

# Nitrosoureas Inhibit the Stathmin-Mediated Migration and Invasion of Malignant Glioma Cells

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## Abstract

**Malignant gliomas are the most common primary intrinsic brain tumors and are highly lethal. The widespread migration and invasion of neoplastic cells from the initial site of tumor formation into the surrounding brain render these lesions refractory to definitive surgical treatment. Stathmin, a microtubule-destabilizing protein that mediates cell cycle progression, can also regulate directed cell movement. Nitrosoureas, traditionally viewed as DNA alkylating agents, can also covalently modify proteins such as stathmin. We therefore sought to establish a role for stathmin in malignant glioma cell motility, migration, and invasion and determine the effects of nitrosoureas on these cell movement-related processes. Scratch wound-healing recovery, Boyden chamber migration, Matrigel invasion, and organotypic slice invasion assays were performed before and after the down-regulation of cellular stathmin levels and in the absence and presence of sublethal nitrosourea ([1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea]; CCNU) concentrations. We show that decreases in stathmin expression lead to significant decreases in malignant glioma cell motility, migration, and invasion. CCNU, at a concentration of 10  $\mu\text{mol/L}$ , causes similar significant decreases, even in the absence of any effects on cell viability. The direct inhibition of stathmin by CCNU is likely a contributing factor. These findings suggest that the inhibition of stathmin expression and function may be useful in limiting the spread of malignant gliomas within the brain, and that nitrosoureas may have therapeutic benefits in addition to their antiproliferative effects. [Cancer Res 2008;68(13):5267-72]**

## Introduction

Although the initial treatment for many malignant gliomas is maximal surgical resection, the widespread migration and invasion of tumor cells into the surrounding brain tissue render a complete removal virtually impossible. Patients are therefore commonly treated with radiotherapy and chemotherapy in efforts to inhibit tumor cell proliferation and increase survival (1). The inhibition of cell migration and invasion is, in principle, likely to prevent the spread of tumors into eloquent brain regions and, in turn, preserve neurologic function. Clinical attempts to specifically effect such inhibition have, however, been limited (2).

Stathmin regulates dynamic instability, the growth and shrinkage of microtubules, by stimulating microtubule plus end

catastrophes and sequestering  $\alpha$ - $\beta$  tubulin dimers (3). Consequently, stathmin has direct effects on cellular processes such as migration, division, and growth cone guidance by influencing the association of microtubules with the actin cytoskeleton (4). Consistent with this, stathmin has been shown to influence sarcoma cell migration and invasion (5). Nitrosoureas such as ([1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea]; CCNU) have previously been shown to decompose under physiologic conditions to chloroethyl carbonium ions and reactive organic isocyanates. The former alkylate DNA and cause interstrand cross-links, whereas the latter carbamoylate proteins on  $\text{NH}_2$ -terminal amino groups and the amino groups of lysine side chains (6). Stathmin, which has a high percentage of lysine residues (16%), readily undergoes CCNU-mediated carbamoylation with a subsequent inhibition in microtubule depolymerization activity (7). We therefore hypothesized that CCNU, via its inhibitory effect on stathmin, could have an inhibitory effect on tumor cell migration and invasion that is independent of its antiproliferative activity.

To test this hypothesis, we first sought to establish a correlation between stathmin expression level and cell motility, migration, and invasion. We then analyzed the effects of sublethal concentrations of CCNU as well as temozolomide on these cell movement-related processes. Temozolomide was examined because of its increasing use in the treatment of patients with malignant gliomas (8). Finally, to establish a direct association between CCNU and stathmin, we assessed the effects of CCNU on the ability of stathmin to prevent the polymerization of microtubules, a well-characterized function of stathmin. The direct significance of our findings on malignant glioma therapies is discussed.

## Materials and Methods

**Cell culture.** The U251-STMNi and U251-LacZi cell lines were derived as previously described (7). Briefly, parental U251 cells (American Type Culture Collection) were transfected with pERV3 (Stratagene) using the Lipofectamine 2000 transfection reagent (Invitrogen). Stable transfectants, selected on the basis of G418 (Invitrogen) resistance, were then transfected with pEind-RNAi, which contains the hygromycin B resistance gene and either the stathmin-specific short-hairpin RNA (shRNA) oligonucleotide sequence 5'-AGTTGTTGTTCTCTCTATTGCCTTCTGATTGGTCAGAAGGCAATA-GAAGAGAACAACAAC-3' (U251-STMNi cells) or the LacZ-specific shRNA oligonucleotide sequence 5'-CTACACAAATCAGCGATTTCGAAAATCGCTGATTTGTGTAG-3' (U251-LacZi cells), downstream of a modified muristerone A (murA)-responsive U6 promoter sequence. Cells resistant to both G418 and hygromycin B (Invitrogen) were subcloned by limiting dilution.

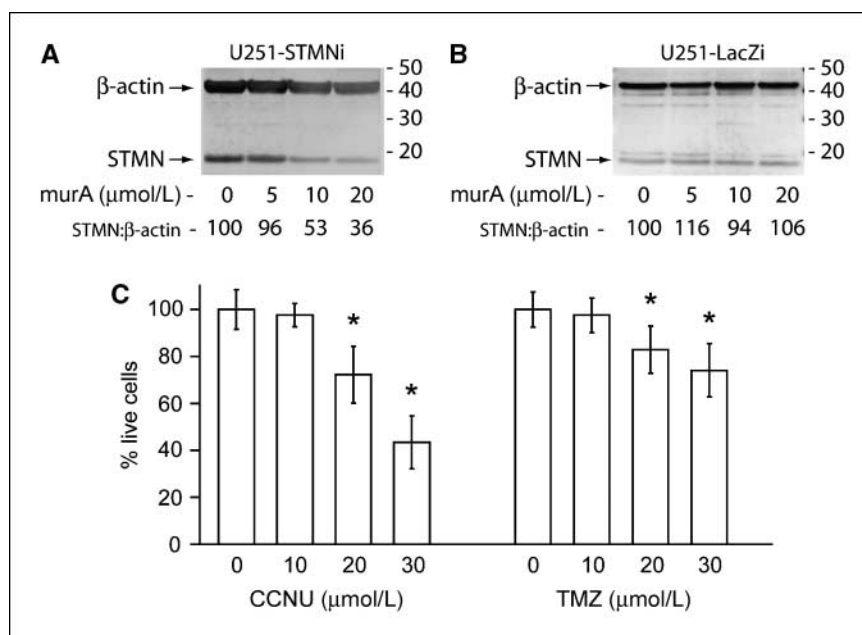
**Cell viability assays.** Cells ( $5 \times 10^4$ ) were seeded into 24-well plates (Corning, Inc.) and treated with DMSO control, 10 to 30  $\mu\text{mol/L}$  CCNU (HNZ Portlink), or 10 to 30  $\mu\text{mol/L}$  temozolomide (Drug Synthesis and Chemistry Branch, National Cancer Institute). After 3 d, cells were trypsinized and counted with a Coulter Particle Counter (Coulter Electronics). Experiments were performed in sextuplicate.

**Note:** X-J. Liang and Y. Choi contributed equally to this work.

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doi:10.1158/0008-5472.CAN-07-6482



**Figure 1.** Stathmin knockdown and drug sensitivity profiles of malignant glioma cell lines. *A* and *B*, evaluation of stathmin expression as determined by immunoblotting in U251-STMNi (*A*) and U251-LacZi (*B*) cells after treatment with increasing concentrations of murA for 72 h, which induces the transcription of stathmin-specific and LacZi-specific shRNAs, respectively. The former causes the knockdown of stathmin expression, whereas the latter serves as a control for the nonspecific effects of murA treatment as well as the transcription of nonspecific shRNAs. Evaluation of  $\beta$ -actin expression was performed on the same blots and used as an internal control to monitor loading and transfer of gel lanes. On the right side of each blot are molecular mass markers in kiloDaltons. Laser densitometric analysis of protein bands was performed, and the ratio of stathmin expression to  $\beta$ -actin expression (STMN: $\beta$ -actin) was determined and normalized to a value of 100 for the nonmurA-treated condition. There is a quantitative decrease in stathmin expression in the U251-STMNi cells after treatment of cells with increasing concentrations of murA (*A*). There is no such effect of murA treatment on stathmin expression in U251-LacZi cells (*B*). *C*, cells were cultured for 72 h in the presence of DMSO vehicle control (0  $\mu$ mol/L), or 10 to 30  $\mu$ mol/L CCNU or temozolomide (TMZ), and viable cells were counted. Cell counts were normalized to the number of vehicle-treated cells, and the mean relative percentages of live cells under each culture condition, indicated along the X axis, is plotted along the Y axis. Experiments were performed in sextuplicate. Columns, mean; bars, SD. \*, drug concentrations in which the mean number of viable cells differs significantly from the mean number of viable cells present in the vehicle control condition ( $P < 0.05$ ).

**Immunoblotting analysis.** Cells cultured for 72 h in the absence or presence of increasing concentrations of murA (Invitrogen), which enhances the activity of a modified U6 promoter and results in the transcription of downstream shRNA oligonucleotide sequences, were solubilized using 1% Nonidet P-40 in TBS (Sigma). Whole cell extracts were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore), which were subsequently probed with primary rabbit antistathmin (Calbiochem; 1:10,000 dilution) and mouse anti- $\beta$ -actin (Sigma; 1:40,000 dilution) antibodies followed by secondary alkaline phosphatase-conjugated goat anti-rabbit and goat anti-mouse IgG antibodies (Promega; 1:7500 dilution). Membranes were developed using DDAO phosphate (Invitrogen) and scanned using an FLA-5100 laser based scanner (Fujifilm). Image processing including laser densitometric analysis of stathmin and  $\beta$ -actin expression levels was performed using MultiGauge version 3.0 imaging software (Fujifilm). Quantitative relative stathmin expression levels were determined by measuring the quantitative light absorbance units minus background (QL-BG) for each of the stathmin and  $\beta$ -actin bands. The ratio of stathmin<sup>(QL-BG)</sup> to  $\beta$ -actin<sup>(QL-BG)</sup> was determined for each sample and normalized to the stathmin<sup>(QL-BG)</sup> to  $\beta$ -actin<sup>(QL-BG)</sup> ratio for the nonmurA-treated sample, which was assigned the value of 100.

**Scratch wound-healing recovery assays.** Cells cultured in the absence or presence of 10  $\mu$ mol/L murA for 72 h were allowed to reach confluence. A 20- $\mu$ L pipette tip (ThermoFisher Scientific) was used to scratch and create a wound in the confluent monolayer. Detached cells were immediately removed by replacement of medium and where indicated, CCNU (10  $\mu$ mol/L), temozolomide (10  $\mu$ mol/L), or both were added. Images were subsequently captured, and then again 24 h later, using an AxioCam MRm camera, an Axiovert 200 microscope, and AxioVision version 4.2 software (all Zeiss). Experiments were performed in triplicate.

**Cell migration assays.** Cells cultured in the absence or presence of 10  $\mu$ mol/L murA for 72 h were added to the upper compartments of 96-well

Boyden chamber plates containing 8- $\mu$ m pore size polycarbonate filters (Neuro Probe). After 16 h of culture in medium containing drug vehicle, CCNU (10  $\mu$ mol/L), temozolomide (10  $\mu$ mol/L), or both CCNU and temozolomide, the polycarbonate filters were removed, fixed with methanol for 10 min, and stained with modified Trypan Blue stain (0.2% in PBS) for 20 min to facilitate visualization and counting of cells. Cells on the upper side of the filter were removed using a rubber scraper, and the migrated cells on the underside of the filter were counted with the aid of a microscope. Experiments were performed in sextuplicate.

**Matrigel invasion assay.** The polycarbonate filter of each Boyden chamber well was coated with Matrigel (BD Biosciences). Cells cultured in the absence or presence of 10  $\mu$ mol/L murA for 72 h were inoculated into the upper chambers, and the chemoattractant rhSDF-1 $\alpha$ /PBSF (pre-B-cell growth-stimulating factor, 100ng/mL; R&D Systems) was added to the bottom chambers. After 24 h of culture in medium-containing drug vehicle, CCNU (10  $\mu$ mol/L), temozolomide (10  $\mu$ mol/L), or both CCNU and temozolomide, the Matrigel-coated polycarbonate filters were removed, fixed, and stained as above. Invaded cells on the underside of the filter were counted as above. Experiments were performed in sextuplicate.

**Glioma invasion in organotypic brain slices.** Brain slices were obtained from 10-d-old NIH Swiss mice and cultured as previously described (9). Glioma cells incubated with Dil (3H-Indolium, 2-[3-(1,3-dihydro-3,3-dimethyl-1-octadecyl-2H-indol-2-ylidene)-1-propenyl]-3,3-dimethyl-1-octadecyl-, perchlorate; 30  $\mu$ mg/mL; Invitrogen) were plated onto 6-well plates at a density of  $5 \times 10^4$  cells/10  $\mu$ L. Upside-down overnight culture of the cell suspensions led to the formation of glioma spheroids ~0.5 mm in diameter. Spheroids were picked and implanted into the corpus callosa of the brain slices with the aid of an inverted microscope equipped with a calibrated micromanipulator. Slices were maintained in culture medium containing no drugs, CCNU (10  $\mu$ mol/L), murA (10  $\mu$ mol/L), or both CCNU and murA. Medium, including drug where

appropriate, were replenished every 3 d. Images were captured after 4 wk and analyzed using the same equipment and software as for the scratch wound–healing assays. Animal studies were carried out in accordance with NIH institutional guidelines for the care and use of animals. Experiments were performed in triplicate.

**In vitro tubulin polymerization assay.** Tubulin polymerization assays were performed using purified rat tubulin as described (10, 11). Six-histidine-tagged recombinant stathmin (5  $\mu\text{mol/L}$ ) was produced as previously described (7), and when indicated, was pretreated for 6 h with CCNU (10  $\mu\text{mol/L}$ ) followed by removal of free CCNU using a Zeba micro desalt column (Pierce). Experiments were performed in triplicate.

**Statistical analysis.** Student's *t* tests were performed where appropriate using JMP 5.1 (SAS Institute, Inc.). *P* value of <0.05 was considered significant.

## Results

**Stathmin knockdown and CCNU inhibit scratch wound healing.** To determine the effects of stathmin knockdown on cell motility, we first generated a U251 malignant glioma–derived cell line in which *murA* enhances the transcription of a stathmin-specific shRNA and induces the knockdown of stathmin expression (U251-STMNi). We also generated a control cell line in which *murA* enhances the transcription of a LacZ-specific shRNA (U251-LacZi). The *murA*-induced knockdown of stathmin protein expression by U251-STMNi cells is dose dependent with a 47% decrease occurring in response to treatment with 10  $\mu\text{mol/L}$  for 72 hours (Fig. 1A). In contrast, treatment of U251-LacZi cells with *murA* concentrations as high as 20  $\mu\text{mol/L}$  do not have appreciable effects on stathmin expression (Fig. 1B). All further experiments involving the use of *murA* were carried out using a concentration of 10  $\mu\text{mol/L}$ .

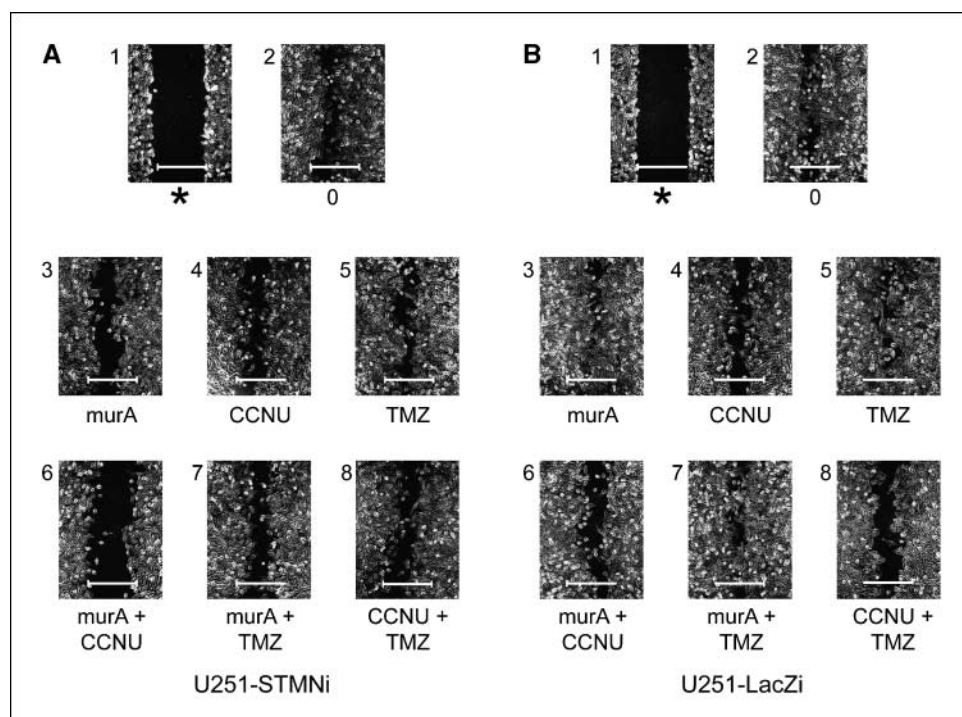
Given the cytotoxic effects of CCNU and temozolomide, we sought to establish experimental conditions under which decreased cell motility rather than decreased cell viability would be the primary potential cause for decreased scratch wound healing.

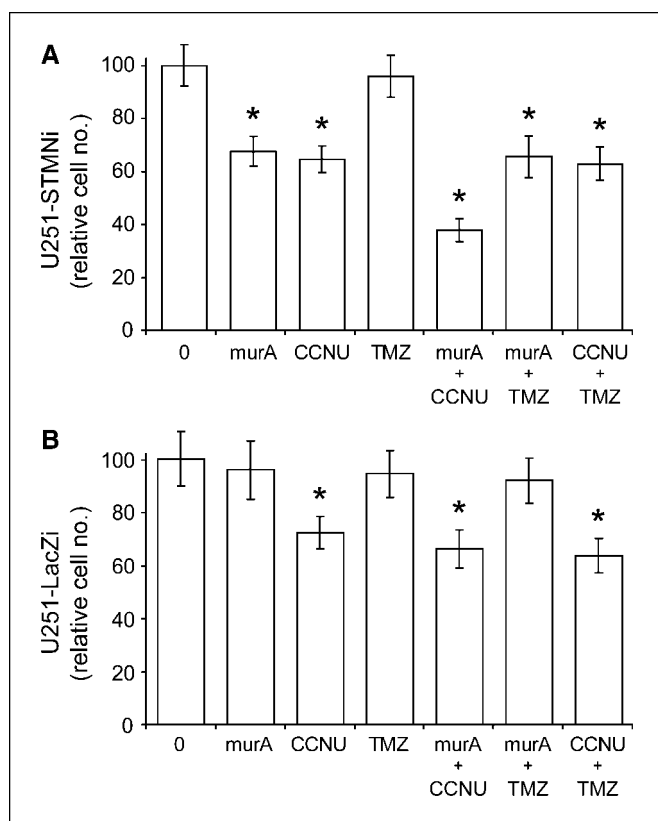
Treatment of cells with 10  $\mu\text{mol/L}$  CCNU does not have a significant effect on cell viability ( $P = 0.6520$ ); however, treatment with 20  $\mu\text{mol/L}$  CCNU leads to a 28% decrease in cell number after 72 hours ( $P = 0.0100$ ; Fig. 1C). Similarly, treatment of cells with 10  $\mu\text{mol/L}$  temozolomide does not have a significant effect on cell viability ( $P = 0.5935$ ); however, treatment with 20  $\mu\text{mol/L}$  temozolomide leads to a 17% decrease in cell number after 72 hours ( $P = 0.0411$ ; Fig. 1C). All further experiments involving CCNU and/or temozolomide were therefore carried out using a concentration of 10  $\mu\text{mol/L}$  to minimize the potential confounding effect of decreased cell viability.

U251-STMNi cells treated with *murA* alone (Fig. 2A3) show decreased wound healing compared with non*murA*-treated U251-STMNi cells (Fig. 2A2). As controls for the effects of *murA* treatment and shRNA expression per se, U251-LacZi cells with (Fig. 2B3) and without (Fig. 2B2) *murA* pretreatment were also assessed, and there seems to be no differences in wound healing. CCNU treatment alone inhibits wound healing by both U251-STMNi (Fig. 2A4) and U251-LacZi (Fig. 2B4) cells, but temozolomide treatment alone does not affect either cell type (Fig. 2A5 and B5). The combination of stathmin knockdown and CCNU treatment seems to be additive in inhibiting U251-STMNi (Fig. 2A6) but not U251-LacZi (Fig. 2B6) cell wound healing. There is no further decrease in wound healing in either cell type due to the addition of temozolomide to *murA* or CCNU (Fig. 2A7, A8, B7, and B8).

**Stathmin knockdown and CCNU inhibit cell migration and invasion.** To quantitate the effects seen in the scratch wound–healing assays, Boyden chamber migration assays were performed (Fig. 3). *MurA* induced knockdown of stathmin expression in U251-STMNi cells causes a 32% decrease in migration ( $P = 0.0008$ ), whereas CCNU treatment causes a 35% decrease ( $P < 0.0001$ ). Treatment of U251-STMNi cells with the combination of *murA* and CCNU causes a 62% decrease in migration, which is significantly greater than that seen after treatment with the former ( $P = 0.0002$ ) or the latter ( $P = 0.0004$ ) alone. In contrast, temozolomide

**Figure 2.** Scratch wound–healing assay. A and B, U251-STMNi (A) and U251-LacZi (B) cells grown to confluence were scratched to create a wound and then washed with medium to remove loosened cells. Drug vehicle (A2, A3, B2, and B3), 10  $\mu\text{mol/L}$  CCNU (A4, A6, A8, B4, B6, and B8), and/or 10  $\mu\text{mol/L}$  temozolomide (A5, A7, A8, B5, B7, and B8) were added to cultures as indicated. Cultures were photographed subsequently and then again 24 h later to assess the degree of wound healing. Cells cultured in the presence of 10  $\mu\text{mol/L}$  *murA* (A3, A6, A7, B3, B6, and B7) were done so for 72 h before scratching, and *murA* was not replaced after the removal of loosened cells. \*, representative photographs of the respective cell lines immediately after scratching and washing (A1 and B1). White scale bar, 500  $\mu\text{m}$ . Experiments were performed in triplicate and representative results are shown.





**Figure 3.** Boyden chamber migration assay. *A* and *B*, U251-STMNi (*A*) and U251-LacZi cells (*B*) that had migrated through Boyden chamber membranes during the course of a 16-h incubation period were counted and normalized to the number of migrating untreated U251-STMNi and U251-LacZi cells, respectively. Where indicated, cells were exposed to CCNU or temozolomide at the start of the 16-h assay period. Cells treated with murA were done so for 72 h before the initiation of the assay. Various treatment conditions are indicated along the X axis, and the normalized mean relative cell numbers are plotted along the Y axis. Experiments were performed in sextuplicate. Columns, mean; bars, SD. \*, drug treatment conditions in which the mean number of migrating cells differs significantly from the mean number of migrating cells in the vehicle control condition ( $P < 0.05$ ).

treatment by itself has no significant effect on migration ( $P = 0.1092$ ) and does not enhance the effects of murA ( $P = 0.6701$ ) or CCNU ( $P = 0.4027$ ) either (Fig. 3A). CCNU treatment of U251-LacZi cells causes a 30% decrease in migration ( $P = 0.0008$ ), but murA treatment of these cells has no significant effect when used alone ( $P = 0.5960$ ) and does not enhance the effects of CCNU ( $P = 0.1297$ ). Temozolomide alone also has no significant effects on the migration of U251-LacZi cells ( $P = 0.3839$ ) and also does not enhance the effects of CCNU either ( $P = 0.1138$ ; Fig. 3B).

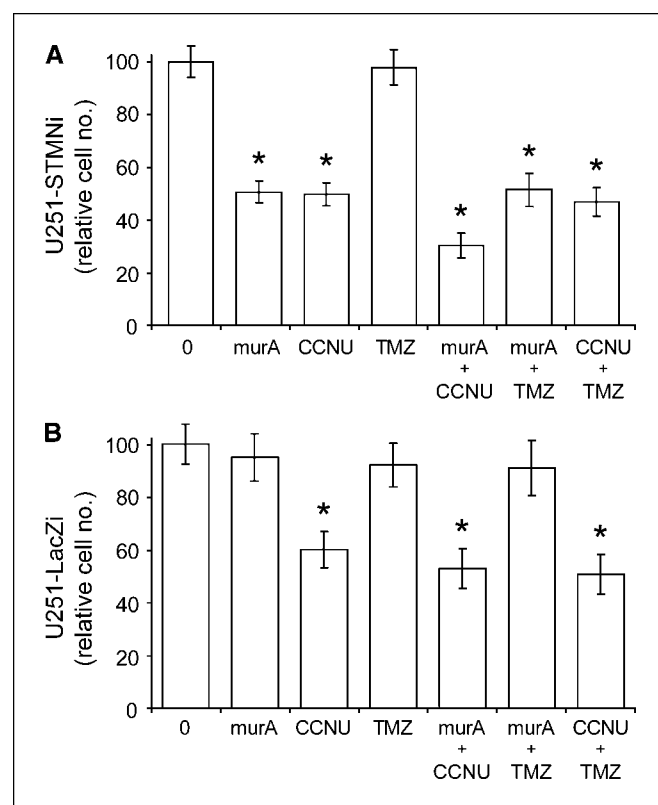
The movement of cells through Matrigel, an *in vitro* model of basement membrane invasion, was evaluated. In U251-STMNi cells, murA-induced stathmin knockdown inhibits invasion by 49% ( $P < 0.0001$ ), whereas CCNU treatment does so by 50% ( $P < 0.0001$ ). The combination of the two inhibits invasion by 70% ( $P < 0.0001$ ), which is significantly greater than that seen in response to either stathmin knockdown ( $P = 0.0013$ ) or CCNU treatment ( $P = 0.0014$ ) alone (Fig. 4A). CCNU treatment of U251-LacZi cells causes a 40% decrease in invasion ( $P = 0.0003$ ), but murA treatment of these cells has no significant effects ( $P = 0.0756$ ; Fig. 4B). Again, temozolomide has no effects by itself and does not enhance the effects of either murA treatment or CCNU treatment in either U251-STMNi or U251-LacZi cells (all  $P > 0.0821$ ; Fig. 4A and B).

We also assessed the movement of tumor cells within organotypic slice preparations, a model for the *in vivo* invasion of tumor cells into surrounding brain tissue. As expected, U251-STMNi cell invasion is decreased by murA, CCNU, and the combination of murA and CCNU (Fig. 5A). U251-LacZi cell invasion is also decreased by CCNU, but murA treatment does not by itself seem to have any appreciable effects nor enhance the effects of CCNU in this cell type (Fig. 5B).

**CCNU directly inhibits stathmin function.** Stathmin prevents microtubule polymerization by sequestering  $\alpha$ - $\beta$  tubulin dimers (3). To determine if CCNU could inhibit this stathmin function, tubulin polymerization assays were performed. The steady-state concentration of polymerized microtubules in a tubulin-containing solution is decreased 63% by the addition of native stathmin. This stathmin-mediated decrease is abrogated by 59% if the stathmin is pretreated with 10  $\mu$ mol/L CCNU (Fig. 6). CCNU by itself has no significant effects on tubulin polymerization (data not shown).

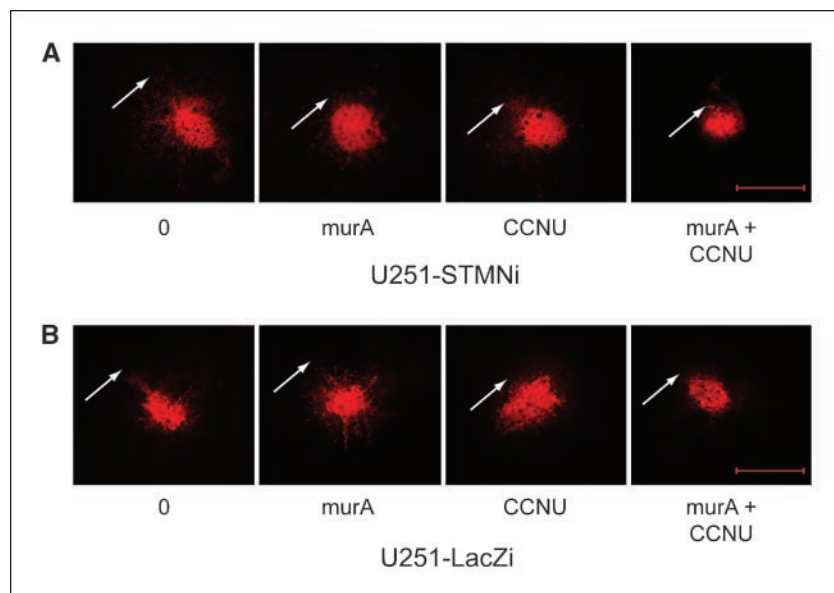
## Discussion

The infiltration of malignant gliomas into eloquent brain regions compromises neurologic function and is a major cause of



**Figure 4.** Matrigel invasion assay. *A* and *B*, U251-STMNi (*A*) and U251-LacZi cells (*B*) that had invaded through Matrigel-coated Boyden chamber membranes during the course of a 24-h incubation period were counted and normalized to the number of invading untreated U251-STMNi and U251-LacZi cells, respectively. Where indicated, cells were exposed to CCNU or temozolomide at the start of the 24-h assay period. Cells treated with murA were done so for 72 h before the initiation of the assay. Various treatment conditions are indicated along the X axis, and the normalized mean relative cell numbers are plotted along the Y axis. Experiments were performed in sextuplicate. Columns, mean; bars, SD. \*, drug treatment conditions in which the mean number of invading cells differs significantly from the mean number of invading cells in the vehicle control condition ( $P < 0.05$ ).

**Figure 5.** Organotypic slice invasion assay. *A* and *B*, Dil-labeled U251-STMNi (*A*) and U251-LacZi (*B*) tumor cell spheroids were implanted into organotypic brain slice cultures and treated with 10  $\mu\text{mol/L}$  murA and/or 10  $\mu\text{mol/L}$  CCNU as indicated below each photomicrograph. Red Dil-labeled tumor cells were monitored for invasion into surrounding tissues. *A*, murA-treated U251-STMNi cells exhibited less invasion into surrounding tissues when compared with untreated cells. CCNU treatment also inhibited invasion of U251-STMNi cells into surrounding tissues, and the effects of murA and CCNU seemed to be additive. *B*, in contrast, murA treatment of U251-LacZi cells did not have any appreciable effects on the invasion of cells into surrounding tissues. CCNU treatment did, however, inhibit the invasion of U251-LacZi cells. MurA treatment did not augment the inhibitory effects of CCNU on U251-LacZi cell invasion. *White arrows*, extent of tumor cell invasion into surrounding tissues. *Red scale bar*, 500  $\mu\text{m}$ . Experiments were performed in triplicate and representative results are shown.



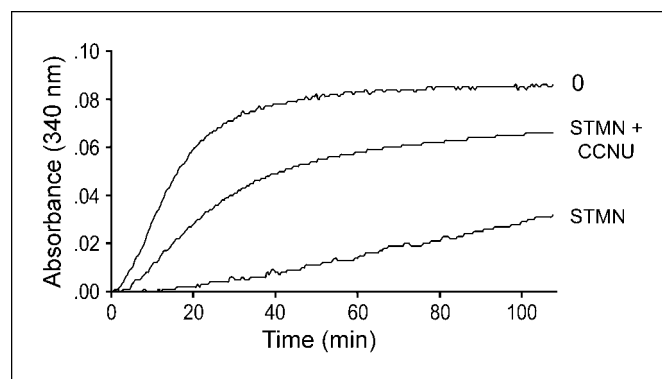
morbidity. Although most malignant glioma therapies are intended to be cytostatic or cytotoxic, therapies intended to inhibit tumor cell migration and invasion are also likely to be beneficial. With this in mind, we investigated the roles and interactions of stathmin and CCNU in the directed movements of malignant glioma cells. The results presented above show that both decreases in stathmin expression and treatment with CCNU can inhibit the migration and invasion of malignant glioma cells. The concentration of CCNU used throughout these studies (10  $\mu\text{mol/L}$ ) was specifically selected because it has no significant effects on cell survival and thereby minimizes decreased cell viability per se as a potential confounding effect on cell motility related functions. Ten  $\mu\text{mol/L}$  is also the approximate mean peak plasma concentration achieved in patients after p.o. administration of CCNU at a dose of 15 mg/kg (12).

A plausible mechanism by which CCNU can retard migration and invasion is through the direct inhibition of stathmin function as shown in the tubulin polymerization assay. Consistent with this model, temozolomide, an imidazotetrazine DNA alkylating agent with no direct effects on stathmin function (7), has no effects on cell migration or invasion. Temozolomide also does not act to enhance the inhibitory effects of stathmin knockdown or CCNU treatment on these cell movement related functions.

The median survival of malignant glioma patients treated with radiotherapy plus temozolomide has been shown to be significantly greater than that of patients treated with radiotherapy alone ( $P < 0.001$ ; ref. 13). In contrast, the addition of nitrosoureas to radiotherapy has not been shown to provide a significant overall survival benefit ( $P = 0.108$ ; ref. 1). One implication of these two studies is that temozolomide is more effective than nitrosoureas in inducing lethal DNA cross links in proliferating tumor cells. Although this is likely the case, our results indicate that nitrosoureas are superior to temozolomide in inhibiting tumor cell motility. A possible therapeutic strategy would then be radiotherapy plus the combination of temozolomide and a nitrosourea, the former to control proliferation and the latter to control invasion.

In a recent phase II clinical trial of radiotherapy and temozolomide plus CCNU, malignant glioma patients whose tumors had promoter methylation of the O<sup>6</sup>-methylguanine-DNA methyltrans-

ferase (MGMT) gene had a median progression-free survival of 19 months (14). In a phase III clinical trial of patients also with MGMT promoter methylated tumors, the median progression-free survival after radiotherapy and temozolomide treatment was 10.3 months (13). The almost 2-fold increase in progression-free survival with the addition of CCNU is consistent with a model in which progression is due to a variable combination of local and/or distant tumor recurrence defined as expanding and/or new areas, respectively, of contrast enhancement on magnetic resonance imaging. The latter requires the movement of tumor cells away from the original tumor site. Due to its inhibitory effects on migration and invasion, CCNU may delay this infiltration of tumor cells into surrounding brain tissues and, consequently, the appearance of new areas of contrast enhancement. When eloquent brain regions are spared, there may also be a delay in the deterioration of neurologic function. A phase III clinical trial



**Figure 6.** Tubulin polymerization assay. The polymerization of tubulin into microtubules was initiated by warming a tubulin-containing solution from 0°C to 37°C. The polymerization reaction was carried out in the absence of stathmin (0), the presence of native unmodified stathmin (STMN), or the presence of 10  $\mu\text{mol/L}$  CCNU-pretreated stathmin (STMN + CCNU). The concentration of polymerized microtubules present in the solution was monitored over time (X axis) for up to 120 min by determining the absorbance (Y axis) at 340 nm in a temperature-controlled 96-well microplate spectrophotometer. Unmodified stathmin inhibits microtubule formation but modification of stathmin by 10  $\mu\text{mol/L}$  CCNU abrogates this inhibition. Experiments were performed in triplicate and representative results are shown.

assessing the addition of nitrosoureas to radiotherapy and temozolomide should therefore be considered, given our experimental data and the promising results of the phase II clinical trial described above.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Acknowledgments

Received 12/7/2007; revised 4/18/2008; accepted 4/22/2008.

**Grant support:** Intramural Research Program of the NIH, National Institute of Neurological Disorders and Stroke and National Institute of Child Health and Human Development.

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We thank A. Sedlock for providing technical assistance, G. Park for critical review of the manuscript, and L. Wu and J. Isaac for advice on the organotypic slice cultures.

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