Combination Chemotherapy in Vitro Exploiting Glutamine Metabolism of Human Glioma and Medulloblastoma

Glenn Dranoff, Gertrude B. Elion, Henry S. Friedman, and Darell D. Bigner

ABSTRACT

The human glioma-derived cell line D-54 MG and the human medulloblastoma-derived cell line TE-671 have been shown to be sensitive in culture to the pharmacological interference with glutamine metabolism by acivicin, 6-diazo-5-oxo-L-norleucine, and methionine sulfoximine. Using as a guide the multiple contributions of glutamine to the biosynthesis of proteins, purines, and pyrimidines, we now have identified six additional antimetabolites active against these lines in vitro at clinically relevant concentrations. The 50% growth-inhibitory levels of the drugs against D-54 MG in 6-day continuous exposure experiments were: L-asparaginase, 0.057 IU/ml; 5-fluorouracil, 0.5 μg/ml; 6-mercaptopurine, 0.8 μg/ml; actinomycin D, 0.0007 μg/ml; N-phosphonacetyl-L-aspartic acid, 2.3 μg/ml; and 5-azacytidine, 0.2 μg/ml (3-day exposure). The corresponding 50% growth-inhibitory values in TE-671 were: L-asparaginase, 0.54 IU/ml; 5-fluorouracil, 1.5 μg/ml; 6-mercaptopurine, 4.7 μg/ml; actinomycin D, 0.00044 μg/ml; N-phosphonacetyl-L-aspartic acid, 4.5 μg/ml; and 5-azacytidine, 0.49 μg/ml. Dipyrudamole up to 10 μg/ml was inactive against both lines. The isobologram method was used to evaluate the effectiveness of several two-drug combinations which were biochemically designed. The sums of the optimal fractional inhibitory concentrations for the pairs were: acivicin plus L-asparaginase, 0.14; acivicin plus methionine sulfoximine, 0.40; 6-diazo-5-oxo-L-norleucine plus methionine sulfoximine, 0.60; acivicin plus 6-mercaptopurine, 1.0, all in TE-671; and acivicin plus 5-fluorouracil, 0.79, in D-54 MG. Our findings suggest that an antimetabolite regimen exploiting glutamine sensitivity might improve the chemotherapy of some human gliomas and medulloblastomas.

INTRODUCTION

The limitations of current chemotherapy in modifying the natural history of primary central nervous system neoplasms are well recognized (31). The addition of carmustine (bischloroethyl-nitrosourea) to whole brain irradiation only slightly prolongs the median and long-term survival of postsurgical patients with malignant gliomas (12). Adjuvant chemotherapy is not consistently beneficial for patients with medulloblastoma, and although several compounds produce temporary regressions in recurrent disease, cures are rarely effected (36).

Stem cell heterogeneity is a critical factor underlying chemotherapeutic failure (27), since tumor lineage, mutation, and ter-

1 This work was supported by NIH Grants PO1 NSCA 200223-01 and CA 11898 and by Duke Comprehensive Cancer Center Developments Funds (CA 14236).
2 Recipient of an Association for Brain Tumor Research Fellowship in memory of Bryce Davis and an American Cancer Society Junior Clinical Faculty Fellowship.
3 To whom requests for reprints should be addressed.
4 The abbreviations used are: DON, 6-diazo-5-oxo-L-norleucine; PALA, N-phosphonacetyl-L-aspartic acid; FIC, fractional inhibitory concentration.

MATERIALS AND METHODS

Tumor Lines. Cultured cell lines derived from a human glioma and medulloblastoma were used. D-54 MG is the Duke University subline of A-172 established by G. Todaro (10) from a human glioblastoma multiforme. Its morphological, karyotypic, and selected biochemical characteristics have been defined (2). TE-671 is a line initiated by McAllister (21) from a human medulloblastoma and further characterized by Friedman et al. (9).

The cell lines were grown in monolayer culture in Eagle’s glutamine- and glutamic acid-free minimal essential medium (Grand Island Biological Co., Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum, 10 μg/ml N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid buffer, and 4 μg/ml in a humidified 5% CO₂ atmosphere at 37°C. At confluence, cells were mechanically harvested with a Pasteur pipet following digestion with 0.125% trypsin:0.02% EDTA. All cell lines have been tested to ensure the absence of HeLa cell, inter-, or intra-cell line contamination, and Mycoplasma infection (2).

Chemicals. Aciclovir, DON, PALA, and 5-azacytidine were provided generously by the Developmental Therapeutics Program, National Cancer Institute. 6-Mercaptopurine was a gift from the Burroughs Wellcome Co., Research Triangle Park, NC. L-Asparaginase and actinomycin D were obtained from Merck, Sharp, and Dohme, West Point, PA. 5-Fluorouracil was purchased from Roche Laboratories, Nutley, NJ. L-Methionine sulfoximine and dipyrudamole were bought from Sigma Chemical Co., St. Louis, MO.

Efficacy Studies. Two × 10⁵ cells were plated in 35-mm² wells containing 2 ml of glutamine- and glutamic acid-free minimal essential medium supplemented with 10% heat-inactivated fetal calf serum, 10 μg/ml N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid buffer, and 0.5 μm...
glutamine, and varying concentrations of inhibitor. The fetal calf serum was stored at 4°C for 2 wk prior to use and contributed less than 0.06 mm glutamine (determined with the Beckman Model 6300 high-performance amino acid analyzer; Beckman Instruments, Inc., Berkeley, CA) to the total level in the experimental solutions. This value is not included in the reported concentration of this substrate. The asparagine content was less than 0.03 mm, all derived from the fetal calf serum. The osmolality in the dishes ranged between 277 and 296 mOsmol (determined with an Advanced osmometer; Advanced Instruments, Newton Highlands, MA). Medium was replaced on alternate days. Viable cell counts, as assessed by trypan blue exclusion, were determined on Day 6. One or two drops of 5% trypan blue were added to 1 or 2 ml of medium, and 20 to 200 cells were counted, depending upon growth. Each point was run in triplicate. Mean values are expressed. 6-Mercaptopurine was first dissolved in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide was less than 0.003% (v/v) and had no effect on cell viability. For the 5-azacytidine experiments, medium was furnished on Day 0, and viable cell counts were measured on Day 3.

Combination Studies. The isobologram method of Elion (7) was used to evaluate the effectiveness of two antimetabolites in combination. Dose-response curves of each agent alone in 0.5 mm glutamine were defined in a 6-day continuous exposure experiment, as described above. Three concentrations of the second inhibitor were run concurrently with three concentrations of the first inhibitor, generating three additional dose-response curves of the combinations. A percentage of control growth (no drugs) allowing analysis of the greatest number of curves simultaneously was chosen. The concentrations of drugs, alone and in combination, corresponding to this level of growth were determined. FICs were calculated by dividing the concentration of the drug in the combination by the concentration of the drug alone required to produce the selected percentage of control growth.

RESULTS

Efficacy Studies. The effects of several antimetabolites on the growth in culture of the human medulloblastoma-derived line TE-671 were investigated (Chart 1). The concentrations of the various drugs producing 50% growth inhibition in 0.5 mm glutamine, the level in cerebrospinal fluid (23), were determined (Table 1). The duration of drug exposure was 6 days, since previous experiments established that, by this time, there was a dose-response relationship between glutamine concentration and growth of TE-671 in vitro (6). The short half-life of 5-azacytidine in solution, 65 h at 25°C (22), however, required abbreviating its exposure to only 3 days. Dipyridamole up to 10 μg/ml was inactive. Increasing the concentration of actinomycin D to 0.01 μg/ml resulted in the killing of virtually all 2 x 10⁵ tumor cells plated per dish. 5-Azacytidine at 10 μg/ml was weakly cytotoxic, achieving a cell kill of approximately 50%.

The 50% growth-inhibitory values for these compounds against the human glioma-derived line D-54 MG were defined in similar experiments (Table 1). D-54 MG was more sensitive than TE-671 to all of the active inhibitors except actinomycin D. Dipyridamole had no effect up to 10 μg/ml. Actinomycin D at 0.01 μg/ml destroyed almost all the glioma cells. 5-Fluorouracil and 5-azacytidine at 3.16 μg/ml and L-asparaginase at 0.632 IU/ml were slightly cytotoxic, producing 25 to 50% kill.

Combination Studies. Since the antimetabolites were selected within a strategy to exploit glutamine sensitivity, it was of interest to examine the degree of potentiation achieved by coupling these agents with a glutamine antagonist. Dose-response curves in TE-671 of acivicin and L-asparaginase, individually and in combination, were established (Chart 2). The concentrations of the drugs at which growth was inhibited to 33% of control were identified, and fractional inhibitory concentrations were derived (Table 2). This required extrapolating the dose-response curve of acivicin from 36 to 33% of control growth. The FICs for each pair of inhibitors were plotted, and intersecting lines were drawn (Chart 3). Since the resulting curve was left of the line connecting unity on both axes, the combination of acivicin and L-asparaginase was synergistic. The FICs for the most effective balance of the drugs, indicated by the point of intersection, were 0.03 for acivicin and 0.11 for L-asparaginase. In terms
of concentrations, the equivalent values were 0.0038 μg/ml and 0.066 IU/ml, respectively. The sum of the optimal FICs, a quantitative measure of drug potentiation with a total of 1 denoting addition and lower values synergy, was 0.14.

The isobologram method was applied in evaluating the effectiveness of several other antimetabolite combinations (Table 3). The percentages of control growth selected for analysis ranged from 40 to 56, depending upon the shapes of the dose-response curves. The glutamine antagonist DON and glutamine synthetase inhibitor methionine sulfoximine, whose individual dose-response curves previously have been reported (6), were moderately synergistic in TE-671, the sum of their optimal FICs being 0.60. Acivicin and methionine sulfoximine in the medulloblastoma were more synergistic at 0.40. The difference in potentiation between the two pairs of drugs was due largely to the relative contributions of methionine sulfoximine, as its optimal FIC with acivicin was only 0.03 in comparison to 0.20 with DON. Mildly synergistic was the combination of acivicin and 5-fluorouracil, their optimal FICs totalling 0.79. The balance between the two compounds was skewed, with the respective FICs being 0.65 and 0.14. Acivicin and 6-mercaptopurine together were strictly additive.

DISCUSSION

The susceptibility of a tumor cell to the pharmacological interference with glutamine metabolism furnishes considerable insight into targeting chemotherapy more effectively. The several functions of glutamine in protein, purine, and pyrimidine biosynthesis suggest that these critical pathways for cellular growth may be exploitable with additional antimetabolites. We previously established that the human glioma-derived line D-54 MG and the human medulloblastoma-derived line TE-671 are sensitive to glutamine antagonists in culture (6). We therefore used this conceptual framework as a basis for identifying the activity of l-asparaginase, 5-fluorouracil, 6-mercaptopurine, actinomycin D, 5-azacytidine, and PALA. Comparison of the 50% growth-inhibitory values of the compounds in a physiological level of glutamine (Table 1) with their achievable plasma concentrations in patients (Table 4) indicates that the experiments potentially are clinically relevant.

An advantage of selecting drugs through a biochemical strategy is the possible potentiation accomplished by using the inhibitors in combination. The synergism we observed between the glutamine synthetase inhibitor methionine sulfoximine and the glutamine antagonists DON and acivicin illustrates the principle of sequential blockade. The very low concentration of methionine sulfoximine required with acivicin is particularly encouraging in view of the former’s dose-dependent central nervous system toxicity (19).

The coupling of acivicin and l-asparaginase exemplifies the concept of complementary inhibition. Since some tumor cells become resistant to l-asparaginase through increased asparagine synthetase activity, combination therapy with a glutamine antagonist may help circumvent treatment failure (13, 14, 24). Antitumor effects have also been improved by administering l-asparaginase concurrently with actinomycin D or 6-mercaptopurine (16). The sensitivity of D-54 MG and TE-671 in culture to l-asparaginase is intriguing in the context of the drug’s reported activity against meningeval leukemia and notable central nervous system toxicity (4).

### Table 2

<table>
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<tr>
<th>Combination</th>
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<th>Optimal concentrations (μg/ml)</th>
<th>% of control growth</th>
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<td>DON + methionine sulfoximine</td>
<td>0.40, 0.20</td>
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<td>Acivicin + methionine sulfoximine</td>
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<td>0.40</td>
<td>0.013, 1.026</td>
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<tr>
<td>Acivicin + 5-fluorouracil</td>
<td>0.65, 0.14</td>
<td>0.79</td>
<td>0.016, 0.074</td>
<td>47</td>
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<td>Acivicin + 6-mercaptopurine</td>
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* a Drugs were tested in a 6-day continuous exposure experiment after plating 2 x 10⁶ cells in 0.5 μM glutamine. All combinations were run in TE-671 except for acivicin plus 5-fluorouracil which was evaluated in D-54 MG.
* b Calculated by dividing the concentration of the inhibitor in the combination by the concentration of the inhibitor alone required to produce a selected percentage of control growth.
* c Units for l-asparaginase are IU/ml.
* d This value varied depending on the shapes of the dose-response curves.
The susceptibility of our human anaplastic glioma and medulloblastoma-derived lines to a number of pyrimidine antagonists provides important opportunities to assess the capabilities of combination therapy. The interaction of aciclovir and 5-fluorouracil is synergistic, with the optimal I.C. 50 of 5-fluorouracil equal to 0.14. A Phase I study of azotomycin, a glutamine antagonist, and 5-fluorouracil against colon carcinoma yielded encouraging results (33). The growth inhibition induced by PALA in D-54 MG and TE-671 is in accordance with the drug’s prior demonstrated activity against the glioma 26 model (17). The interaction of PALA and aciclovir is synergistic in vitro as well (20) and capable of counteracting in vivo the resistance to PALA in a variant of the Lewis lung carcinoma (18).

Other agents that have important relationships to glutamine antagonists include 6-mercaptopurine and actinomycin D. Our finding that aciclovir and 6-mercaptopurine are additive in TE-671 is consistent with the observed higher remission rate in acute leukemia with 6-mercaptopurine and DON together compared to 6-mercaptopurine alone (28). As aciclovir and actinomycin D have been shown to be synergistic against hepatomas (32), this combination may extend the range of concentrations in which actinomycin D is effective against our tumor lines.

We now have identified eight sites of metabolic vulnerability in human glioma and medulloblastoma-derived cell lines. Since the underlying theme among the agents is a close relationship to glutamine metabolism, many potentially powerful combinations may be designed. Their ultimate success in vivo will depend upon careful attention to pharmacokinetics and the selectivity of their interactions. As our tumor lines may be grown in athymic mice s.c. and intracranially, systematic testing of these drugs will allow better definition of the blood-brain barrier as a mechanism for the creation of pharmacological sanctuaries (30). The diversity of compounds and possibilities for potentiation represent a rational approach to the heterogeneity characteristic of primary central nervous system neoplasms. An antimetabolite regimen may improve the chemotherapy of some gliomas and medulloblastomas by complementing the sensitivity, kinetic, and resistance profiles of current alkylating agents.

REFERENCES

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