this minority lesion must contribute disproportionately to cellular sensitivity to temozolomide. There is also considerable evidence from established cell lines from glioma and from other types of cancer that there is a marked degree of cross-resistance between the chloroethylnitrosoureas and temozolomide (see Figure 3 and Baer *et al.*[17]) and that levels of ATase correlates with temozolomide resistance in these cell systems. It has also been shown that it is possible to sensitize cells to temozolomide by using approaches designed to reduce the cellular levels of ATase.[17,25]

Deficiencies in the mismatch repair (MMR) system produce resistance to temozolomide, but not to nitrosoureas,[26] and it has been suggested MMR mutations confer resistance to temozolomide which

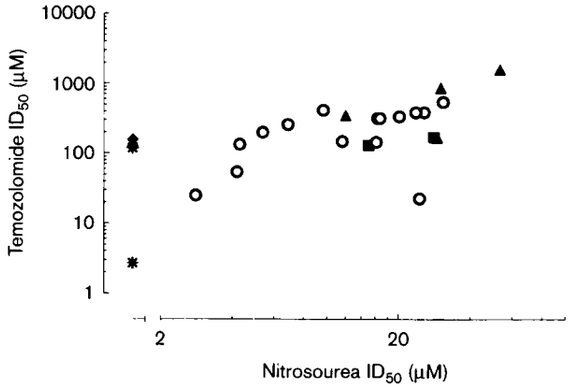

Figure 3. Cross-resistance patterns between temozolomide and CCNU in cultures derived from tumors of a variety of different histological types. (○) Data from present study. (■) Established glioma cell lines.[17] (▲) Colon cancer cell lines.[18] (◆) Established bladder cell lines.[31] (✳) Established testicular teratoma cell lines.[31]

is independent of the ATase activity within the cells.[18] Although there is no data on the MMR status of these short-term lines derived from glioma, microsatellite instability is a feature of some high-grade astrocytomas[27] and alterations in the expression of MMR genes seem to be quite frequent events in gliomas.[28] Clearly the role of MMR in modulating resistance to methylating agents requires further elucidation in malignant glioma. It is interesting that in the present study, there are no cultures which display marked resistance to temozolomide in the absence of resistance to CCNU, an observation consistent with the hypothesis that ATase levels alone modulate temozolomide sensitivity in unselected short-term cultures from malignant glioma. One the other hand, one cell line, IN949, displays significant sensitivity to temozolomide but marked resistance to CCNU. It is tempting to suggest that this cell line cannot have high levels of ATase and that its resistance to CCNU is mediated by some other mechanism. This line is also likely to be of importance in studies which are aimed at the investigation of the mechanisms of nitrosourea cytotoxicity and how this might be modulated. In a separate panel of short-term gliomas cell cultures we have shown that a small proportion of these cultures are resistant to CCNU but have no detectable ATase activity.[23] It is probable that these lines are similar to IN949. There have also been reports that nitrosourea resistance is not modulated by ATase levels in medulloblastoma cell lines.[29]

The microtitration assay used in this study has been shown to correlate closely with monolayer clonogenic assays.[30] Because of this close correlation it is possible to directly compare the results of the present study with published data from clonogenic assays carried out with other types of cancer. This will provide information on the relative sensitivity of malignant glioma against other types of neoplasm. The published ID_{50} values were taken from the literature from two studies, one which used two established cell lines derived from malignant gliomas[17] and one which used colon carcinoma cell lines.[18] Both these studies also reported the sensitivity of their cell lines to either CCNU or BCNU. Data was also available from a further study which compared the sensitivity of cell lines derived from testicular teratoma and bladder cancer to temozolomide, although this study did not examine the sensitivity of these lines to chloroethyl nitrosoureas.[31] Only samples that had been derived from pre-therapy tumor samples were included. No cell lines which were derived from post-treatment samples or which have been artificially made resistant to cytotoxic drugs were included in this review. The data from this correlative study is presented in Figure 3. The sensitivity of established cell lines from malignant glioma are very similar to the short-term lines in the present study to both CCNU and temozolomide. Colon carcinoma cell lines were comparatively resistant to the drugs, and both bladder cell lines and one testicular teratoma cell line were as resistant to temozolomide as the cell lines from malignant glioma, whilst the other cell line derived from a testicular teratoma was markedly sensitive to temozolomide. This degree of sensitivity to temozolomide was similar to that seen in the our most sensitive cell lines derived from malignant glioma including the cell line IN949 which was resistant to CCNU but sensitive to temozolomide. For most cell lines, irrespective of the tissue of origin, there appeared to be considerable cross-resistance between nitrosoureas and temozolomide.

Temozolomide appears to have rather poor activity against short-term cultures derived from human malignant glioma with significant cytotoxicity being achieved in only about 20% of cultures, a figure which correlates well the observed clinical response rate. The marked cross-resistance of this agent with CCNU *in vitro* suggests that it will limit the role of this agent either in combination with nitrosoureas or for the treatment of recurrent disease following nitrosourea treatment. There seems as great a need as ever to develop new cytotoxic drugs with low systemic toxicity for treating patients with malignant cerebral glioma.

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