Chemosensitization of Glioblastoma Cells to Bis-dichloroethyl-nitrosourea with Tyrphostin AG17

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ABSTRACT

Recent data have suggested that mitochondria play a supportive role in maintaining the tumorigenic phenotype. Indeed, antimitochondrial agents have been hypothesized to be potential chemosensitizers to human malignancy. We assessed the utility of this approach by characterizing the antimitochondrial activity of 3,5-di-tert-butyl-4-hydroxybenzylidene-malononitrile (AG17), in combination with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) in two human glioblastoma cell lines. AG17 (NSC 242557) is a tyrphostin that has been thought to have some antimitochondrial activity, with limited tyrosine kinase antagonism, and was used at noncytotoxic and nongrowth-inhibitory concentrations (0.25 μM). Glioblastoma cells were incubated in AG17, and changes in mitochondrial activity were determined. Tumor cells became auxotrophically dependent on uridine and pyruvate, indicating the lack of a functioning respiratory chain. Despite this, cells continued to exhibit no growth-inhibitory effects. Exposure to AG17 was associated with significant depolarization of the mitochondrial membrane potential and decreases in mitochondrial mass in both glioblastoma cell lines, correlating with the finding of auxotropic dependence. In contrast, normal human astrocytes treated with the same dose of AG17 did not show changes in growth, mitochondrial membrane potential, or mass. Indeed, auxotropic dependence on uridine and pyruvate could not be established in these cells. Glioblastoma cells became significantly more responsive to BCNU chemotheraphy with AG17 pretreatment; a linear relationship was noted that correlated the number as well as percentage of polarized mitochondria with glioblastoma cell survival at the highest dose of BCNU used (144 μg/ml). Normal human astrocytes did not change with regard to the dose response to BCNU with previous incubation with AG17. No difference was found in the type of cellular death (apoptosis) in either of the glioblastoma cell lines, with BCNU treatment alone, or with the combination AG17 and BCNU, despite the decrease in polarized mitochondria and mitochondrial mass. AG17 has antimitochondrial properties when used at low dose in human glioblastoma, which are relatively specific to tumor cells when compared with normal astrocytes. The use of AG17 as a chemosensitizer, with drugs such as BCNU, offers a new and possibly effective approach to be developed in patients with glial tumors.

INTRODUCTION

An estimated 28,600 new cases of primary brain tumors were diagnosed in 1995, most being of astrocytic type ("gliomas"). Approximately 60% of gliomas comprise the most malignant grade astrocytic tumor, GBM (1). Despite the use of multimodality therapy (debunking surgery and radiation and cytotoxic chemotherapy), GBM is uniformly fatal, with a median survival of 51 weeks and a 2-year survival of <15% (2, 3). Thus, despite their relatively low incidence, gliomas represent a significant percentage of cancer-related death, attesting to the high degree of inherent malignancy.

As a result, given the limitations of conventional therapy to date, mechanistic approaches are required for GBM and other malignant astrocytic brain tumors. A greater understanding at the molecular level of the pathogenesis and behavior of these malignant neoplasms should allow more effective therapeutic interventions for individuals with disease and preventive strategies for those at increased risk. It would be hoped that the expected consequences of this type of intervention would include restoration of differentiation, cell cycle checkpoints, and genomic stability, with blockade of autocrine loops and selective death of malignant cells.

The mitochondria have been thought to be involved in neoplasia for some time (4). Early studies in diverse types of cancer cells revealed impairment of oxphos, with preferential use of glycolysis for the generation of energy (ATP). In fact, most studies have revealed that rapidly growing tumors virtually always have a reduction of mitochondria of 50% or more, with substantial rates of glycolysis (compare oxphos; Ref. 4). Human brain tumors have shown similar results in both pathologically high- and low-grade tumors (5). Indeed, inhibition of glycolytic pathways by targeting hexokinase has resulted in diminished growth in glioma xenograft models (6). In addition, we have

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The abbreviations used are: GBM, glioblastoma multiforme; oxphos, oxidative phosphorylation; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; AG17, 3,5-di-tert-butyl-4-hydroxybenzylidene-malononitrile; NHA, normal human astrocyte; NAO, nonyl-acridine orange.

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noted other changes of mitochondria in gliomas, including mitochondrial DNA alterations, with specific changes in gene copy number (amplification and/or deletion), and localization/transposition of mitochondrial DNA sequences into the nucleus of the tumor cell (7, 8). Cisplatin-resistant ovarian carcinoma cells have been found to have an increase in mitochondrial mass (9) and increases in mitochondrial membrane potential (10). These changes are unrelated to the mitochondrial role in energy production, which is diminished (11). Finally, in yeast, mitochondria are important in resistance to bleomycin, with specific areas of the mitochondrial genome required to produce a bleomycin resistance phenotype (12).

These results are especially interesting in the context of recent studies, suggesting that the mitochondria are important in the control and execution of apoptosis, in a mitochondrial membrane-dependent and -independent manner (13–16). Given our previous data, the experimental evidence that the drug resistance phenotype could be related to the mitochondrial genome and that the mitochondria may be involved in regulating apoptosis, we assessed the utility of using an antimitochondrial agent as a "chemosensitizer" to standard cytotoxic agents used in glioma treatment (BCNU). A tyrphostin was used, which has been characterized previously at higher doses to inhibit mitochondria (tyrphostin AG17; Ref. 17).

**MATERIALS AND METHODS**

**Drugs.** AG17 (NSC 242557) was obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute. AG17 stock solutions were dissolved in DMSO (Sigma Chemical Co., St. Louis, MO). DMSO was kept to <0.6% of the total culture medium. BCNU was obtained from the manufacturer (Bristol Myers Squibb, Princeton, NJ) and diluted in absolute ethanol and subsequently in PBS (pH 7.0) before use (usually within 1 h). BCNU treatment was performed during the last 2 h of AG17 pretreatment.

**Cell Culture.** Two glioblastoma cell lines (DBTRG-O5MG and DK-MG) were the generous gift of Dr. Carol Kruse (University of Colorado, Denver, CO) and have been characterized previously (18, 19). Tumor cells were maintained in RPMI 1640 with 10,000 units of penicillin-streptomycin and 10% FBS; 4.5 g/l glucose, 50 μg/ml uridine, and 1 mm pyruvate were routinely included in the medium unless otherwise specified. All tumor cell culture materials were obtained from Life Technologies, Inc. (Gaithersburg, MD). NHAs were obtained from Clonetics Corp. (San Diego, CA) and maintained in the supplied Basal Media with supplements. Axitrophic dependence on uridine and pyruvate were performed with the CellTiter 96 aqueous assay kit (Promega), following the manufacturer’s instructions as noted. Data points represent the mean results of at least three different experiments.

**Respiratory Chain Competence and Chromatin Condensation Assays.** Cells that lack a respiratory chain require the supplementation of uridine and pyruvate in the cell medium (20), due to the requirement of an intact respiratory chain to activate dihydroorotate dehydrogenase (an enzyme of pyrimidine biosynthesis located in the inner mitochondrial membrane), and the inability of mtDNA-less cells to oxidize cytoplasmically produced NADH. Cells without functional oxphos will undergo apoptosis when supplements are removed; hence, incubation of cells in a uridine- and pyruvate-free environment is a sensitive manner to determine functional competence of mitochondrialy mediated oxphos. Tumor cells cultured in the presence of AG17 and supplements for 30 days were incubated in otherwise complete medium but without uridine and pyruvate. Assessment of cell growth, morphological change, use of medium, and survival were made. Evidence of chromatin condensation of cells obtained from tumor culture in unsupplemented medium, indicating apoptosis, was performed according to Schwartz et al. (21), using acridine orange and ethidium bromide (Sigma; both at 100 μg/ml) dissolved in PBS (pH 7.0), and added as a 1:25 v/v ratio to cells obtained as noted. At least 200 cells were assessed per experiment.

**Mitochondrial Membrane Potential Assessment.** Mitochondrial membrane potential (ΔΨm) was measured using the lipophilic cation JC-1 (Molecular Probes, Eugene, OR). JC-1 can enter cells, and selectively mitochondria, and has been used to assess ΔΨm in a variety of different models (22). In brief, JC-1 is a monomer at 527 nm after being excited at 490 nm; with polarization of ΔΨm, J-aggregates are formed that shift emission to 590 nm. This can be detected on a flow cytometer by assessing the green signal (at 527 nm) and green-orange signal (at 590 nm) simultaneously, creating an index of the number of cells with polarized and depolarized mitochondria. Both DBTRG-O5MG and DK-MG were stained by obtaining 1 × 10⁶ cells in fresh complete medium; JC-1 was added at 10 μg/ml, and cells were incubated for 10 min in the dark at room temperature. Cells were then washed with ice-cold PBS (pH 7.0) twice and trypsinized. After resuspension in 400 μl of PBS, cells were immediately analyzed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA). At least 5 × 10⁵ cells were analyzed in the list mode to determine the percentage of cells with polarized and depolarized mitochondria. Results reported are the mean of at least three experiments.

**Mitochondrial Mass Determination.** Mitochondrial mass was determined by using the fluorescent stain NAO, which binds the mitochondrial phospholipid cardiolipin in an energy-independent manner, and has been extensively used to provide an index of mitochondrial mass (23). Cells (1 × 10⁶) were incubated with 30 μM of NAO in complete medium for 10 min at room temperature in the dark, washed with ice-cold PBS (pH 7.0) twice, trypsinized, and resuspended in 400 μl of PBS. Cells were immediately analyzed using a log scale photomultiplier to

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4 Unpublished observations.
detect green fluorescence. Results reported are the mean of at least three experiments.

**Statistics.** Student's t test was used to compare groups. Results are reported using two-sided evaluations.

**RESULTS**

**AG17 Dose Determination**

To assess the chemosensitization abilities of AG17, we determined the dose of drug that was not toxic to either the tumor cells or NHAs. Both brain tumor cell lines had a doubling time of 22–24 h; the NHAs had a doubling time of 47 h. In none of the cell populations (DBTRG-O5MG, DK-MG, or NHA) was there a significant diminution of growth rate when compared with untreated controls. Although the dose of 0.25 μM was not found to affect cell growth in the time period of the reported experiments, in cells treated more than 30 days, both the NHA untreated control and AG17-treated NHA cells began growing more slowly. The NHA eventually underwent cell death subsequently, most likely due to reaching cellular senescence; no differences in the time period to death were noted between the treated and untreated NHAs. Higher doses of AG17 (>0.5 μM) were associated with progressive cell death in both the tumor cells and normal astrocytes (results not shown). Because antagonism of tyrosine kinases is associated with the growth-inhibition effect of AG17 (17), it was assumed that such kinase activity was unaffected in these cells.

**Mitochondrial Function in AG17-treated and Untreated Cells**

**Auxotrophic Dependence on Uridine and Pyruvate.** To characterize the tumor cells that had been treated with AG17 with regard to mitochondrial function, assays of auxotrophic dependence on uridine and pyruvate were performed. Cells that were treated for 30 days with AG17 were evaluated for survival in medium lacking uridine and pyruvate, with determination of the presence and mechanism of cell death. AG17-treated DBTRG-O5MG cells stopped proliferating 1 day after supplements were removed and progressively became detached from the tissue culture flask. Over 7 days, virtually all cells were no longer attached. These cells were collected and stained with ethidium bromide and acridine orange and showed chromatin condensation indicative of apoptosis. Similarly, when DK-MG cells were treated for 30 days with AG17 and supplements were withdrawn, there was no additional cellular proliferation; after 15 days without supplements, all cells had detached from the tissue culture flask. Staining of these cells with ethidium bromide and acridine orange also showed chromatin condensation, again indicative of apoptosis. Table 1 shows the results of quantitation of the apoptotic nuclei. With no AG17 treatment, between 0.5 and 5% of cells showed evidence of apoptosis. Similarly, in cells treated with 0.25 μM AG17 for 30 days with uridine and pyruvate supplementation, <10% (1–6%) of cells had apoptotic nuclei. In contrast, in the tumor cell lines, cells that detached after supplementation was withdrawn showed >90% apoptotic nuclei; the NHAs revealed only 2% of cells with apoptotic nuclei after supplements were withdrawn. In both cases, parallel cultures of tumor cells that were untreated and unsupplemented did not die. Hence, the DBTRG-O5MG and

**Table 1**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>No AG17&lt;sup&gt;a&lt;/sup&gt;</th>
<th>With supplements</th>
<th>Supplements withdrawn</th>
</tr>
</thead>
<tbody>
<tr>
<td>O5MG</td>
<td>6/210 (3)</td>
<td>3/212 (1)</td>
<td>186/200 (93)</td>
</tr>
<tr>
<td>DK-MG</td>
<td>10/201 (5)</td>
<td>14/230 (6)</td>
<td>193/213 (91)</td>
</tr>
<tr>
<td>NHA</td>
<td>1/200 (0.5)</td>
<td>3/200 (1.5)</td>
<td>4/206 (2)</td>
</tr>
</tbody>
</table>

<sup>a</sup> O5-MG and DK-MG are tumor cell lines; NHAs are normal human astrocytes.

With regard to mitochondrial function, assays of auxotrophic mitochondria in the AG17-untreated state, compared with AG17-treated and untreated NHAs began undergoing senescence subsequently, and as a result, further delineation of the time to reach auxotrophy was not possible.

**Determination of Mitochondrial Membrane Potential.**

Cells were then evaluated for changes in mitochondrial membrane potential (ΔΨm) by determining fluorescence ratio to JC-1 dye. These results are seen in Fig. 1 and summarized in Table 2. NHAs were associated with 70 ± 1% (SE) polarized mitochondria in the AG17-unaffected state, compared with 57.5 ± 0.7% and 56 ± 0.8% polarized mitochondria from the DBTRG-O5MG and DK-MG cells, respectively (P < 0.01 for both. Student's t test, compared with NHAs). Treatment of DBTRG-O5MG and DK-MG with AG17 for 1, 2, 3, and 30 days was associated with increases in the number of depolarized mitochondria, as determined by cytofluorimetric analysis using JC-1 (Fig. 2). In general, a 1-day exposure to AG17 was not associated with a significant change in numbers of depolarized mitochondria in either DBTRG-O5MG or DK-MG. For DBTRG-O5MG, exposure to 2, 3, and 30 days of AG17 was associated with a 35–55% decrease in the number of polarized mitochondria when compared with the untreated control (Table 2). In a similar fashion, DK-MG at 2, 3, and 30 days of exposure to AG17 was associated with decreasing amounts of polarized mitochondria with between a 32 and 65% decrement from the untreated control. In Fig. 1, note the progressive shift in JC-1 fluorescence with increasing time of incubation in AG17 in the representative cytofluorimetric contour plots toward the depolarized state (smaller y-axis values). In contrast, the NHA cells did not reveal substantial shifts in JC-1 fluorescence when evaluated by flow cytometry and were not different when evaluated for changes in percentage of cells that were depolarized (Fig. 1 and Table 2). This result correlates well with the finding that AG17 auxotrophy was difficult to establish in these cells.

**Determination of Mitochondrial Mass.** To determine whether the changes in ΔΨm were due to loss in mitochondrial
Fig. 1 Cytofluorometric determination of mitochondrial membrane potential in NHAs and glioma cells. JC-1 orange fluorescence (polarized mitochondria) is represented in the vertical axis; JC-1 green fluorescence (total mitochondria) is noted in the horizontal axis. In the DK-MG and DBTRG-05MG tumor cells, note progressive decrease in the number of polarized mitochondria with increasing time of exposure to AG17 (noted time periods in days). In contrast, the NHAs did not change with regard to number of polarized mitochondria, with 30-day incubation in AG17. Representative data are of at least three experiments and correlate with Table 2.

Table 2  JC-1 staining: Relative number of polarized mitochondria (% total)

<table>
<thead>
<tr>
<th></th>
<th>Untreated</th>
<th>1 day (%Δ)</th>
<th>2 days (%Δ)</th>
<th>3 days (%Δ)</th>
<th>30 days (%Δ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O5MG</td>
<td>57.5 ± 0.7</td>
<td>54 ± 1.4 (−6)</td>
<td>37.5 ± 0.7* (−35)</td>
<td>28.5 ± 0.9* (−51)</td>
<td>26 ± 1.4* (−55)</td>
</tr>
<tr>
<td>DK-MG</td>
<td>56 ± 0.8</td>
<td>54 ± 0.1 (−4)</td>
<td>38.5 ± 1.0* (−32)</td>
<td>29 ± 1.0* (−48)</td>
<td>19.5 ± 2.0* (−65)</td>
</tr>
<tr>
<td>NHA</td>
<td>70 ± 1</td>
<td>70 ± 3 (100)</td>
<td>70 ± 3 (100)</td>
<td>70 ± 3 (100)</td>
<td>70 ± 3 (100)</td>
</tr>
</tbody>
</table>

* P < 0.05, compared with untreated control.

mass, we evaluated the staining pattern of NAO in the treated and untreated tumor and NHA cells. Fig. 2 and Table 3 show the results of these evaluations. Mitochondrial mass was found to be substantially higher in NHAs than either DBTRG-05MG or DK-MG. NHAs had a relative mitochondrial mass of 168 ± 7, compared with 9.68 ± 0.5 and 9.75 ± 0.6 in the DK-MG and DBTRG-05MG tumor cells (P < 0.01, NHA versus DBTRG-05MG and DK-MG, Student's t test; Table 3). With AG17 treatment, the NHA mitochondrial mass did not change significantly after 30 days (150 ± 3, P > 0.05, Student's t test). Tumor cells, however, responded differently to AG17 pretreatment. In both tumor cell lines, mitochondrial mass was unchanged after 1 day of AG17 treatment but subsequently fell to between 50 and 87% (DBTRG-05MG) and 49 and 89% (DK-MG; Table 3). As was noted in the assessment of mitochondrial membrane potential, longer AG17 exposure was associated with a larger magnitude of mitochondrial mass decrease (Table 3). Fig. 2 shows representative NAO fluorescence in the NHAs and
Untreated 1 day 2 days 3 days 30 days

DK-MG

DBTRG-O5MG

NHA

Fig. 2 Cytofluorimetric determination of mitochondrial mass. NAO staining of cardiolipin shows progressive decreases in signal with increases in time of AG17 exposure in the DBTRG-O5MG and DK-MG cells. No change in mitochondrial mass was noted with AG17 treatment for 30 days in the normal human astrocytes. Representative data are from at least three experiments and correlate with Table 3.

Table 3 NAO staining: Relative number of mitochondria

<table>
<thead>
<tr>
<th></th>
<th>Untreated</th>
<th>1 day (%)</th>
<th>2 days (%)</th>
<th>3 days (%)</th>
<th>30 days (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O5MG</td>
<td>9.75 ± 0.06</td>
<td>10.35 ± 0.1 (6)</td>
<td>4.86 ± 0.1&lt;sup&gt;a&lt;/sup&gt; (−50)</td>
<td>4.54 ± 0.06&lt;sup&gt;a&lt;/sup&gt; (−50)</td>
<td>1.22 ± 0.01&lt;sup&gt;c&lt;/sup&gt; (−87)</td>
</tr>
<tr>
<td>DK-MG</td>
<td>9.68 ± 0.5</td>
<td>10.65 ± 0.2 (10)</td>
<td>4.93 ± 0.1&lt;sup&gt;a&lt;/sup&gt; (−49)</td>
<td>4.56 ± 0.04&lt;sup&gt;a&lt;/sup&gt; (−53)</td>
<td>1.08 ± 0.01&lt;sup&gt;c&lt;/sup&gt; (−89)</td>
</tr>
<tr>
<td>NHA</td>
<td>168 ± 7</td>
<td></td>
<td></td>
<td></td>
<td>150 ± 3 (−11)</td>
</tr>
</tbody>
</table>

<sup>a</sup> p < 0.02, compared with untreated control.
<sup>b</sup> p < 0.01, compared with untreated control.
<sup>c</sup> p < 0.05, compared with untreated control.

tumor cells; note the left shift of the signal in tumor cells treated with AG17, with no shift in NHA cells. Hence, the change in ∆Ψm noted in the tumor cells was at least partially if not wholly due to decreases in mitochondrial mass.

BCNU Dose Response

Fig. 3 shows the results of pretreatment with AG17 and survival of tumor cells to escalating doses of BCNU. DBTRG-O5MG and DK-MG cells were treated up to 30 days with the nontoxic and nongrowth-inhibitory dose of 0.25 μM AG17. There was a progressive decrease in the survival per BCNU dose with increasing time of exposure to AG17 in both glioma cell lines. In AG17 untreated cells, the BCNU dose of 144 μg/ml (D<sub>max</sub>) was associated with a cell survival of 65 ± 4% in the DBTRG-O5MG cells; previous exposure to AG17 decreased the number of BCNU surviving cells to 43 ± 6% (1 day), 21 ± 2% (2 days), 10 ± 3% (3 days), and 0.04 ± 0.01% (30 days). At 2, 3, and 30 days of exposure to AG17, the BCNU D<sub>max</sub> survival was significantly different than the control untreated cells (p < 0.01, Student's t test). In a similar fashion, the
Fig. 3 BCNU dose response. A, DK-MG. Cells were treated with BCNU with either no treatment or 1, 2, 3, and 30 days in 0.25 μM AG17. With increasing time in AG17, cells became more sensitive to BCNU. B, DBTRG-O5MG. Similar results were noted with increasing time in AG17 and increasing sensitivity to BCNU. Data points represent the results of at least three experiments. *, P < 0.05; **, P < 0.01, compared with untreated control; Student's t test.

Fig. 4 Correlation of Survival at Dmax with mitochondrial alterations. DBTRG-O5MG and DK-MG were correlated with the percentage of polarized mitochondria.

DK-MG cells were associated with increases in sensitivity to BCNU after exposure to AG17. Untreated DK-MG cells were associated with a survival at BCNU Dmax of 61 ± 8%, whereas pretreatment with AG17 shifted the survival to 58 ± 8% (1 day), 46 ± 6% (2 days), 22 ± 8% (3 days), and 0.08 ± 0.01% (30 days); differences between AG17-treated and untreated cells at Dmax were noted to be significant at 3 days (P < 0.05, Student's t test) and 30 days (P < 0.01, Student's t test). Exposure of both sets of tumor cells to 2, 3, and 30 days of 0.25 μM AG17 was also associated with increased sensitivity to lower doses of BCNU (Fig. 3). Incubation of the NHA cells with AG17 was not associated with a difference in response to BCNU; both treated and untreated cells had an LD50 of approximately 40 μg/mL.

Fig. 4 shows the relationship between Dmax survival versus the percentage of active polarized mitochondria. A linear correlation was noted between the number of mitochondria as well as percentage of polarized mitochondria and BCNU Dmax cellular survival. Both these variables had strong correlative relationships, suggesting that decreases in mitochondrial mass and percentage of polarized mitochondrial...
dria are associated with an increase in sensitivity to the cytotoxic agent BCNU.

**Type of Cell Death from BCNU of AG17-treated and Untreated Cells**

Recent studies have shown that the mitochondria are active participants in chemotherapy-induced apoptosis, which is mitochondrial membrane potential dependent (15, 16) and independent (13, 14). Because the mitochondrial membrane potential was diminished with AG17 treatment, we assessed the type of cell death in the tumor cells that had been pretreated with AG17 and subsequently with BCNU. Table 4 shows these data. Tumor cells that were untreated underwent apoptosis (with morphological changes and chromat condensation) when exposed to BCNU; with AG17 pretreatment, BCNU induced a similar pattern of cellular death. No difference in BCNU-induced cell death type was noted with 1-, 2-, 3-, or 30-day AG17 pretreatment from untreated cells. The percentage of apoptotic cells noted with AG17/BCNU treatment, when compared with the cell survival data, suggests that the majority of cells dying with treatment are undergoing apoptosis. Hence, in the glioma cells studied, BCNU-induced apoptosis is not changed when mitochondrial membrane potentials and mitochondrial mass are diminished. Thus, decreases in polarized and total mitochondria, with increases in sensitivity to cytotoxic agents, still results in an apoptotic death after exposure to the agent.

**DISCUSSION**

Mitochondrial modification in tumor cells has been shown to effect changes in the response to a cytotoxic environment, distinct from roles in energy production and a multidrug resistance genotype (9-11, 24). The present study shows that in glioblastoma cells, chemosensitization with tyrphostins such as AG17, with its antimitochondrial effects, can be effected with standard cytotoxic agents used in this tumor type (nitrosourea BCNU). These cells treated with AG17 continue to be able to undergo apoptosis, despite substantial decreases in \( \Delta \psi_m \) and mitochondrial mass.

This type of antimitochondrial approach using AG17 has the advantage of limited toxicity to normal astrocytes, which substantially broadens the therapeutic index of such a treatment regimen. Indeed, our use of nonlethal and growth-inhibitory doses of AG17 was intentional to avoid normal tissue toxicity. In addition, the AG17 dose used was also designed to minimize possible synergism between the cytotoxic effects of AG17 alone with those of BCNU, which would complicate the interpretation of any chemosensitization effect. The ability of all cells to propagate (in supplemented medium) for over a month while exposed to 0.25 \( \mu \text{M} \) AG17 without cytotoxicity and growth inhibition suggests that our observations were not due to synergistic effects. However, it is noted that AG17 has in vitro activity per se when used at higher doses (17, 25); hence, our study using very low doses of the drug may underestimate the effects of combination chemotherapy on glioblastoma cells. Nevertheless, our observations using nonlethal doses of the antimitochondrial agent AG17 to decrease mitochondrial mass and the number of polarized mitochondria is associated with increasing sensitivity of these cells to BCNU treatment, with limited changes in the corresponding normal cells (astrocytes).

The chemosensitization effects noted most likely relate, at least in part, to the effects of AG17 on the mitochondria. In addition to being able to determine a dose of drug that was not growth inhibitory or cytotoxic, we could identify a mechanism by which cells would spontaneously die an apoptotic death, e.g., by withdrawal of uridine and pyruvate supplements. The auxotrophic dependence on uridine and pyruvate is a sine qua non for respiratory deficient cells, and our demonstration of such auxotrophy is a strong argument that low-dose AG17 does indeed effect a loss of functional mitochondria. Because most cancer cells maintain energy production using glycolysis, rather than mitochondrial mediated oxphos, it is not surprising that cells could grow in the absence of functional mitochondria if supplemented with uridine and pyruvate supplements (5, 20). Although it is possible that other effects of AG17 may also be involved in the mechanism of chemosensitization (i.e., the tyrosine kinase antagonist activity), previous studies have shown that decreases in tyrosine kinase activity are correlated with growth inhibition (17). Because our cells were treated with doses that were not growth inhibitory and previous studies have shown no change in phosphotyrosine mass even at in higher doses (17), the effects of AG17 seem less well correlated with inhibition of signal transduction than with changes in mitochondrial function, which occurred early. Furthermore, the lack of effect of AG17 on NHAs and the inability to establish auxotrophic dependence in these cells also support the mitochondrial involvement in the chemosensitization effect. These data thus indicate that the chemosensitization effect is related to the antimitochondrial effects of AG17. Our previous studies chemosensitizing breast carcinoma and cisplatin-resistant U-937 cells using ethtidum bromide to deplete functional mitochondria and data from butyrate-induced apoptosis studies are in agreement with and support the present observations (26, 27).

The mechanism by which AG17 is antimitochondrial has yet to be defined (17, 25). Although some tyrphostins act to inhibit mitochondrial function, typically these agents use higher concentrations of drug to produce such an effect, which is much higher than those that antagonize tyrosine kinase (17). Initial

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**Table 4 Number of apoptotic nuclei with AG17 (0.25 \( \mu \text{M} \)) pretreatment and BCNU (144 \( \mu \text{g/ml} \)) administration**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Untreated*</th>
<th>1 day*</th>
<th>2 days*</th>
<th>3 days*</th>
</tr>
</thead>
<tbody>
<tr>
<td>OSMG</td>
<td>42/211 (20)</td>
<td>78/216 (36)</td>
<td>132/220 (60)</td>
<td>221/230 (96)</td>
</tr>
<tr>
<td>DK-MG</td>
<td>32/230 (14)</td>
<td>62/201 (31)</td>
<td>101/202 (50)</td>
<td>195/201 (97)</td>
</tr>
</tbody>
</table>

* Number of apoptotic nuclei/total number of nuclei counted; number in parentheses are percentages.

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studies (28, 29) showed that AG17 is a potent uncoupler of oxphos in low micromolar amounts. Our observations indicate that in the least, AG17 does affect the mitochondria by decreasing the numbers of polarized mitochondria, probably by diminishing the overall mitochondrial mass. Whether AG17 alone affects the mitochondria directly or acts upstream to alter different cellular metabolic processes that change mitochondrial function is unknown. It is of note, however, that antimitochondrial agents with antitumor activity can have direct effects on the organelle, which have been well described (28, 30). Indeed, certain types of cancers take up these compounds, such as the lipophilic cations, in a facile manner, related to increased anionic tumor $\Delta \Psi_m$ (30–34); this negative $\Delta \Psi_m$ also results in the retention of these drugs within the mitochondria and may be responsible for the antitumor effects seen in the present and previous studies (31). Our study shows that tumors such as GBM are also responsive to the antimitochondrial approach when combined with standard cytotoxic chemotherapy. Indeed, as well, submicromolar concentrations of AG17 per se were cytotoxic to both glioblastoma cell lines evaluated in this study.4

There are several advantages in using AG17 and related compounds in brain tumors. A prominent reason of the failure of chemotherapeutic approaches in treatment relates to the intrinsic drug resistance of glioma cells with isolation of the brain from drug (via the blood brain barrier; Ref. 35). Whereas agents used in gliomas such as BCNU are clearly lipophilic, it has been seen that their effects clinically are limited, and only rarely do complete responses occur (1, 2). Both in vitro and in vivo evidence suggests that this is related to exposure and access problems by drug (35). The present study shows that the BCNU dose-response can be shifted in GBM cells by pretreatment with AG17, increasing the sensitivity of the tumors to chemotherapy. Such increases in sensitivity should make the tumor more accessible to therapeutic intervention with drugs such as BCNU, which are clearly effective against brain tumors in vitro. An additional advantage is that AG17 is, in itself, a lipophilic agent, with no impediment to access the brain/tumor from the blood-brain barrier. Finally, AG17 is well tolerated by astrocytes. Hence, combination chemotherapy with AG17 and BCNU is a novel approach that may increase the utility of chemotherapeutic options in gliomas and other brain tumors. Further studies will be interesting to determine the viability of such an approach in malignant brain neoplasms.

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Chemosensitization of glioblastoma cells to bis-dichloroethyl-nitrosourea with tyrphostin AG17.

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