Drug cytotoxicity at elevated temperature

In vitro study on the U-87MG glioma cell line

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The malignant glioma cell line U-87MG was used for 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), aziridinylbenzoquinone (AZQ), cis-diaminodichloroplatinum (II) (cis-DDP), and spirohydantoin mustard (SHM) treatments at 37° and 42°C. With the exception of SHM, all drugs killed a greater proportion of cells at the higher temperature, as assessed by the colony-formation assay. Drug-dose enhancement ratios were 1.6, 2.8, 2, and 1:1 for BCNU, AZQ, cis-DDP, and SHM, respectively. Because methods to heat discrete volumes of brain are now available, we conclude that hyperthermic increase of BCNU, AZQ, and cis-DDP cytotoxicity might have therapeutic application for malignant gliomas.

KEY WORDS • hyperthermia • drug-hyperthermia interaction • glioma • brain neoplasm

Malignant gliomas continue to resist therapeutic efforts despite combination protocols involving surgery, radiation therapy, and chemotherapy. However, in these combined trials, treatments are administered sequentially with time intervals separating each modality, usually not less than 1 week. Experimental evidence pointing to powerful interactions between irradiation, chemotherapy agents, and hyperthermia suggests that their simultaneous administration might achieve better results. As a step in that direction the in vitro cytotoxicity of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), aziridinylbenzoquinone (AZQ), cis-diaminodichloroplatinum (II) (cis-DDP), and spirohydantoin mustard (SHM) in a glioma cell line at 37° and 42°C was studied.

Materials and Methods

Cell Line

The malignant glioma cell line U-87MG was used for this study. This is one of a number of cell lines derived from human malignant gliomas by J. Pontén and associates from 1966 to 1969, and was donated by Dennis Deen, Ph.D., Brain Tumor Research Center, University of California at San Francisco. The cells were grown and stored in frozen vials. From this stock, cells were thawed and used for the present experiments. The cells were grown in 75-sq cm flasks using antibiotic-free Eagle's minimum essential medium (MEM) supplemented with 20% fetal bovine serum and nonessential amino acids and incubated at 37°C in 5% CO₂.

Treatment

Forty-eight hours before an experiment, 5 × 10⁵ cells were seeded in 25-sq cm flasks and incubated. On the day of the experiment, cultures were reaching confluence. The flask was sealed with Parafilm and immersed in a circulating water bath at 42°C. The water bath temperature was monitored with an American Society for Testing and Materials 64C mercury thermometer, calibrated by National Bureau of Standards procedures. Control preparations were kept at 37°C.

For this study, BCNU, AZQ, cis-DDP, and SHM were obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. Approximately 5 mg of drug was weighed and solubilized with the appropriate solvent to yield a 1-mg/ml solution. Absolute alcohol, dimethyl sulfoxide (DMSO), Hanks' balanced salt solution, and DMSO were used, respectively, for BCNU, AZQ, cis-DDP, and SHM. The drug solution was micropipetted to the culture medium of each flask. Maximum concentration
of alcohol or DMSO was 0.3%. All drug treatments lasted 1 hour.

**Colony-Formation Assay**

After treatment, the cells were washed, trypsinized, and counted. Two to three cell dilutions at progressively higher cell concentrations were then plated in quadruplicate using 60-mm dishes containing heavily irradiated 9L feeder cells. Colonies were stained and counted after 14 days of incubation. Treatment results are given as survival fractions: ratios between colony counts of treated cells and control cells. Survival fractions were plotted against dose on semilogarithmic paper.

**Results**

Figure 1 shows the response of U-87MG cells to 42°C treatments from 1 to 3 hours. The slope of the curve begins to flatten by the 2-hour point, suggesting development of thermotolerance. Cell survival curves obtained after graded doses of drug were added to the cells are shown in Fig. 2. The curves corresponding to treatments at 42°C are normalized for about 10% cell kill of the control preparation treated with heat alone. With the exception of SHM, the cytotoxic effect of the drugs was increased at 42°C.

Dose enhancement ratios (ratios between the drug dose necessary to kill 90% of the cells at 37°C and at 42°C) are listed in Table 1. For the control cells, the colony yield was 48.8 ± 6.5 colonies/100 cells plated (mean ± standard error of the mean).

**Discussion**

As early as 1960, Woodhall and collaborators reasoned that because the rate of most metabolic and enzymatic cellular processes is temperature-dependent “it can be assumed that the effect of any anti-tumor substance upon a tumor cell will be increased in the presence of a hyperthermic environment.” Their report on experiments conducted in rabbits and on the treatment of patients suffering from head and neck cancer by a heated blood-drug perfusate was the first to suggest that hyperthermia does enhance drug effects. In 1972, Ben-Hur, et al., reported on thermally enhanced radiosensitivity of cultured cells and pointed out that the

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**TABLE 1**

**Drug enhancement ratio for each drug tested**

<table>
<thead>
<tr>
<th>Drug</th>
<th>DER</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCNU</td>
<td>1.6</td>
</tr>
<tr>
<td>AZQ</td>
<td>2.8</td>
</tr>
<tr>
<td>cis-DDP</td>
<td>2.0</td>
</tr>
<tr>
<td>SHM</td>
<td>1.0</td>
</tr>
</tbody>
</table>

* Drug enhancement ratio (DER) = dose necessary to kill 90% of the cells at 37°C/dose necessary to kill 90% of the cells at 42°C. For explanation of drugs see text.
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search for synergistic effects between temperature and various lethal treatments on mammalian cells was promising. They surmised that if similar effects could be found in vivo, hyperthermia could be used to localize other lethal treatments and mentioned chemotheraphy as a specially important example.1 Since then, numerous reports (reviewed in several recent publications) have established that temperatures above 37°C potentiate the lethal effect on cells of radiation and most chemotherapy agents, both in vitro and in animal tumor models.1,10,17,26 The need to develop new treatments for malignant gliomas, the availability of a safe method for heating discrete volumes of brain using microwave techniques, and the evidence already gathered experimentally point to the importance of studying heat-drug and heat-radiation interactions with potential therapeutic implications for malignant gliomas.20,21,24,30 In the clinical situation, local brain hyperthermia could increase focally the cell killing effect of a drug with no added systemic toxicity.

Our experiments were designed to determine if human glioma cells would be more susceptible to cell killing at 42° than at 37°C when treated with drugs relevant to treatment of malignant gliomas: BCNU and cis-DDP, which are currently used in the treatment of malignant gliomas; AZQ, which is undergoing clinical trials; and SHM, a drug specially created to enter the central nervous system.3,8,18,19,23,24 We chose 42°C on the assumption that it is the lowest acceptable temperature in the therapeutic range and that if there is drug-dose enhancement at 42°C there might also be enhancement at 43° or 44°C.6,10,16 Drug concentrations were low so as to simulate achievable drug concentrations in human gliomas.11,13-15,22,25

The drug-dose enhancement ratios of 1.6 and 2 for BCNU and cis-DDP were expected. The dose enhancement ratio of BCNU for a 37° to 42°C temperature in the 9L rat gliosarcoma line is 1.7.4 and ratios between 1.4 and 3.6 were found by Fisher and Hahn6 for cis-DDP at 42°C. More surprising are the lack of SHM enhancement and the high enhancement ratio of AZQ.

With few exceptions there is no satisfactory explanation for thermal enhancement of drug effects.10 Before any drug-cell interaction, the drug solution may undergo physicochemical changes due to the elevated temperature. BCNU as such is an inactive product but undergoes physicochemical changes within the cell culture medium rapidly loses cytotoxic properties and is hydrolyzed in aqueous solutions to two active short-lived moieties and the hydrolysis is temperature-dependent. Enhancement ratios for BCNU can, at least partially, be explained on the basis of this physicochemical change in the drug solution, an increased number of active BCNU intermediates hitting the target per time interval.4,10,25 Deen, et al.3 showed that SHM in cell culture medium rapidly loses cytotoxic properties at rates that increase with temperature; at 37°C, cytotoxicity in 9L cells was completely eliminated by a 30-minute preexposure to cell culture medium. The half-life of the drug at 37°C in culture medium is 5 minutes (D Deen, unpublished data). It is conceivable that, at 42°C, SHM is so rapidly degraded that it creates a concentration-time product identical to that at 37°C for 1-hour exposures.

Hahn10 has proposed that the increased cytotoxicity at elevated temperatures for the majority of the alkylating agents is a result of the increased deoxyribonucleic acid (DNA) alkylation kinetics expected at these temperatures. The four drugs used in this study are all DNA-alkylating and cross-linking agents.12,27,28 An alternative or additional explanation is that hyperthermia would change the configuration of the chromatin, favoring the formation of cross-links between the two DNA strands, a lethal cellular event.4,10 In a highly speculative vein, we suggest that the elevated dose enhancement ratio of AZQ might be due to the additional damage inflicted on the cell by toxic radicals: AZQ is metabolized in the microsomes to a semiquinone able to generate oxygen radicals and peroxides.27 Overwhelming the antioxidant defenses might render the cell more susceptible to heat damage.7

Whatever mechanisms of heat-enhanced drug cell kill might be at work, the results of the present in vitro study provide a rationale for testing clinical protocols for malignant gliomas using hyperthermia in combination with BCNU, cis-DDP, and AZQ.

References


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