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Preclinical report

Response of short-term cultures derived from human malignant glioma to aziridinylbenzoquinone, etoposide and doxorubicin: an *in vitro* phase II trial

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The relative resistance of malignant glioma to chemotherapy makes the identification of new cytotoxic drugs critically important. The use of short-term cultures derived from these tumors to screen drugs at doses that can be attained within human intracranial tumors provides a model system that should be capable of identifying effective drugs suitable for clinical evaluation. The sensitivity of a panel of short-term cultures derived from 22 malignant astrocytoma and four malignant oligodendroglioma was assessed to aziridinylbenzoquinone (AZQ), etoposide and doxorubicin (DOX) using a [^{35}S]methione uptake assay. The ID_{50} of each culture was compared to the levels of drug which could be achieved in the tumor using standard doses. There was marked heterogeneity between cultures in response to each drug. Whilst there was no evidence that cultures derived from grade III astrocytoma were more sensitive to any of the drugs than cultures derived from grade IV astrocytoma, cultures derived from oligodendroglioma tended to be more sensitive to the alkylating agent AZQ, but not to either of the other drugs. The sensitivity of these short-term cultures at concentrations that can be achieved *in situ* corresponded well with the clinical efficacy of AZQ and etoposide. Although DOX appeared to be toxic to human gliomas cells *in vitro*, its limited penetration into the intact brain would seem to preclude its use i.v., but it is likely to be effective if local drug delivery techniques could be employed. The study suggests that short-term cultures derived from malignant glioma should be used to screen investigational agents for potential clinical efficacy. [© 2001 Lippincott Williams & Wilkins.]

Key words: Astrocytoma, aziridinylbenzoquinone, chemosensitivity, doxorubicin, etoposide, oligodendroglioma.

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Introduction

Human malignant astrocytoma (WHO grades III and IV) is an intractable disease that is only modestly sensitive to a limited number of cytotoxic drugs. Only the chloroethylnitrosoureas like BCNU and CCNU and the methylating agent procarbazine produce significant increases in survival when employed as an adjuvant to radiotherapy.[1] Typically, between 20 and 40% of patients show evidence of objective radiological or clinical response and perhaps 15–30% of patients will survive more than 2 years after surgery.[2] These poor results indicate that there is a need to identify new cytotoxic drugs with activity against these tumors. Their comparative rarity makes the design and execution of effective clinical trials difficult and time consuming. As short-term cultures can be produced from virtually all good-quality biopsies of adult malignant astrocytoma and can be shown to be composed of replicating neoplastic astrocytes, this makes it possible to carry out rapid, large-scale drug screening using panels of these cultures—in effect an '*in vitro* phase II trial'. This should rapidly identify promising compounds for clinical trial.

Over the last few years, phase II clinical trials have been carried out in patients with malignant glioma using a number of experimental drugs including aziridinylbenzoquinone (AZQ; Diaziquone), etoposide and doxorubicin (DOX). AZQs are alkylating agents which possess significant activity against a variety of *in vivo* screening systems,[3] and possess the molecular characteristics of low molecular weight, low ionization in solution and high lipid solubility (log $p < 0.5$), necessary for central nervous system (CNS) penetration.[4] Unfortunately, they are usually poorly soluble in aqueous systems, making pharmaceutical formulation difficult. Subsequent rational chemical

synthesis produced a number of compounds which were more soluble, but which retained good antitumor activity.[5,6] One of these compounds, AZQ, which was particularly effective in model screens, was selected for clinical study.[7] This drug and its metabolites can readily be identified by high-performance liquid chromatography in blood, cerebrospinal fluid (CSF) and tumor tissue,[8,9] making evaluation of drug penetration into the CNS and brain tumor tissue possible. In phase II trials involving patients with malignant glioma, 20–25% of evaluable patients showed definite tumor regression and about one-third had stable disease.[8,10] Etoposide is lipid soluble and penetrates into the CSF, attaining levels 2–10% of the corresponding plasma levels.[11] Studies indicate that etoposide passes readily in brain tumor tissue producing levels between 3.5 and 74.6% of the concurrent plasma levels and concentrations as high as 5.9 μg/g in intracerebral tumor tissue following i.v. administration at a dose of 100 mg/m.[12] DOX does not penetrate into the intact CNS. It binds plasma protein increasing its apparent molecular weight and this factor coupled with its log octanol/water partition coefficient of -0.1 suggests that there is little or no penetration into the intact CNS.[13] It has not been possible to detect this drug in the CSF of patients undergoing chemotherapy for non-neurological cancer and in such patients whose systemic disease was responding to DOX had progressive metastatic disease within the CNS.[14] However, blood–brain barrier modification has been shown to produce high levels of DOX in experimental animals following intracarotid infusion and because of the characteristic breakdown of the blood–brain barrier within high-grade gliomas, clinical studies have been undertaken to evaluate the efficacy of this drug against malignant glioma.[15]

The purpose of this paper is to explore the heterogeneity in sensitivity of short-term cultures derived from a panel of patients with high-grade malignant astrocytoma and oligodendroglioma to AZQ, etoposide and DOX. This study also addresses the relationship between the response of these cultures *in vitro* and the dose levels which can be attained within intracranial tumors treated clinically.

Materials and methods

Cell culture and media

Cultures were prepared from biopsy samples of 26 cases of adult malignant glioma comprising 15 glioblastoma, seven grade III astrocytoma, one grade IV oligodendroglioma and three cases of grade III oligodendroglioma, and assayed for chemosensitivity between passage levels 5 and 12. Cultures were initiated as previously described.[16] The cultures were routinely fed with Ham's F10 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.[17] Cell counts were routinely carried out using a ZM Coulter counter calibrated for use with human glioma cells.

Drugs

The drugs used in this study together with the range of concentrations used are given in Table 1. The National Cancer Institute (Bethesda, MD) supplied AZQ. The drug was aseptically reconstituted in 0.5 ml of anhydrous *N*,*N*-dimethylacetamide (DMA; Sigma, Poole, UK) and further diluted in 0.01 M phosphate buffer to give a solution which contained 1 mg/ml of AZQ and 5% v/v DMA. This solution was further diluted in Ham's F10 to give a stock solution of 10 μg/ml AZQ and 0.05% v/v DMA. This concentration of DMA did not affect glioma cell growth *in vitro* (data not shown). Bristol Laboratories (Syracuse, NY) supplied etoposide as a reference standard. A stock solution was prepared in Ham's F-10 at a concentration of 100 μg/ml and sterilized by filtration through a 0.22 μm filter. Farmitalia Carlo Erba (Barnet, UK) supplied DOX A stock solution was prepared by dissolving the drug aseptically in Ham's F-10 at a concentration of 1 mg/ml. All stock solutions were aliquoted and stored at -70°C. Drug breakdown was not determined but assumed to be minimal at this temperature. Drug solutions were all diluted to working strength in complete growth medium immediately before use.

Chemosensitivity assay

The chemosensitivity assay used was an intermediate duration [^{35}S]methionine uptake assay, the results of which have been shown to correlate with monolayer clonogenic assay, and has been described in detail elsewhere.[18] Briefly, cultures were trypsinized in exponential growth phase and 96-well microtitration plates (Sterilin; Bibby Sterelin, Stone, UK) seeded with 2×10^3 cells/well. After 24 h incubation at 37°C, the medium was removed and 200 μl of drug dilution was added to four wells. Two wells of each row were left free of drugs to act as controls. The drug dilution was replaced 24 and 48 h later to give a total drug

exposure time of 72 h. Drug solutions were then removed and each well washed 3 times with Hanks' balanced salt solution (HBSS) and then filled with 200 μl of fresh growth medium. Following a recovery period long enough to include one population doubling time, 100 μl of 2 μCi/ml [^{35}S]methionine (SJ 123; Amersham, Little Chalfont, UK) was added to each well and incubated for 4 h. The plates were then washed in HBSS, fixed in methanol, extracted in ice-cold 10% w/v trichloroacetic acid (BDH, Poole, UK), washed in tap water and dried in methanol. Fifty microlitres of toluene-based scintillation fluid (NE233; Nuclear Enterprises) was added to each well and the plates dried by centrifugation. Autofluorograms were prepared by placing a sheet of X-ray film (Kodak Xomat, Hemel Hempstead, UK) under each plate and exposing them for 24–48 h at −70°C in the dark as previously described.[19,20] Quadruplicate determinations for each drug were performed on at least two separate occasions. The density of each spot on the autofluorograms was determined using a MR600 microplate reader (Dynatech, Billingshurst, UK). Using the absorbance reading for each well, the incorporation of labeled amino acid was expressed as percentage of the mean of the control in that row and the dose of drug that inhibited protein synthesis by 50% (ID_{50}) determined graphically and the mean value of ID_{50} for each drug calculated. For all experiments cell counts were made daily of replicate plates of each cell line to determine the population doubling time and to ensure that cultures remained in exponential growth throughout the assay.

Results

AZQ

The range sensitivity of the 26 cultures treated with AZQ is shown in Figure 1. The overall range of ID_{50}s was 0.015–1.2 μg/ml, an 80-fold range. Cultures derived from grade IV tumors had ID_{50}s between 0.015 and 1.2 μg/ml (an 80-fold range), and those cultures derived from grade III tumors between 0.042 and 0.7 μg/ml (a 17-fold range). The median ID_{50} for the grade III tumors was 0.072 μg/ml and for grade IV tumors was 0.079 μg/ml, a difference which was not statistically significant (p=0.85, two-tailed Mann–Whitney U-test). Cultures derived from oligodendroglioma were more sensitive to AZQ than cultures derived from astrocytomas (p=0.046, two-tailed Mann–Whitney U-test).

Etoposide

The responses of 26 cultures to etoposide are shown in Figure 2. The overall range of ID_{50}s was between 0.023 and 5.0 μg/ml, a 217-fold range. Cultures derived from grade IV tumors had ID_{50}s between 0.023 and 5.0 μg/ml with a median of 0.41 μg/ml, whilst cultures derived from grade III tumors had ID_{50}s between 0.16 and 2.6 μg/ml, a 16-fold range with a

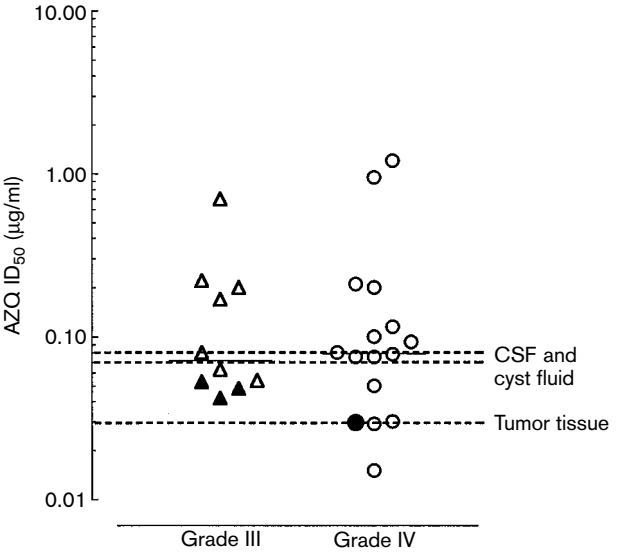

Figure 1. Sensitivity of short-term cultures derived from grade III and grade IV malignant glioma to AZQ. Each symbol represents the ID_{50} of an individual culture. Open triangles represent cultures derived from grade III tumors and circles represent cultures derived from grade IV tumors. Filled symbols represent the ID_{50}s of cultures derived from malignant oligodendroglioma. Horizontal lines representing medians omitted for clarity. The concentrations of AZQ that can be attained within either tumor tissue[9] or CSF and cyst fluid[8,34] are superimposed on the graph.

Table 1. Drugs used in the study and the range of concentrations tested

Drug	Stock solution	Initial dilution	Subsequent dilutions	Concentration range (μg/ml)
AZQ	1 mg/ml	1/100	1/10	10–0.0001
Etoposide	100 μg/ml	1/10	1/10	10–0.0001
DOX	1 mg/ml	1/100	1/10	10–0.0001

median of 0.29 μg/ml. This difference was not statistically significant (p=0.98, two-tailed Mann–Whitney U-test) Cultures derived from oligodendroglioma were no different in their sensitivity to etoposide than cultures derived from astrocytoma (p=0.81, two-tailed Mann–Whitney U-test).

DOX

The distribution of sensitivity of 27 cultures to DOX is shown in Figure 3. The overall rage of ID_{50}s is between 0.0013 and 0.12 μg/ml, a 92-fold range. For cultures derived from grade IV tumors, the range was 0.0013–0.083 μg/ml, a 64-fold range, whilst the ID_{50}s of cultures derived from grade III tumors ranged between 0.0025 and 0.12 μg/ml, a 48-fold range. The median ID_{50} for cultures derived from grade IV tumors was 0.017 and 0.02 μg/ml for grade III tumors. This difference was not statistically significant (p=0.92, two-tailed Mann–Whitney U-test). There was also no evidence that cultures derived from oligodendroglioma were more sensitive to DOX than cultures derived from astrocytoma (p=0.92, two-tailed Mann–Whitney U-test).

Summary

There was no relationship between age and sensitivity to AZQ, etoposide or DOX (data not shown), or between sensitivity to any of these drugs and culture doubling time. There was no evidence of cross-resistance between any of these drugs and or with other alkylating agents including CCNU, procarbazine or the vinca alkaloid, vincristine (data not shown here, but reported elsewhere).[21]

Discussion

The concept of an '*in vitro* phase II' trial is attractive, as it will enable rapid identification of drugs which are likely to be effective in the clinic. This should provide a time saving over conventional drug development programmes based on clinical studies by eliminating ineffective compounds at an early stage and before clinical evaluation has been started. However, the

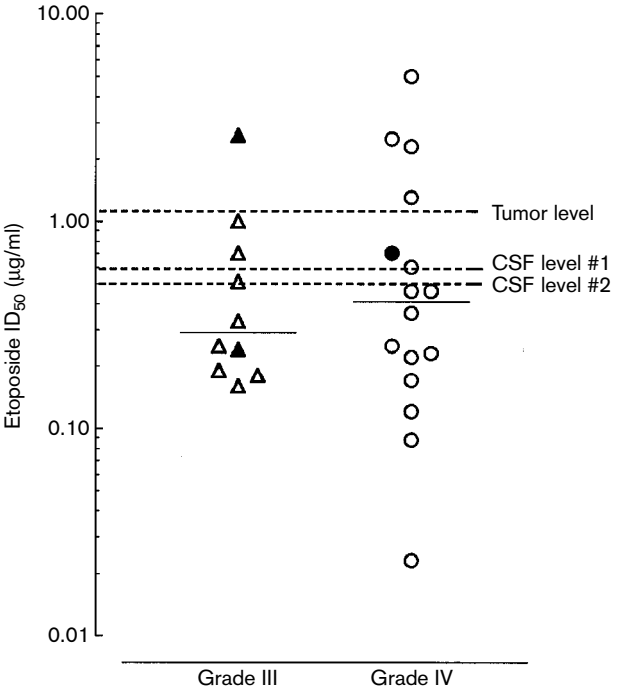

Figure 2. Sensitivity of short-term cultures derived from grade III and grade IV malignant glioma to etoposide. Each symbol represents the ID_{50} of an individual culture. Open triangles represent cultures derived from grade III tumors and circles represent cultures derived from grade IV tumors. Filled symbols represent the ID_{50}s of cultures derived from malignant oligodendroglioma. Horizontal solid lines represent the median ID_{50} for each grade of tumor. The concentrations of etoposide that can be attained either within tumor tissue[12] or the CSF[37] are superimposed on the graph.

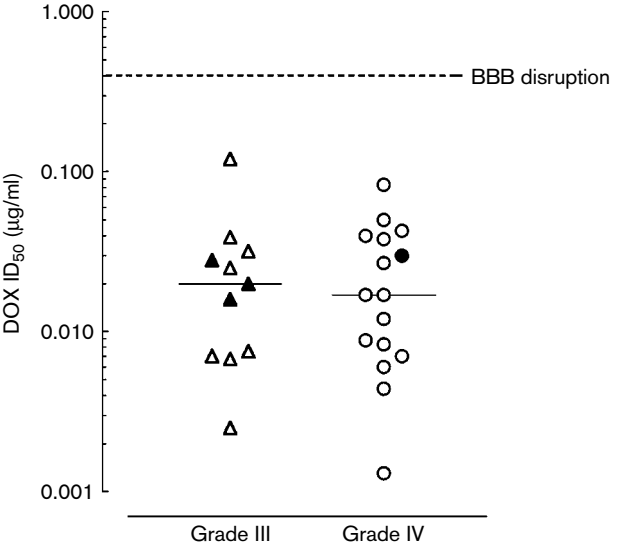

Figure 3. Sensitivity of short-term cultures derived from grade III and grade IV malignant glioma to DOX. Each symbol represents the ID_{50} of an individual culture. Open triangles represent cultures derived from grade III tumors and circles represent cultures derived from grade IV tumors. Filled symbols represent the ID_{50}s of cultures derived from malignant oligodendroglioma. Horizontal solid lines represent the median ID_{50} for each grade of tumor. The concentration of DOX that can be attained following blood–brain barrier (BBB) disruption in experimental animals[41] is superimposed on the graph.

design and interpretation of '*in vitro* phase II trials' requires some basic information about the target cells and the drugs being tested. It is essential that the cells that are screened are representative of the tumors being tested and without evidence of overgrowth of adventitious agents like fibroblasts. The target cells should not display the marked sensitivity to chemotherapy that is a feature of some of the rodent model screens that have been employed as drug screens in the past. Secondly, there must be some evidence of the intratumoral levels that a particular drug can attain. This may require direct measurement of drugs administered before operation and not rely on theoretical predictions of brain tumor penetration based on the molecular characteristics of the drug. For example, not all small molecular weight, lipid-soluble cytotoxic drugs are automatically effective against malignant glioma. They must produce toxicity at levels that can be attained *in situ*.

The use of short-term cell cultures derived from adult malignant astrocytoma provides a good model system to carry out an '*in vitro* phase II trial'. It is possible to produce such cultures from virtually all surgical biopsies of malignant astrocytoma[22] and there is little doubt that such cultures are composed of populations of cells which are representative of the tumor of origin. Cells in these cultures display astrocytic markers like glial fibrillary acidic protein (GFAP), glutamine synthetase and β-alanine-sensitive blockade of high-affinity uptake of GABA.[23] They are demonstrably neoplastic in nature as the cells contain complex numerical and structural chromosomal abnormalities,[24–26] and typical neoplastic features including growth in reduced serum concentrations and on confluent monolayers of normal cells,[27] expression of plasminogen activator,[23] angiogenic activity on chick chorioallantoic membrane[23] and 'invasive' behavior in chick heart fragment[28] or rat brain aggregate confrontation assays.[29]

The efficacy of AZQ has been assessed in a number of phase II trials in patients with recurrent malignant astrocytoma.[30–33] The clinical response rate for patients with recurrent malignant astrocytoma ranged between 6 and 26% with a mean response rate of about 11%. The relationship between the ID_{50}s for each of the cultures and the intratumoral levels of AZQ remaining 1–2 h after administration at a variety of dose levels is shown in Figure 1. The intratumoral levels were determined by administering [^{14}C]AZQ at doses of 2–4 mg/m to brain tumor patients immediately prior to craniotomy and analyzing samples taken at operation for unchanged [^{14}C]AZQ.[9] AZQ attained levels of 0.03 μg/ml in tumors. Only four of 26 cultures (15%), three derived from grade IV astrocytoma and one from a grade IV oligodendroglioma, had ID_{50}s which lay at or below this level of AZQ. In clinical trials where higher doses of AZQ were used (15–20 mg/m^2), AZQ attained concentrations between 0.07 and 0.08 μg/ml glioma cyst fluid[8] or in CSF.[34] Although the levels of drug in CSF or cyst fluid are very likely to be higher than can be attained in tumors, taking 0.07 μg/ml as a cut-off level, then five of 10 (50%) cultures derived from grade III tumors and five of 16 (31%) derived from grade IV tumors had ID_{50}s which would lie below pharmacologically achievable levels. It is noteworthy that of the 10 cultures with ID_{50}s below this level, four of them are derived from malignant oligodendroglioma, suggesting that there may be value in considering clinical trials that include AZQ to patients with these tumors. Although only a very small numbers of patients with recurrent oligodendroglioma have been included in phase II trials using AZQ, some of these patients did achieve objective clinical responses or stable disease. As there is now compelling evidence that many patients with anaplastic oligodendroglioma responded to combination chemotherapy with procarbazine, CCNU and vincristine,[35] and that oligodendrocytes show sensitivity to drugs like BCNU *in vitro*,[36] it would be of considerable importance to investigate if there is a more widespread sensitivity of these tumors to other alkylating agents.

Etoposide attains high levels in plasma with peak plasma levels of the order of 30 μg/ml. It penetrates well into intracerebral tumors attaining levels between 0.5 and little over 1.0 μg/ml.[12] Penetration into the CSF is also good and in patients with recurrent glioma, CSF levels following 600–1000 mg/m^2 etoposide are of the order of 0.5–0.6 μg/ml, which are similar to those achieved within brain tumor tissue.[37] At this cut-off concentration, eight of 26 (69%) of cultures had ID_{50}s below this concentration. Seven of 10 (70%) cultures derived from grade III tumors and 10 of 16 (63%) cultures derived from grade IV tumors had ID_{50}s below this concentration. Stewart and colleagues[12] have reported intratumoral levels slightly in excess of 1 μg/ml in patients with malignant brain tumors. Using this cutoff point, only five of 26 (19%) of cultures would not respond to etoposide. Interestingly, cultures derived from oligodendroglioma did not seem to be especially sensitive to either etoposide or DOX (see below), suggesting that their unique chemosensitivity is limited to alkylating agents.

The clinical response to etoposide in recurrent malignant astrocytoma has been investigated by a number of workers and attempts have been made to carry out long-term, prolonged treatment with this drugs[38] or using very high doses.[37] There response

rate appears certainly to be higher than for AZQ and the proportion of patients showing either partial response or stable disease ranges between 21 and 48%.[39] Studies that use combinations of etoposide with drugs like cisplatin have even higher response rates of the order of 40–50%.[40]

A higher proportion of cultures have ID_{50}s below the levels of etoposide which can be achieved clinically than with AZQ and this is consistent with the clinical evidence that a higher response rate can be achieved with etoposide than with AZQ. Typically, only 10% of patients respond to AZQ, and between 20 and 50% respond to etoposide. In contrast to both AZQ and etoposide, which penetrate to an extent into brain tumor tissue, the exact extent to which DOX penetrates the intact CNS following i.v. injection is unknown, although evidence suggests that penetration is minimal.[13] However, if DOX is administered at doses of 1–5 mg/kg to rats or dogs by the intracarotid route with blood–brain barrier modification, it can attain levels as high as 0.45 μg/g of brain tissue although such global exposure to DOX was not with out toxicity.[14] Even if 1/10th of this level could be attained in patients with malignant gliomas appreciable clinical benefit would be expected as all but three cultures, two from grade IV astrocytomas and one from a grade III astrocytoma, has ID_{50}s below this level. However, clinical trials using i.v. administration of DOX in patients with recurrent malignant astrocytoma have been undertaken and the drug has proved completely ineffective in this setting.[15] Nevertheless, DOX is remarkably effective against cells derived from both malignant astrocytoma and oligodendroglioma *in vitro* with the most resistant cultures having ID_{50}s at or below 100 ng/ml and nearly half the cultures had ID_{50}s below 20 ng/ml. Its failure to produce clinical responses must be related to its non-penetration into brain tumors following i.v. administration because of the blood–brain barrier. There is, however, renewed interest in developing methods of local delivery of high concentrations of chemotherapy which both spare the patient systemic toxicity but also circumvent the blood–brain barrier.[42] Recent trials have demonstrated the potential of biodegradable polymers like poly[1,3(bis-carboxyphenoxy-propane/sebacic acid) anhydride (Gliadel) wafers to effectively deliver a number of drugs within brain tumor resection cavities[43,44] and this approach might be applicable to delivering drugs like DOX effectively to brain tumors.

It should be borne in mind that the cultures used in this study were derived from tumors taken at the time of diagnosis from patients who had not received chemotherapy or radiotherapy. Most patients with recurrent malignant glioma who are included in phase II trials of investigational agents do so following treatment with radiotherapy and often chemotherapy before relapse. Recent studies have indicated that the cellular composition of recurrent malignant glioma differs from that seen in the tumor at the time of diagnosis. Whether this occurs through natural biological evolution of the tumor or as a consequence of therapy is unclear, but use of samples taken at diagnosis may overestimate the response rate of tumors treated at recurrence. We are investigating this using paired samples taken from the same patient at both the times of diagnosis and recurrence.

Although there appears to be a relationship between sensitivity *in vitro* to both AZQ and etoposide *in vitro*, and the clinical efficacy of these drugs as evidenced by phase II clinical trial, the concept of an *in vitro* phase II trial needs further validation using a larger number of drugs with differing clinical effectiveness against malignant glioma and where there is reasonably accurate evidence of the concentration of drug that can be achieved clinically.

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