**In Vitro versus in Vivo Correlations of Chemosensitivity of Human Medulloblastoma**

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**ABSTRACT**

An *in vitro* clonogenic assay was used to test the chemosensitivity of the human medulloblastoma cell line TE-671. Dose-response relationships for reduction in colony formation were generated for cyclophosphamide, vincristine, Adriamycin, 1,3-bis(2-chloroethyl)-1-nitrosourea (NSC 499962), and 1,4-cyclohexadiene-1,4-dicarbamic acid, 2,5-bis(1-aziridinyl)-3,6-dioxodithierylster (NSC 182986); and the *in vitro* drug dose at which there is a 75%, 50%, or 10% reduction in the number of colonies in comparison to controls (ID$_{0}$s) were derived from these data. Methotrexate produced no colony reduction at any dose tested up to 1000 $\mu$g/ml. The *in vitro* results were compared to growth delays in s.c. TE-671 xenografts in athymic mice treated with the same agents. Agents with an ID$_{50}$ less than assumed *in vivo* plasma drug concentrations were all active *in vivo*, whereas two of the three agents with an ID$_{50}$ greater than assumed *in vivo* plasma drug concentrations demonstrated no *in vivo* activity. These results suggest that for these agents, the relationship between the ID$_{50}$ of the drug and its *in vivo* concentration allows *in vitro* clonogenic assay results to agree with *in vivo* growth delay responses.

**INTRODUCTION**

Conventional therapy for medulloblastoma consists of surgical resection and subsequent whole neuraxis irradiation, yielding a 5-year survival rate of approximately 40 to 50% (5, 8). The role of adjuvant chemotherapy is undefined with discrepant results reported in 2 large studies. The European study compared conventional radiation with radiation and CCNU$^\text{a}$-vincristine, noting an advantage with the addition of chemotherapy (7). The Children’s Cancer Study Group compared conventional radiation with disease and CCNU-vincristine-prednisone, and could find no difference in disease-free survival (15). Experimental chemotherapeutic studies of medulloblastoma have been hampered by the lack of an *in vitro* or *in vivo* tumor model. We and others have reported the development of an *in vitro* and *in vivo* chemosensitivity model using the human medulloblastoma cell line TE-671 (17, 24, 35, 39) and preliminary therapeutic studies in athymic nude mice (18). We now report the *in vitro* chemosensitivity of TE-671 using a clonogenic assay and compare these results with xenograft growth delay produced by the same drugs in the animal model. We found that the *in vitro* clonogenic assay results correlated well with the *in vivo* growth delay responses.

**MATERIALS AND METHODS**

**Cell Line.** The human medulloblastoma cell line TE-671 established from a cerebellar tumor (24) was grown in cell culture as described previously (17).

**Clonogenic Assay.** Drug assays were performed after initial studies had established a linear relationship between the number of cells plated and the number of resulting colonies, allowing the selection of an optimal final cell concentration (5 x 10$^4$ cells/60-mm dish). For all experiments except with methotrexate, cells were grown to 80% confluence, mechanically harvested with a Pasteur pipet, washed, and resuspended in Eagle’s minimal essential medium to a concentration of 1 x 10$^6$ cells/ml after passage through a 27-gauge needle. These cells were incubated for 1 hr at 37$^\circ$ in room air with a drug or drug vehicle, washed twice, and resuspended in Richter’s (26) zinc option minimal essential medium (Grand Island Biological Co., Grand Island, NY) supplemented with 20% heat-inactivated fetal calf serum to a concentration of 1 x 10$^6$ cells/ml. Two agarose-based solutions were prepared previously in a 44$^\circ$ water bath. For the base layer, 1.4% agarose (SEA-KEM-ME; FMC Corp., Rockland, ME), 5X-zinc option, distilled H$_2$O, fetal calf serum, and 5.5% sodium bicarbonate were mixed at a v:v ratio of 30:12:18:12:3, respectively, yielding a final agarose concentration of 0.56%. Three-ml aliquots were pipetted to each 60-mm Petri dish. For the top layer, the same reagents were mixed at a v:v ratio of 9.1:8:22:9:8:2, respectively, yielding a final agarose concentration of 0.26%. Two volumes of this solution were mixed with 1 volume of the cell suspension, achieving a final agarose concentration of 0.17%. A final cell concentration of 5 x 10$^5$ cells/dish in 1.5 ml of this solution was plated over the base layer using 4 plates/drug or vehicle. For experiments with methotrexate, similarly harvested cells were grown in the continuous presence of methotrexate or vehicle at the same agarose concentrations. Plates were incubated for 14 days. Subsequently, the number of colonies (>50 cells) on each dish was counted using a Wild microscope (Heerbrugg, Switzerland). The mean percentage of colony formation was calculated from the number of colonies counted per dish/number of cells plated per dish. Response was assessed by the ratio of treated versus control colony formation.

The following agents were tested *in vitro*: BCNU; AZQ; methotrexate (NSC 740); vincristine (NSC 67574); Adriamycin (NSC 123127); and 4-hydroxy-cyclophosphamide, the primary metabolite of cyclophosphamide active in vitro (kindly provided by Dr. M. Colvin, Johns Hopkins Oncology Center). All drug solutions were freshly prepared on the day of assay. AZQ, vincristine, and 4-hydroxy-cyclophosphamide were dissolved in Eagle’s minimal essential medium, and serially diluted to desired concentrations. Adriamycin was dissolved in 0.9% NaCl solution (saline) and serially diluted in Eagle’s minimal essential medium. Methotrexate was dissolved in zinc option-20% fetal calf serum and serially diluted. BCNU was dissolved in absolute ethanol and serially diluted with Eagle’s minimal essential medium. Mixtures (1:1) of drug solution and cell suspension were used to achieve a 1-ml cell suspension containing 10$^6$ cells with

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3 The abbreviations used are: CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (NSC 79037); BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea (NSC 499962); AZQ, 1,4-cyclohexadiene-1,4-dicarbamic acid, 2,5-bis(1-aziridinyl)-3,6-dioxodithierylster (NSC 182986); ID$_{50}$, ID$_{0}$, ID$_{10}$ in *in vitro* drug dose at which there is a 75, 50, or 10% reduction in the number of colonies in comparison to controls, respectively.

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the desired final drug concentration.

Calculation of In Vitro Parameters. Parameters of the dose-response curves were derived by linear regression analysis of the relationship between percentage of colony formation (treated colonies/control colonies × 100) and log drug concentration. To obtain the individual percentage of colony formation values for the different drug concentrations, the number of colonies for each treated dish was divided by the mean number of colonies of the control dishes. The slope value of each log dose versus response curve was then used to compute the ID_{75} and the ID_{50} by substituting 75 or 50% for percentage of colony formation and solving the regression equation for drug concentration.

Animals. Male or female athymic BALB/c mice (nu/nu genotype, 6 weeks or older) were used for all in vivo studies. Animals were maintained as described previously (9).

In Vivo Studies. Chemotherapy of the human medulloblastoma cell line growing s.c. in athymic nude mice was performed as described previously (18). Briefly, groups of 10 randomly assigned mice were treated i.p. with chemotherapeutic compounds when the median tumor volume as determined by caliper measurement exceeded 200 cu mm. The doses of compounds used were: BCNU, 75 mg/sq m for one dose; cyclophosphamide, 513.9 mg/sq m daily for 2 doses; vincristine, 7.31 mg/sq m for one dose; AZQ, 26.5 mg/sq m for one dose; Adriamycin, 36 mg/sq m for one dose; and methotrexate, 24 mg/sq m daily for 5 doses. These doses represented 75% of the calculated 10% lethal dose (18). For each experiment, one group of matching animals served as a control and received the drug vehicle on an identical schedule.

In vivo response was assessed by the median difference in days between the median of treated animals' and the median of control animals' tumors to reach a volume of 1000 cu mm. Statistical significance was determined by the Wilcoxon rank sum test.

All 6 agents tested in vitro were also tested in vivo; 4-hydroxy-cyclophosphamide, the primary metabolite of cyclophosphamide active in vitro, was used in the clonogenic assay to avoid the need for hepatic activation.

Pharmacokinetic Simulations. The time courses of expected drug concentrations were simulated using pharmacokinetic parameters which were either determined by us, i.e., for AZQ in athymic mice (30), or derived from data available in mice in published reports. The data for Adriamycin were in athymic mice (22), but in laboratory mice for cyclophosphamide (2), methotrexate (34), and vincristine (14). No data were available for BCNU in mice; data obtained in Fischer rats (23) were therefore used for that drug. The simulations were carried out using the appropriate coefficients and exponents adjusted for the doses used in our in vivo studies (20). The time-averaged plasma drug concentrations over the first hr, C_{mean}, were subsequently computed on the basis of the respective areas under the plasma concentration versus time curve (20).

RESULTS

The results of the in vitro dose-response curves are represented in Charts 1 and 2. Reduced colony formation was observed with increasing drug concentrations for all drugs except methotrexate. The calculated parameters of the dose-response relationships, i.e., slope, ID_{75}, and ID_{50}, are listed in Table 1. Excluding methotrexate, the dose-response curve was steepest for BCNU and shallowest for vincristine, but the ID_{75} and ID_{50} were lowest for Adriamycin and highest for BCNU. Also listed in Table 1 are the estimated in vivo plasma drug concentrations averaged over the first hr, and the results of the study on the in vivo growth delays of implanted medulloblastoma after the drug treatments. Chart 3 shows pharmacokinetic simulations of the time course of expected drug concentrations in mice over the first hr after the doses used in the in vivo studies.

The 3 drugs (4-hydroxy-cyclophosphamide, Adriamycin, and AZQ) with ID_{75} < C_{1hr} each produced a statistically significant growth delay in vivo. No documented in vivo growth delay was
**Experimental Chemosensitivity of Human Medulloblastoma**

**Table 1**

<table>
<thead>
<tr>
<th>Drug</th>
<th>In vitro dose-response curves</th>
<th>Results of in vivo drug studies</th>
<th>Significance of in vivo drug activity (T-C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adriamycin</td>
<td>Slope: 55.9</td>
<td>ID₅₀ (µg/ml): 0.071</td>
<td>ID₇₅ (µg/ml): 0.205</td>
</tr>
<tr>
<td>Vincristine</td>
<td>35.8</td>
<td>0.110</td>
<td>0.504</td>
</tr>
<tr>
<td>AZQ</td>
<td>50.8</td>
<td>0.590</td>
<td>1.83</td>
</tr>
<tr>
<td>4-Hydroxycyclophosphamide</td>
<td>48.0</td>
<td>0.550</td>
<td>1.83</td>
</tr>
<tr>
<td>BCNU</td>
<td>69.9</td>
<td>3.52</td>
<td>8.02</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>&gt;1000</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a* T-C, difference in days between the median of treated animals and the median of control animals to reach a volume of 1000 cu mm; *p < 0.05*; *No, p > 0.05.*

Fifty and 75% reduction in colony formation was not achieved at any drug concentration tested (maximum concentration, 1000 µg/ml).

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**DISCUSSION**

Our use of an *in vitro* clonogenic assay has demonstrated the dose response of the human medulloblastoma cell line TE-671 to 6 antineoplastic agents. These results provide analysis of the cellular sensitivity of the cell line to these drugs, allowing correlation with observed or predicted *in vivo* plasma drug levels and therapeutic efficacy.

Published criteria for determining appropriate *in vitro* drug concentrations predictive of *in vivo* activity include the average drug concentration over 1 hr at the peak of the plasma clearance curve (4), one-tenth peak plasma drug concentration (37), and a sensitivity index based on the area under the cell survival curve (29). Chang (12) has recently demonstrated in a pancreatic adenocarcinoma model the relationship between the ID₇₅, calculated from the log dose-response curve, and the *in vivo* plasma drug concentration that was estimated from either human pharmacokinetic data (1) or the 10% lethal dose in animal studies (38). Our data suggest that, for this established human medulloblastoma tumor line, drugs with average *in vivo* plasma drug concentrations over the first hr higher than the *in vitro* ID₅₀ or ID₇₅ will demonstrate an *in vivo* antitumor activity as measured by significant tumor growth delay.

The present findings are of potential clinical significance, since not only do they indicate that results of the *in vitro* clonogenic assays provide information as to whether a given drug shows an effect, but they also indicate what approximate range of drug concentrations has to be achieved *in vivo*. When making comparisons between *in vitro* and *in vivo* antitumor drug concentrations, however, numerous factors must be considered, e.g., protein binding, tissue distribution, mechanism of action, and the pharmacokinetics of the drug. In the present study, we used the estimated average *in vivo* plasma concentrations for the comparisons. Other pharmacokinetic parameters need to be evaluated, and precise *in vitro/in vivo* correlation will require measure-
ments of achievable murine drug plasma levels and ideally also of tumor drug levels. AZQ was the only drug in the present study with a measured in vivo plasma drug concentration, and this drug, active in vivo, demonstrated an ID<sub>50</sub> lower than the average in vivo plasma drug concentration over the first hr. Nevertheless, further studies will need to demonstrate the overall relationship between parameters of the in vitro dose-response curve and measured (rather than estimated) in vivo plasma drug concentration.

The most important function of an in vitro assay is accurate prediction of in vivo response. Salmon et al. (29) and Von Hoff et al. (37) have shown a good correlation between decreased colony formation of biopsy derived cells in short-term culture and a patient's tumor response, especially in predicting resistance. Nevertheless, as indicated by Selby et al. (32) "although a predictive value of negative test equal to 90 percent seems impressive, it appears less so when the prevalence of drug resistance is 80 percent." Direct correlations between in vitro and in vivo experimental models with established cell lines and stable transplantable tumors allow easier analysis, although all chosen indexes of correlation must by design be arbitrary compared to clinical situations. Previous work has demonstrated the significant in vitro sensitivity-in vivo response correlations observed in human melanoma (21, 36), human pancreatic carcinoma (4, 11, 13), and human colon adenocarcinoma (40). Nevertheless, many human tumor models reflecting a range of types of human cancer may need to be tested to ensure that significant in vitro-in vivo correlations exist.

It is possible that the in vitro-in vivo model described here may allow screening of agents with efficacy in the therapy of medulloblastoma, but this remains to be established. In the absence of randomized Phase III clinical trials showing the unequivocal value of single or combination adjuvant drug therapy, it is difficult to relate our in vitro-in vivo results with TE-671 to clinical results with medulloblastoma. Nevertheless, in Phase II trials with cyclophosphamide, Allen and Helson (3) have shown responsiveness that would agree with our predicted efficacy of that agent. Clinical results with vincristine and nitrosoureas are more difficult to evaluate. We showed in vivo responsiveness with vincristine, but none with BCNU. One major clinical study showed efficacy of vincristine-CNU combination (7), and another study showed no effect (15). AZQ demonstrated achievable ID<sub>50</sub> levels in vivo with moderate albeit significant growth delay in vivo. Only limited clinical information is presently available with AZQ, but early results show some effect in individual patients using dosage regimens which result in plasma concentrations of AZQ of approximately ID<sub>50</sub> to ID<sub>90</sub> levels for 1 to 6 hr (31). Early studies have shown no efficacy for methotrexate-containing regimens and the possibility of severe neurological toxicity (25). Methotrexate demonstrated no in vitro or in vivo activity in our system, and this knowledge might have precluded use of this toxic agent.

The precision of our results with TE-671 may be influenced by the use of a continuous cell line with relative growth and karyotypic stability (17). While beneficial to this analysis, this may not reflect the genotypic and phenotypic heterogeneity of medulloblastoma in patients. Although there is increasing evidence that human cancers, especially solid tumors, are composed of subpopulations of cells that are heterogeneous in the expression of genotypic and phenotypic characteristics (6, 10, 16, 33), cellular heterogeneity has not yet been examined as extensively in medulloblastoma as in many other human tumors. Nevertheless, abundant evidence exists that there is a broad range of morphological and immunocytochemical characteristics of human medulloblastoma (19, 27, 28), and there is no reason to suspect that medulloblastoma is any less heterogeneous than other human tumors. Although this in vitro-in vivo model may allow screening of agents effective against medulloblastoma, tumor heterogeneity would argue against the use of only one continuous cell line. However, no other medulloblastoma lines are currently available, and such testing must await establishment of new permanent lines or analysis of short-term cultures. Ultimately, the only true test of the predictiveness for drug effectiveness in human medulloblastoma by this experimental model, either in vitro or in vivo, will be the selection of drugs that lead to effective clinical therapy.

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