Marked Inhibition of Tumor Growth in a Malignant Glioma Tumor Model by a Novel Synthetic Matrix Metalloprotease Inhibitor AG3340

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ABSTRACT
Synthetic matrix metalloprotease (MMP) inhibitors have activity against a variety of tumors in preclinical models but have not been studied in gliomas. We determined the effect of AG3340, a novel synthetic MMP inhibitor with \( K_i \) values against gelatinases in the low picomolar range, on the growth of a human malignant glioma cell line (U87) in SCID-NOD mice. Mice were injected s.c. with U87 cells. Tumors were allowed to grow to a size of approximately 0.5 \( \times \) 0.5 cm (after about 3 weeks), and the mice were randomized to receive either: (a) 100 mg/kg AG3340 in vehicle; or (b) vehicle control (0.5% carboxymethyl cellulose, 0.1% pluronic F68), both given daily i.p. Tumor area was measured twice weekly, and animals were sacrificed when moribund, or earlier if premorbid histology was examined. In vivo inhibition of tumor growth was profound, with AG3340 decreasing tumor size by 78% compared with controls after 31 days (when controls were sacrificed; \( P < 0.01 \), Wilcoxon test). Control animals survived 31 days after the i.p. injections began, and AG3340 mice survived 71 days, representing a >2-fold increase in survival associated with tumor growth delay. Histological examination found that AG3340-treated tumors were smaller, had lower rates of proliferation, and significantly less invasion than control-treated tumors. Hepatic or pulmonary metastases were not seen in either group. In a separate experiment, the tumors were smaller and sampled after a shorter duration of treatment; the changes in proliferation were more marked and occurred earlier than differences in tumor invasion between the two groups. Furthermore, in vitro cell growth was not inhibited at AG3340 concentrations of <1 mM. AG3340 markedly increased survival in this in vivo glioma model. Treatment with AG3340 may be potentially useful in patients with malignant gliomas.

INTRODUCTION
Human malignant glial tumors comprise the majority of primary brain tumors. Glial cancer patients have a dismal prognosis; their median survival is about 12 months, and long-term survivors are rare (1). The hallmarks of these neoplasms are that they display a high degree of vascularity and invasion, with tumor cells commonly invading several centimeters beyond the main tumor mass at diagnosis (2–4), thus rendering surgical cure impossible. Because MMPs are being increasingly implicated in the extracellular matrix remodelling required for glioma invasion and angiogenesis (5–7), we considered MMP inhibition, by using a synthetic inhibitor, to be a potentially important new therapeutic strategy.

The MMPs are the principal secreted proteinases required for extracellular matrix degradation in a variety of physiological and pathological tissue remodelling processes, including wound healing, embryo implantation, tumor invasion, metastasis, and angiogenesis (8–10). At least 18 MMPs have been described (7, 11), which are subdivided into the collagensases, stromelysins, gelatinases, and membrane-type MMPs (12). Their activities are tightly controlled at the levels of gene transcription, zymogen activation by proteolysis, and inhibition of active forms by the TIMPs (13). There is a wealth of evidence for an association between either deregulated production or activation of MMPs and aggressive or invasive behavior in a variety of human cancers (14–18). Similarly, evidence that MMPs are involved in invasion and angiogenesis in gliomas comes from observations
that gelatinase-A (MMP-2), gelatinase-B (MMP-9), and MT1-MMP have been found in several glioma cell lines and surgical specimens (19–35), that gelatinase-A or gelatinase-B protein is localized to the tumor microvasculature (28, 29, 34), and that the amount of MMP overexpression correlates with glioma grade (28, 29, 35).

A number of low molecular weight synthetic MMP inhibitors are under development by the pharmaceutical industry. In general, these have a peptide backbone similar to the cleavage site on collagen that binds the MMP, and they contain a hydroxamate group that coordinates the catalytic zinc ion in the active site (36). Several promising studies have been published that find antitumor activity of synthetic MMP inhibitors in a variety of in vivo tumor models. The most widely studied of these is batimastat (also called BB-94; British Biotech Ltd., Oxford, United Kingdom), which produced prolonged survival in an ovarian tumor xenograft (37); inhibited metastasis of melanoma (38), breast (39), and colon cancer (40) cell lines; and inhibited growth of a colon cancer xenograft (41), a breast cancer cell line (42), and a hemangioma (43). Other synthetic MMP inhibitors (Celltech Therapeutics, Ltd., Slough, United Kingdom) inhibit the growth of prostate, melanoma (44), and lung cancer cell lines (45). There are no published studies regarding the use of synthetic MMP inhibitors in malignant gliomas.

AG3340 is a novel synthetic MMP inhibitor (Agouron Pharmaceuticals) that selectively inhibits critical MMPs at picomolar concentrations. AG3340 has one of the lowest $K_s$ for gelatinase-A and gelatinase-B (50–150 pm). It has a low molecular weight ($M_r$ 423.5), is lipophilic, and crosses the blood-brain barrier. These properties are critical if AG3340 is to be used clinically in the treatment of gliomas; preliminary results in a number of tumor models show AG3340 has antitumor activity (46–50). In the present study, we were interested in determining whether AG3340 would inhibit glioma invasion or growth of the human malignant glioma cell line U87. We observed a marked reduction in tumor invasion and a profound inhibition of tumor growth and cellular proliferation with AG3340 treatment.

**MATERIALS AND METHODS**

**U87 Cell Line.** This human malignant glioma cell line was obtained from American Type Culture Collection (American Tissue Culture Collection, Rockville, MD). The cells were grown in DMEM containing F12/10% FCS; cells were passaged when they reached ~80% confluence, harvested by trypsin treatment, and replaced in DMEM-F12/10% FCS.

**Tumor Implantation.** Female SCID-NOD mice (Cross Cancer Institute, Edmonton, Alberta), 6–8 weeks of age, were used. For injection into animals, cells were grown to ~80% confluence, harvested, counted using a hemocytometer, rinsed twice in PBS (Life Technologies, Inc.), and resuspended at a density of 2.0 × 10$^6$ cells/50 μl in PBS. For s.c. injection, the cell suspension was drawn up to 0.2 ml in a syringe for each injection, with a 26-gauge, 3/8-inch intradermal needle, and 2.0 × 10$^6$ injected s.c. into a volume of 50 μl above the midfemur region of the left hind limb of the mouse. So that we studied only established and growing tumors, the lesions were allowed to grow until their average size was approximately 0.5 × 0.5 cm (after about 3 weeks). The mice were then randomized to treatment with either vehicle control or AG3340 on day 0. Tumor cross-sectional area was measured twice weekly and calculated by multiplying the length × width. On the day of sacrifice, tumor height was also measured, and tumor volumes were estimated by multiplying length × width × height × 0.5 (42). Tumor areas for each treatment group were averaged and compared using the Wilcoxon test. The Wilcoxon test was used because of the heterogeneity of variance of tumor area between these two groups. We performed two series of experiments (series 1 and 2) to characterize tumor growth and histologically analyze the tumors produced (Fig. 1). Animals were sacrificed as a group when they became moribund (i.e., had trouble with ambulation or feeding). In series 1, the purpose of the experiments was to compare tumor size between the AG3340 and control groups. To control for the duration of treatment and tumor size when examining the histological characteristics of these tumors, half of the AG3340 group was sacrificed on the same day as the control group (i.e., to control for duration), and the other half of the AG3340 group was allowed to grow until sacrifice was indicated (i.e., when the tumors were the same size as those in the much earlier sacrificed control group). In series 2, the purpose of the experiments was to identify the earlier mechanisms of glioma growth inhibition by examining tumor samples from mice with tumors that were smaller and treated for shorter periods of time than in series 1.

**AG3340 Treatment.** AG3340 was obtained as a white powder from Agouron Pharmaceuticals, Inc. An aqueous suspension was prepared by homogenizing and sonicating in 0.5% carboxymethyl cellulose and 0.1% pluronic F68 (Life Technologies, Inc.). Control solutions of vehicle without AG3340 were also prepared. Animals were injected with either 100 mg/kg AG3340 or vehicle control i.p. daily for 6 days each week until sacrifice; twice the dose was given on the sixth day of each week, and none on day 7.

**Sample Preparation.** Tumors were dissected and excised in situ, briefly washed with saline to remove excess blood, and divided into three parts. We preserved the tumor-host architecture during dissection by collecting both the overlying skin and underlying muscle (and bone where appropriate). One part was placed in 10% formalin, embedded in paraffin, sectioned, and stained with H&E and with antibodies to GFAP (Dako, Copenhagen, Denmark) for routine histological examination. A second part, consisting only of tumor (i.e., without skin, bone, etc.) was snap frozen in liquid nitrogen and stored at ~70°C for zymographic analysis. The final third was placed in Tissue-Tek OCT Compound (Sakura Finetek, Torrance, CA) and then snap frozen and stored at ~70°C. The liver and lungs were also excised, placed in formalin, and processed as described above. Three mice (two in AG3340 and one in the control group) had to be sacrificed on weekends, and their tissue was unavailable for analysis.

**Analysis of Proliferation Using BrdUrd.** We used the technique of immunostaining for BrdUrd as a measure of cellular proliferation in experiment 2 (51). Briefly, mice received
injections of BrdUrd (100 mg/kg) i.p. 1 h before sacrifice; then tumor tissues were collected, fixed in 10% formalin, and sectioned in paraffin. Sections were then deparaffinized and hydrated. Sections were treated with 2 N HCl for 10 min to denature DNA, followed by 0.1 M sodium borate to neutralize the HCl. BSA (0.3%) was then added to block nonspecific binding, and sections were incubated with a mouse monoclonal anti-BrdUrd antibody (1:10, Becton Dickinson) overnight at 4°C. Goat anti-mouse IgG conjugated with biotin (1:100) was added for 60 min, and sections were then incubated with Streptavidin-FITC (1:100), counterstained with propidium iodide (10µg/ml), and mounted with Gelvatol. Sections were visualized using an immunofluorescence microscope equipped with appropriate filters. The number of BrdUrd-positive nuclei was counted in the most cellular areas of the tumor and defined as a proportion of total nuclei stained with propidium iodide in the same fields (×1000; oil immersion) and compared using the χ² test.

Analysis of Apoptosis. Apoptotic cells were labeled using the ApoTag in situ detection kit (Oncor, Oncogene Research Products). Briefly, paraffin slides were deparaffinized followed by digoxigenin-dUTP labeling of apoptotic fragmented DNA 3’OH ends. The subsequent steps involved binding of fluorescein-conjugated anti-digoxigenin antibody and viewing by epifluorescence. Counterstaining was done using propidium iodide. The average number of positive nuclei was recorded and defined as a proportion of total nuclei stained with propidium iodide in three high power (×400) fields.

Analysis of Vascularization. We assessed vascularization in tumors that were processed to paraffin blocks, cut into 8-µm-thick sections, and stained with rabbit antisera to laminin (Sigma). Briefly, laminin antisera stains vascular basal lamina and is a reliable marker of vessel growth in brain tumors. We followed the methods described elsewhere (52), except enzyme digestion was done with trypsin for 2 h. Sections were counterstained with hematoxylin (to optimize assessment of general tissue morphology), dehydrated, and mounted in Permount (Fisher). Development time was the same for controls and experimental slides and performed in parallel. All controls performed were negative. The paraffin section of each tumor with the greatest cross-sectional area was selected, stained as above, assigned a code number by one investigator (Q. S.), and read by an observer (P. F.) blinded to the particular experiment or treatment group. The total number of vessel profiles per high powered field (×250) was counted, and the tumor regions were categorized (e.g., margin, proliferating tumor, necrotic area, and others) in adjacent microscopic fields across the maximum tumor diameter ranging from the tumors inferior border, through the center of the tumor to the region adjacent to the skin. Four to 17 fields/tumor were counted (depending on tumor diameter), spanning the total diameter of solid tumor and excluding the fields where the tumor cells infiltrated into the underlying muscle. For each tumor specimen, the number of laminin-positive profiles/field was averaged and compared using the Wilcoxon rank sums test.

Zymographic Analysis. This in vitro assay uses gelatin-substrate gel electrophoresis to measure the levels of metalloproteinase activity in tumor samples. Frozen tumor tissues from series 1 (n = 32) were pulverized in liquid nitrogen and homogenized in buffer [0.5 M Tris-HCl (pH 7.6), 0.2 M NaCl, 10 mM CaCl₂, and 1% Triton X-100] in an Ultra-Turrax-25 homogenizer. Ten µg of total protein from homogenate supernatants were electrophoresed on 10% SDS polyacrylamide gels containing 1 mg/ml of gelatin. Gels were washed overnight in washing buffer [50 mM Tris-HCl (pH 8.0), 5 mM CaCl₂, and 2.5% Triton X-100] and then incubated for 24 h at 37°C in the above buffer without Triton-X 100 so that renaturation of enzyme could occur. Gels were stained with Coomassie blue and destained. Gelatinolytic activities were visualized as clear bands against a blue background (35). Recombinant human TIMP-1 was a gift of Dr. Andrew Docherty (Celltech, Slough, United Kingdom).
AG3340 Inhibits Glioma Growth

Tumor cell viability and proliferation were studied using AG3340 (10 μM) or vehicle control. BrdUrd labeling (Amersham) was carried out for 60 min at 37°C in DMEM supplemented with 10% FCS. Subsequently, the cells were washed in PBS and fixed in ethanol for 30 min at −20°C. Anti-BrdUrd mouse monoclonal antibodies (Becton Dickinson) were applied for 1 h, and cover slips were washed in PBS. The mouse monoclonal antibody was detected by immunofluorescence with FITC-conjugated rabbit anti-mouse. [3H]Thymidine uptake was also determined as a measure of cellular proliferation. Cells were seeded and allowed 2 h to adhere, 1 ml of feeding medium (containing 10% fetal bovine serum) was added to each well, and various concentrations of AG3340 were added. All experiments were done in replicates of three. After a 48-h incubation, cells were pulsed with 1 Ci of [3H]thymidine/ml for a period of 16 h before being harvested. To measure thymidine incorporation, cover slips were washed four times in PBS, placed in scintillation vials containing 5 ml of scintillation fluid (Ecolume; ICN), and placed in a Bectasen beta counter (LS 5000CE; Beckman).

Plasma Concentrations of AG3340. We determined plasma concentrations of AG3340 in the 16 animals in experiment 2B on the day the animals were sacrificed; the levels at 1 h (n = 4) and 24 h (n = 4) after the last dose of AG3340 were analyzed. Animals were anesthetized with 1 ml of metofane by inhalation (Janssen Pharmaceutical, Canada), and ∼0.8 ml of blood was collected by cardiac puncture, placed in glass tubes with sodium heparin (Becton Dickinson, NJ), then centrifuged at 10,000 rpm for 10 min. The serum was stored at −70°C.

AG3340 and an internal standard (AG3347) were extracted from mouse plasma and analyzed for AG3340 content by UV HPLC analysis. Briefly, 100 μl of plasma were mixed with 1 ml of acetonitrile, vortexed for 1 min, and centrifuged for 15 min at 4000 rpm. The supernatant was removed and dried at 50°C under nitrogen. AG3340 was solubilized in 120 μl of mobile phase prior to injection onto the HPLC column. Chromatographic detection was performed on a reverse phase column (Columbus, 5 μm, C18, 150 × 3.2 mm) with a flow rate of 0.5 ml/min. The mobile phase was 20% acetonitrile/50 mM NH4HPO4/0.01 M triethylamine (pH 3.2). Detection of AG3340 was at 240 nm. The percentage of extraction efficiency of AG3340 was determined using the internal standard, and AG3340 concentrations were calculated from a standard curve prepared at the time of assay. The quality controls for AG3340 did not vary more than 15% of the theoretical value. AG3340 concentrations were back-calculated to ng/ml in plasma.

RESULTS

Effects of AG3340 on Tumor Growth. In mice treated with AG3340 or vehicle control, tumor area was measured twice weekly beginning on day 0 (i.e., 3 weeks later after s.c. implantation, when the tumors were palpable and growing), and the results are shown in Fig. 1A. A significant difference in tumor size between the two groups appeared by day 21 (0.42 cm2) versus 0.75 cm2, P < 0.004, Wilcoxon test) and remained until day 31 [0.65 cm2 (versus 2.93 cm2, P < 0.0009, Wilcoxon test)] when the control group (n = 8) needed to be sacrificed because the mice were moribund. On the same day, several animals from the AG3340 group were sacrificed (n = 3) electively; one animal in the AG3340 group died during an i.p. injection (from an i.p. hemorrhage; autopsy proven) on day 38, and data for this animal were combined with the other AG3340 animals sacrificed on day 31. The others (n = 4) in the AG3340 group were allowed to grow until sacrifice was needed on day 70. The length of survival for the AG3340 group was 2.3 times...

### Table 1 Effects of AG3340 on invasion, proliferation, and vascularity of the human malignant glioma cell line U87

<table>
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<td>Average tumor size</td>
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<td>(cm²; day 70)</td>
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<td>No or minor invasion</td>
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<tr>
<td>(% of tumors)</td>
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<td>43</td>
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<td>Number of mitoses/10⁴</td>
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<tr>
<td>(%; day 31)</td>
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<td>Average number of vessels/10⁴</td>
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**Series 2, experiment 2A**

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<td>(cm²)</td>
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<td>Number of mitoses/10⁴</td>
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<td>BrdUrd-staining nuclei (%)</td>
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**Series 2, experiment 2B**

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<tr>
<td>(days)</td>
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<td>Average size of tumor</td>
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<td>(% of tumors)</td>
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**Histological Examination.** Histological sections were reviewed in a double-blind fashion by an anatomical neuropathologist (N. B. R.), described and compared for series 1 and 2. The following were evaluated: (a) muscle invasion (scored on a 0–4 scale, where 0 was absent and 4 is marked); (b) number of mitoses per high powered field (the average of three randomly selected fields at ×400); (c) presence of metastases in the lungs or liver (0, none; 1, present); (d) amount of vascularity (0–3 scale, where 0 is absent and 3 is prominent); and (e) amount of necrosis (estimation of amount of necrosis as percentage of total tumor). The number of mitoses, tumor volume, proportion of apoptotic nuclei, and proportion of BrdUrd-staining nuclei were compared using Wilcoxon’s two-sample test, and discrete measures (invasion, necrosis, cellularity, and atypia) were compared using Fisher’s Exact test.

**Assessment of In Vitro Cell Growth with Treatment of AG3340.** Tumor cell viability and proliferation were studied in vitro using both BrdUrd and [3H]thymidine labeling. U87 cells were seeded at a density of 10³ cells on glass coverslips (Thermanox; Nalge Nunc International). Cells were treated with either AG3340 (10 μM) or vehicle control. BrdUrd labeling (Amersham) was carried out for 60 min at 37°C in DMEM supplemented with 10% FCS. Subsequently, the cells were washed in PBS and fixed in ethanol for 30 min at −20°C. Anti-BrdUrd mouse monoclonal antibodies (Becton Dickinson) were applied for 1 h, and cover slips were washed in PBS. The...
greater than the control group. The tumor size between the control group on day 31 and the AG3340 mice sacrificed on day 70 was not significantly different [2.93 cm (2) versus 2.38 cm²; P, 0.55, Wilcoxon test]. This experiment was repeated (n = 16) with nearly identical results (Fig. 1B). In series 2, the animals were sacrificed earlier and with smaller tumors, and again significant inhibition of tumor growth was seen (Fig. 1, C and D).

**Effects of AG3340 on Tumor Invasion and Proliferation.** We examined the histological sections on all tumors and found significantly more invasion into the underlying muscle in the control group than the AG3340 group (Table 1). In series 1, the proportion of tumors with no or minor (a score of 0 or 1) muscle invasion was 6 of 14 (43%) in the AG3340 group but only 1 of 15 (6.7%) in the control group (P < 0.035, Fisher’s Exact test). This was true for AG3340 animals that were sacri-
AG3340 inhibited glioma growth in vivo and in vitro. First lane is conditioned from U87 cells in vitro, the next four lanes are extracts from AG3340-treated mice (AG), and the final four lanes are extracts from vehicle control (VC) mice; the tenth lane is conditioned medium from BHK cells expressing human gelatinase-B to indicate the migration of gelatinase-A and gelatinase-B. Note that the human M, 92,000 marker (92 KDa) runs slightly ahead of the M, 105,000 mouse gelatinase-B enzyme. No consistent difference in gelatinase-A or gelatinase-B activity was seen between the control and treatment groups.

Effects of AG3340 on Tumor Vascularity. The mean concentrations of AG3340 detected 1 and 24 h after i.p. administration of AG3340 once daily were 249 nM (range, 67–365 nM) and 66 nM (range, 37–100 nM), respectively; none was detected in the eight vehicle control animals.

DISCUSSION

The systemic administration of AG3340 significantly reduced the rate of tumor growth in this s.c.-implanted glioma tumor model. Both the rate of proliferation and the amount of tumor invasion were markedly reduced in treated animals; changes in proliferation were more marked and occurred earlier than did changes in tumor invasion. Furthermore, growth inhibition was long-lasting.

Overall, these changes produced a significant prolongation of survival in AG3340-treated animals compared with controls. The mechanism of growth inhibition in vivo is unknown. Although there were more apoptotic cells in AG3340-treated tumors compared with tumors from control animals, which were allowed to grow to maximum size, there was no significant difference in apoptosis at earlier times of treatment with AG3340. Neither did we observe a striking increase in apoptosis or a change in tumor vascularity to account for the difference in tumor size. Furthermore, there was no significant inhibition of

**Other Histological Features.** The amount of necrosis was not significantly different between the control and AG3340-treated groups. Similarly, there were no hepatic or pulmonary metastases in either group.

**Effects of AG3340 on Apoptosis.** The terminal deoxynucleotidyltransferase-mediated nick end labeling assay was used to determine whether there was a difference between treatment and control groups in the rate of apoptosis. There was no significant difference in the amount of apoptosis seen between the treatment and control groups in animals sacrificed at the same time (Table 1). In series 1, tumors from mice sacrificed on day 70 had a larger proportion of apoptotic nuclei than those from animals sacrificed on day 31 (7.7 versus 1.86%, P = 0.0467, Wilcoxon test).

**Effects of AG3340 on Tumor Vascularity.** There was no significant difference in the amount of vascularity seen between the treatment and control groups in experiments 1A, 1B, or 2A; we did not perform vascular staining on tumor samples from experiment 2B (Table 1).

**Gelatinase Activity in Tumor Samples.** Considerable variation in gelatinase activity, as visualized by zymography, was seen but did not differ between treatment groups (Fig. 3). Samples from both groups show the presence of inactive gelatinases and a small amount of active forms. That no differences were observed between the AG3340 and control groups is probably due to the dissociation of the inhibitor-MMP complex during sample preparation.

The ability of AG3340 to inhibit gelatinases was confirmed by zymography (Fig. 4). Samples from conditioned medium from a BHK (baby hamster kidney) cell line stably transfected with a human gelatinase-B expression vector were run on a zymographic gel, and gelatinolytic activity was inhibited in vitro by incubating the gels overnight with various concentrations of AG3340. Gelatinolytic activity of all samples was inhibited by 1 nM AG3340; almost total inhibition was seen at concentrations above 1 μM. Inhibition of gelatinases at 10 nM was superior to that achieved with 100 ng/ml of recombinant TIMP-1.

**Effects of AG3340 on In Vitro Cell Growth.** AG3340 did not significantly inhibit cell growth at concentrations of 100 nM to 100 μM using BrdUrd staining, [3H]thymidine labeling, or the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay; significant inhibition of proliferation occurred only at concentrations of 1 μM (Fig. 5).

**Plasma Concentrations of AG3340.** The mean concentrations of AG3340 detected 1 and 24 h after i.p. administration of AG3340 once daily were 249 nM (range, 67–365 nM) and 66 nM (range, 37–100 nM), respectively; none was detected in the eight vehicle control animals.

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The systemic administration of AG3340 significantly reduced the rate of tumor growth in this s.c.-implanted glioma tumor model. Both the rate of proliferation and the amount of tumor invasion were markedly reduced in treated animals; changes in proliferation were more marked and occurred earlier than did changes in tumor invasion. Furthermore, growth inhibition was long-lasting.

Overall, these changes produced a significant prolongation of survival in AG3340-treated animals compared with controls. The mechanism of growth inhibition in vivo is unknown. Although there were more apoptotic cells in AG3340-treated tumors compared with tumors from control animals, which were allowed to grow to maximum size, there was no significant difference in apoptosis at earlier times of treatment with AG3340. Neither did we observe a striking increase in apoptosis or a change in tumor vascularity to account for the difference in tumor size. Furthermore, there was no significant inhibition of
in vivo, inhibiting angiogenesis (53). AG3340 has antiangiogenic effects to affect tumors by blocking invasion and metastasis and by in vivo (38, 41). MMP inhibitors have classically been thought that their actions are more complex; these molecules may affect cellular proliferation and other steps in the multifunctional roles of MMPs, TIMPs, and synthetic MMP inhibitors in cancer biology. The accepted role of MMPs in cancer growth and metastasis is to facilitate extracellular matrix breakdown to allow intravasation and extravasation, but it is becoming clear that their actions are more complex; these molecules may affect cellular proliferation and other steps in the metastatic process (54, 55). Recent evidence from intravital video microscopic examination of cells undergoing extravasation has shown that this process occurs independently of the expression of MMPs and their inhibitors (56). This has led to the suggestion that MMPs may be important in creating and maintaining a favorable growth environment for primary tumors and metastases (54). Several recent reports suggest potential mechanisms through which MMPs may indirectly stimulate growth in vivo by influencing growth factor bioavailability. One such mechanism involves liberation of IGFs from their soluble binding proteins (IGFBPs). MMP-1, MMP-2, and MMP-3 can degrade IGFBP-3, thus releasing active IGF (57). Also, MMP-3 can cleave the membrane-anchored precursor form of heparin-binding epidermal growth factor-like growth factor, releasing active heparin-binding epidermal growth factor that can act on cells in a paracrine or autocrine fashion (58). MMPs may also negate mechanisms designed to moderate cytokine signals, as occurs in cleavage and release of the inactive type II cell surface “decoy” receptor for interleukin 1 (59). Any or all of these types of events may be occurring in the tumor microenvironment but particularly at the tumor-stromal interface. It is attractive to speculate that such mechanisms may explain the paradox of the ability of AG3340 to inhibit tumor growth in vivo without any significant action on proliferation in vitro, because the abundant supply of growth-promoting substances in serum-containing cell culture medium may override subtle cellular control of growth factor bioavailability.

Another potentially confounding factor is that TIMPs might not always act as strict antagonists of tumor metastasis, because a number of studies have reported stimulatory effects of TIMPs on cell growth that may be independent of their MMP-inhibiting capabilities. Several investigators found that TIMP levels were not negatively correlated with the degree of tumor malignancy, as would be expected if decreased TIMP production led to a more malignant phenotype. Somewhat paradoxically, TIMP expression increased with the degree of malignancy in several cancer types (58–63), including gliomas (64). Others have reported that experimental manipulation of TIMPs can inhibit tumor growth; for example, TIMP-2 overexpression reduced melanoma growth but not metastases (65), and transfection of TIMP-2 into transformed fibroblasts reduced metastases as well as tumor growth (66). Finally, TIMP-1 and TIMP-2 are
with TIMP-2, they may be comitogens that depend for their actions on other factors such as insulin (70). It is also possible that synthetic MMP inhibitors may affect tumor growth independent of their MMP-inhibitory activity.

In summary, this study is the first step in demonstrating the potential efficacy of AG3340 in human glioma growth and invasion. We are presently testing this MMP inhibitor in an orthotopic intracerebral human glioma model to determine whether it is effective in that model and reaches sufficient intratumoral concentrations. Finally, we are combining AG3340 with radiotherapy and chemotherapy. It is likely that AG3340 will be the most useful as cancer therapy when combined with conventional treatments.

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