Use of verapamil to enhance the antiproliferative activity of BCNU in human glioma cells: an in vitro and in vivo study

ALFRED P. BOWLES, JR., M.D., COOLEY G. PANTAZIS, M.D., AND WILLIAM WANSLEY, B.S.

Department of Surgery, Section of Neurosurgery, and Department of Pathology, Section of Anatomic Pathology, Medical College of Georgia, Augusta, Georgia

The authors have evaluated the antiproliferative activity of verapamil, alone or in combination with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) in brain-tumor cells. These effects were studied in vitro using four human glioma cell lines and in vivo using glioblastoma multiforme cells transplanted to athymic nude mice. The results showed that verapamil when used alone produced inhibition of tumor growth; however, when verapamil was used in combination with BCNU (in vitro), significant dose-dependent suppression of proliferation occurred in all four cell lines. The in vivo results were far more dramatic. Mice treated with BCNU (25 mg/kg) plus verapamil (50 mg/kg) achieved a 200-fold decrease in tumor growth with a greater than 80% regression in tumor size. Complete cures were achieved in 80% of the mice observed for at least 50 days following the completion of therapy. These findings support the use of verapamil in overcoming drug resistance in malignant brain tumors.

KEY WORDS • brain neoplasm • glioma • verapamil • BCNU • chemotherapy

MALIGNANT gliomas of the brain and spinal cord are among the most difficult human neoplasms to treat successfully and continue to pose major challenges to the surgeon and oncologist alike. Despite recent attempts to improve chemotherapeutic approaches for the treatment of malignant gliomas, results remain limited and palliative. De novo and acquired resistance to cytotoxic drugs is not unique to tumors of the nervous system, and cause major obstacles to the successful treatment of most neoplasms.

Verapamil, a calcium channel blocker which is conventionally used for the treatment of supraventricular tachycardia, has significant effects on membrane ion influx. The use of verapamil with a number of cytotoxic drugs has been recently evaluated and the results have shown that this agent may produce an adjunctive response in mediating the reversal of drug resistance. The biochemical basis for the enhanced antitumor effects of verapamil is not well defined, yet it has been shown that verapamil can increase the cellular accumulation of agents.

The nitrosourea 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) degrades in aqueous solution to form a reactive carbonium ion with alkylating properties and an isocyanate compound with carbamylating activity. As a single agent, BCNU is the most effective and most commonly used drug for the treatment of gliomas, yet clinical responses are variable and poor. Effectiveness is limited by hepatic dysfunction, pulmonary and bone marrow toxicity, and de novo and acquired resistance. Several mechanisms for resistance probably exist, yet its best-outlined mode of action has been linked to a deoxyribonucleic acid (DNA) repair enzyme, O6-guanine-DNA alkyltransferase.

Little is known about the effects of verapamil in combination with chemotherapeutic agents for the treatment of malignant gliomas. To investigate the effectiveness of verapamil in treating malignant gliomas, we studied the in vitro and in vivo antiproliferative activities of verapamil with and without BCNU in four human glioma cells. The in vitro cytostatic activity was determined from the use of a fluorescent microcarrier chemosensitivity assay. In vivo antiproliferative activity was determined from the use of xenografts in athymic nude mice. The present study describes the enhanced antiproliferative properties of BCNU against brain tumors when used in combination with verapamil.
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Materials and Methods

Cell Cultures

Three established human glioma cell lines and one human primary culture were used for the in vitro chemosensitivity analysis of BCNU and verapamil. The established cell lines were obtained from the American Type Culture Collection and consisted of three different glioblastoma multiforme cell lines: U87, U373, and T98G. The primary cell culture was obtained from biopsied fragments of the resected tumor from a patient on our neurosurgery service. The tumor was diagnosed histopathologically as a fibrillary astrocytoma (AA), Kernohan grade II. All tumor cells were maintained by continuous passage in Dulbecco's minimum essential medium (DMEM) with 10% fetal bovine serum (FBS), supplemented with essential amino acids and vitamins, pyruvate, and glutamine. Cultures were maintained at 37°C in an incubator with a humidified atmosphere of 5% CO₂/95% air.

Reagents

A stock solution of hydroethidine (7 mg/ml) was prepared by dissolved 1.4 mg of hydroethidine in 200 μl of N,N-dimethyl-formamide. From this, 20 μl was added to 2 ml of phosphate-buffered saline (PBS) to give a final concentration of 0.7 mg/ml. Solubilized concentrations of BCNU were prepared by dissolving sterile lyophilized aliquots in absolute ethanol and then diluting the concentrate in PBS to the desired concentrations. A stock solution of verapamil was provided in a sterile vial containing 2 mg drug in 5 ml sterile water. Desired concentrations were diluted in sterile PBS.

In Vitro Chemosensitivity Assay

A fluorescent microcarrier technique was used to determine the cytostatic activity of BCNU and verapamil. Frozen cell lines (U87, U373, and T98G) were thawed and resuspended in DMEM with 10% FBS. Freshly isolated tumor tissue (cell line AA) was dissected under a laminar flow hood and enzymatically digested in a cocktail of collagenase and trypsin (25 mg/100 ml), washed in PBS, then added to a flask containing DMEM and 10% FBS as a monocellular suspension. Cytodex microcarriers were preswollen and autoclaved, then incubated with tumor cells in a ratio of 1 to 2 x 10³ cells per bead. The cells and microcarriers were gently swirled and incubated at 37°C with anchorage of cells onto the beads achieved within 4 hours. One hundred microcarriers with 1 to 2 x 10⁴ attached cells were then transferred to the wells of microtest plates and incubated with the desired concentrations of verapamil and BCNU. The cell suspensions were treated with BCNU (50 μg/ml) and concentrations of verapamil ranging from 2 to 200 μg/ml. After 72 hours of incubation, the cells were stained with hydroethidine for 1 hour and transferred to filter well plates. The cells were washed three times in PBS, then were concentrated to the center of the wells by constant vacuum, and the total particle-bound fluorescence was determined by front-face fluorimetry. The results were expressed as relative fluorescence units (RFU).

Hydroethidine readily enters and accumulates in viable metabolically active cells without being toxic. Once internalized into the cell, hydroethidine may stain the cytoplasm blue or the DNA red. Since the relative fluorescence is proportional to the number of metabolically viable cells, the percent of cytostatic activity (CA) was calculated from the formula:

\[
\%CA = \frac{C - E}{C - S} \times 100,
\]

where C is the RFU in the tumor cells incubated with control medium, E the RFU in tumor cells incubated with test reagents, and S the fluorescence of the background. The background fluorescence is represented by the RFU of the wells treated with 0.2 M nitric acid. This concentration of acid destroyed all of the cells, which was confirmed by the uptake of trypan blue. The relative fluorescence of the acid-treated wells represents the nonspecific staining of the cellulose acetate manifold of the filter well plates. In all experiments the background fluorescence was found to be less than 5% of control.

Tumors and Mice

Athymic homozygous (nu/nu) nude mice of the Balb/c strain were used for these studies. The mice were maintained in a 12/12-hour light/dark-cycle environment and given food ad libitum. The mice initially weighed 20 to 25 gm at the beginning of the chemotherapy course. The U87 cell line (glioblastoma multiforme) was initially injected (2 x 10⁶) into the right flank of four mice. The resultant tumors were serially transplanted and maintained in vivo. The U87 cells (2 x 10⁶ cells) in 0.1 ml of DMEM plus 10% FBS were inoculated subcutaneously into the right flank of the mice. The animals were examined until 6- to 8-mm tumors were palpated, at which time they were treated as described below.

In Vivo Evaluation of Antitumor Activity

A single intraperitoneal dose of BCNU (25 mg/kg, or 0.75 of the calculated 10% lethal dose) and an intraperitoneal injection of verapamil (50 to 100 mg/kg) were administered on the 1st day. Subsequent to these injections, verapamil (25 to 50 mg/kg) was given intraperitoneally every other day for 20 days. Control
TABLE 1

Inhibition of growth of tumor cells by BCNU (50 μg/ml) in the absence or presence of verapamil*

<table>
<thead>
<tr>
<th>Tumor Cell Line</th>
<th>Without Verapamil</th>
<th>With Verapamil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 μg/ml</td>
<td>20 μg/ml</td>
</tr>
<tr>
<td>AA</td>
<td>30.4 ± 8.5</td>
<td>60.4 ± 7.2♀</td>
</tr>
<tr>
<td>U87</td>
<td>18.5 ± 8.2</td>
<td>60.2 ± 7.2♀</td>
</tr>
<tr>
<td>U373</td>
<td>30.4 ± 8.6</td>
<td>50.4 ± 5.0♀</td>
</tr>
<tr>
<td>T98G</td>
<td>37.5 ± 8.6</td>
<td>55.5 ± 4.2♀</td>
</tr>
</tbody>
</table>

* Values are mean % cytostasis activity ± standard error of the mean of four replicates. Significance of difference of data vs. same tumor line without verapamil treatment: ♀ = p < 0.01; ♂ = p < 0.001; ♀ = p < 0.03; ♂ = p < 0.004.

FIG. 1. Graph showing the in vitro cytostatic activity of BCNU. Four glioma cell lines were incubated with increasing concentrations of BCNU from 50 to 200 μg/ml. The percentage of cell growth inhibition (% cytostatic activity) was calculated by comparing the uptake of hydroethidine in quadruplicate with both BCNU and control cultures. The in vitro therapeutic concentration of BCNU was 50 to 100 μg/ml, and the cytostatic activity values for 50 μg/ml BCNU varied from 18% to 25%. Variations of the mean did not exceed 15%.

FIG. 2. Graph showing the in vitro cytostatic activity of verapamil. Four glioma cell lines were treated with increasing concentrations of verapamil and the percentage of cell growth inhibition (% cytostatic activity) was calculated from the uptake of hydroethidine. Concentrations of verapamil (x axis) are represented logarithmically. The cytostatic values represent the mean of four replicates. Variations from the mean did not exceed 10%.

mice received intraperitoneal injections of sterile PBS every other day throughout the treatment period. The extent of tumor growth was measured with sterile metric calipers every other day.

Estimations of the tumor weight (w) were made from the linear measurements using the formula:

\[ w = A^2 \times B \times 0.5, \]

where A represents the width in mm and B the length in mm. To standardize the variability in tumor weights among the different test groups at the initiation of treatment, the relative tumor weights (RTW’s) at different times were estimated using the formula:

\[ RTW = w_i/w_0, \]

where \( w_i \) is the mean tumor weight of a group of mice at a given time and \( w_0 \) the initial mean tumor weight for that group. The in vivo cytotoxic effects were determined by comparing the retardation of tumor growth and tumor doubling time in the control and treatment groups. The cytotoxic effect was also expressed as the largest \( C_{RTW}/D_{RTW} \) value, where \( D_{RTW} \) denotes the RTW’s of the drug-treated groups and \( C_{RTW} \) the RTW’s of the control groups. The ratio of the doubling time (TD) of the drug-treated tumors and control was calculated from the formula:

\[ TD = \frac{\ln^2}{\ln(w_i - \ln w_0/i)}, \]

where \( i \) is the number of days of treatment and \( \ln = e^{2.71828} \).

Statistics

In the in vitro experiments, the control and treatment groups of individual cell lines were treated in quadruplicate and the variability of the mean of the cytostatic values was expressed as the standard error of the mean (SEM). The in vivo experimental groups consisted of five mice each for the control group and for each treatment group. The variability about the mean of the RTW’s was expressed as the SEM. Statistical significance of the results was determined with the two-tailed Student t-test.

Results

The results of the drug sensitivity assays are shown in Figs. 1 and 2 and Table 1.

Effect of BCNU Alone on Cell Proliferation

In all of the cell lines examined, BCNU inhibited growth in a dose-dependent fashion in doses from 25 to 100 μg/ml (Fig. 1). Cytostasis was most pronounced in the T98G cell line and least effective in the U87 cell line. At BCNU doses of 50 μg/ml, cytostasis varied from 18.5% to 37.5%.

Effect of Verapamil Alone on Cell Proliferation

The effect of verapamil on cell proliferation is shown in Fig. 2. At verapamil doses of 2 μg/ml, cytostatic values ranged from 4.5% to 27.5%; these values were

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modestly increased at 20 \( \mu \text{g/ml} \). When the concentration of verapamil was increased to 200 \( \mu \text{g/ml} \), significant growth inhibition occurred in all cell lines.

Effect of BCNU Plus Verapamil on Cell Proliferation

The inhibition of tumor growth by 50 \( \mu \text{g/ml} \) of BCNU in the presence or absence of verapamil is shown in Table 1. The addition of verapamil at nontoxic (2 \( \mu \text{g/ml} \)) and higher (20 \( \mu \text{g/ml} \)) doses significantly increased the cytostatic activity in all four cell lines. A fourfold increase in the cytostatic activity was achieved when verapamil (20 \( \mu \text{g/ml} \)) was added in the treatment of the U87 cell, and a threefold increase in the astrocytoma primary culture (AA). Both U87 and AA were the cell lines most resistant to the effects of BCNU or verapamil when treated alone. In the more sensitive cell lines (U373 and T98G) a 2- to 2.4-fold increase was achieved. The effects of verapamil on the cytostatic activity of BCNU in the U87 and AA cell lines appeared to be synergistic, whereas the effects in the U373 and T98G were additive.

In Vivo Antitumor Effects of BCNU and Verapamil in U87

Because of the significant antiproliferative effects of BCNU in combination with verapamil achieved in vitro, we studied the antitumor activity in vivo with the transplanted U87 tumor. The in vivo results are summarized in Figs. 3 and 4 and Table 2.

The groups of five mice transplanted subcutaneously with \( 1 \times 10^6 \) U87 cells were treated as described in the

### TABLE 2

<table>
<thead>
<tr>
<th>Drug</th>
<th>( C_{RTW}/D_{RTW} )</th>
<th>( T_D ) (treated)/( T_D ) (control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCNU &amp; verapamil</td>
<td>200</td>
<td>( \infty/3.85 )</td>
</tr>
<tr>
<td>verapamil</td>
<td>2.0</td>
<td>4.35/3.85 = 1.13</td>
</tr>
<tr>
<td>BCNU</td>
<td>3.6</td>
<td>5.55/3.85 = 1.44</td>
</tr>
</tbody>
</table>

* \( C_{RTW}/D_{RTW} \) = relative tumor weight for control/drug-treated groups; \( T_D \) (treated)/\( T_D \) (control) = doubling time of drug-treated tumors/control tumors.

Materials and Methods section. All of the control animals grew well-circumscribed, spherically shaped, solid masses during the 3-week study period (Fig. 4). The masses fluctuated in size for variable periods after implantation, yet once the diameter of 5 mm was reached, growth was consistently linear until treatment Day 10 (28 days after implantation) when exponential growth began. After a single BCNU injection of 25 mg/kg, tumor growth was significantly retarded (p < 0.01) and decreased 3.6-fold (Fig. 3 and Table 2), although total tumor regression did not occur. At doses of 25 mg/kg, BCNU was well tolerated without apparent toxicity. Verapamil alone (25 or 50 mg/kg) produced significant inhibition of tumor growth (p < 0.01), although not to the same extent as BCNU alone. Tumor growth was decreased approximately twofold compared to the control group (Table 2) yet, as with BCNU alone, tumor growth persisted. Initial intraperitoneal injections of verapamil (50 mg/kg), followed by 25 mg/kg every other
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day were well tolerated without significant toxicity. Unfortunately, when the mice were treated with 100 mg/kg verapamil followed by 50 mg/kg, significant toxicity occurred; 50% of the mice were dead by 4 days after the initiation of treatment.

As judged from the changes in the RTW's, the ratio of the tumor doubling times, and \( C_{RTW}/D_{RTW} \) (Figs. 3 and 4 and Table 2), treatments with BCNU plus verapamil were completely effective in limiting tumor growth. A greater than 200-fold decrease was elicited, with greater than 80% regression in tumor growth. An excellent curative effect was shown with BCNU plus verapamil, with complete cure achieved in four of the five mice observed at least 30 days following the completion of therapy.

Discussion

Verapamil, in combination with a number of chemotherapeutic agents, has recently been shown to enhance cytotoxicity by reversing acquired drug resistance in many human cell lines, including nonsmall-cell lung cancer, colorectal carcinoma, leukemia, and neuroblastoma, and the antitumor activity has been achieved without enhancing myeloid toxicity.\(^9,13,18,19\) The greatest adjunctive effect of verapamil has been shown in drug-resistant lines, with lesser effects in sensitive lines.\(^5\) The antitumor activity produced from verapamil has been achieved in cells with the multidrug-resistance phenotype mediated by the mdr-1/P-170 efflux pump. It has been suggested that verapamil increases intracellular accumulation by preventing drug efflux.\(^3,19\) Drug efflux is an energy-dependent process and the candidate for the protein responsible for accelerated efflux is a plasma membrane glycoprotein with a relative molecular mass of 170,000 to 180,000 found in different multidrug-resistant human and animal cell lines.\(^5,18\) Verapamil causes an increase in the phosphorylation of this glycoprotein with resultant inhibition of drug efflux.\(^6\)

Although antitumor activity has been enhanced in a number of tumors, the majority of the successful results have come from \( \textit{in vitro} \) models involving animal cell lines. Human tumors studied have yielded less favorable results. Goodman, \textit{et al.},\(^5\) have shown that verapamil failed to have a major modulatory effect on enhancing \( \textit{in vitro} \) growth-inhibitory activity of vinblastine or Adriamycin in 53 different tumors. Results suggest that resistance may involve many mechanisms and that the selection of drugs for certain tumors is of importance.

We investigated the antiproliferative activity of verapamil alone and in combination with BCNU in brain-tumor cells. Clinical responses have been achieved with BCNU (generally short-term) in some patients with malignant gliomas. With the therapeutic limitations of systemic toxicity with BCNU, certainly the possibility exists that by increasing cell accumulation of drug, improved results could be achieved.

Our \( \textit{in vitro} \) studies have shown that verapamil significantly increased the cytostatic activity of BCNU in four human malignant glioma cell lines. Cell suspensions were treated with 30 \( \mu \text{g/ml} \) of BCNU alone or with either 2 or 20 \( \mu \text{g/ml} \) of verapamil. The \( \textit{in vitro} \) therapeutic concentration of BCNU that corresponds to the \( \textit{in vitro} \) estimate that may be achieved clinically is 50 to 100 \( \mu \text{g/ml} \).\(^12\) Significant increases in antiproliferative activities were achieved with nontoxic concentrations of verapamil (2 \( \mu \text{g/ml} \)). Clinically achievable and tolerable plasma levels of verapamil in patients vary from 250 to 4000 ng/ml.\(^3,5,15\) Increased activity was more pronounced in the U87 cell line, with a fourfold increase, whereas only a two- to 2.4-fold increase was achieved in the U373 and the T98G cell lines. Other studies have similarly shown that the effect of verapamil was greatest in the resistant cell lines.\(^2,5,11\) The \( \textit{in vitro} \) results are clinically relevant because the
concentrations of verapamil that were used are similar to the pharmacological plasma levels that have been achieved in patients without cardiac toxicity. With the use of a xenogenic model, therapeutic cures in athymic mice were achieved when the transplanted tumor was treated with BCNU and verapamil. These two agents, used separately, limited the growth of the human-derived transplanted glioma. In contrast, BCNU in combination with verapamil eliminated gross tumor was treated with BCNU and verapamil. These in athymic mice were achieved when the transplanted tumors were used in combination with BCNU. Although verapamil may effect the mdr-1/P170 efflux pump, the proposed mechanism of activity is different in malignant gliomas. Perhaps the activity is linked to the DNA repair enzyme, O6 guanine-DNA alkyltransferase, or involves a mechanism that affects a completely different system. Overall, our findings support the use of calcium channel blockers in eliminating drug resistance in malignant brain tumors. Hopefully, on the basis of continued clinical investigation, verapamil may play an important role in the chemotherapeutic management of malignant brain tumors.

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References


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Address reprint requests to: Alfred P. Bowles, Jr., M.D., Department of Surgery, Section of Neurosurgery, Medical College of Georgia, Augusta, Georgia 30912.