Hepatocyte growth factor, also known as scatter factor (HGF/SF), is a 90,000 Da heterodimeric growth factor first discovered for its ability to stimulate cell motility. Its receptor, c-Met, is a receptor tyrosine kinase (RTK) that is activated upon HGF/SF binding. Together, HGF/SF and c-Met comprise a well-characterized ligand/receptor complex involved in multiple cellular functions, including proliferation, survival, motility, and morphogenesis (1, 2). This pathway has been directly implicated in tumor growth and progression in a wide variety of human cancer types, including glioblastoma multiforme (GBM). Globally, cancers of the brain and nervous system account for 1.7% of new disease, representing 189,000 cases annually and resulting in 142,000 deaths. More than one third of these cases are GBM, the most common and deadly form of glioma (3). The current treatment for GBM includes a combination of debulking surgery when possible, treatment with temozolomide, an imidazotetrazine derivative of the DNA-alkylating agent dacarbazine that crosses the blood-tumor barrier, and radiation therapy. Even with this recently approved regimen, prognosis for GBM patients remains poor with a mean survival of 14.6 months from the time of diagnosis and a less than 30% survival rate at 2 years (4). Temozolomide combined with radiation is currently the best treatment option and the standard of care for GBM as well as anaplastic astrocytoma and metastatic melanoma (reviewed in ref. 4). However, the poor survival rate in GBM shows the critical unmet medical need for novel therapeutics for treatment of this disease.

Previous studies have shown expression of the c-Met receptor in 100% of glioblastoma cases, and the expression of the HGF/SF ligand occurs in ~80% of cases analyzed (5), suggesting the possible formation of functional HGF/SF:c-Met autocrine loops that may drive glioma growth and progression. Therefore, inhibition of the HGF/SF:c-Met axis may provide increased therapeutic efficacy to this highly refractory disease in combination with the current temozolomide/radiotherapy regimen. As HGF/SF is the only known ligand for the c-Met receptor, neutralization of HGF/SF using an antibody therapy...
may be an attractive mechanism by which to accomplish inhibition of this axis.

Docetaxel is a commonly used semisynthetic taxoid that acts by disrupting the microtubule network in cells that is essential for cell division, thereby inhibiting mitosis. Docetaxel is approved for use in breast (6, 7), prostate (8, 9), and non–small cell lung cancers (10, 11), and more recently head and neck cancer (12, 13). Multiple previous studies have shown both the expression of HGF/SF and c-Met in these diseases (14–17). This suggests that there may also be clinical opportunities in multiple cancers for docetaxel combined with anti-HGF/SF neutralizing antibodies.

The U-87 MG cell line is a human glioblastoma-derived cell line that has been previously described to contain a HGF/SF-c-Met autocrine loop (18). Multiple HGF/SF- and c-Met–neutralizing agents have been shown to have efficacy in this or other glioblastoma models that contain an HGF/SF-c-Met autocrine loop when grown as a xenograft, including ribozymes that target HGF/SF or c-Met (19–21), antagonistic fragments of HGF/SF such as NK4 (22), and a combination of three antibodies against HGF/SF that when combined are able to neutralize HGF/SF binding to c-Met (23). More recently, studies have shown that single neutralizing HGF/SF antibodies can also completely inhibit U-87 MG xenograft growth in vivo, grown either as a s.c. xenograft (24) or as an orthotopic model (25). These studies show that the U-87 MG model is appropriate for testing neutralizing anti-human HGF/SF agents.

Here, we further explore the antitumor effects of a fully human anti-HGF/SF–neutralizing antibody, AMG 102, in combination with either docetaxel or temozolomide using the U-87 MG cell line in vitro and in vivo. Temozolomide and docetaxel both possess direct cytotoxic effects but act via different biological mechanisms. AMG 102 was able to increase the inhibitory effect of either docetaxel or temozolomide in vitro and in vivo. In the case of docetaxel, we present evidence that this increase in cell death was mediated through apoptosis in vitro. Despite published reports using leukemic cell lines in vitro, suggesting apoptosis may be a mechanism of temozolomide-mediated cytotoxicity (26), we found no evidence of apoptosis in the temozolomide-treated cells either alone or in combination with anti-HGF/SF antibodies in U-87 MG cells, suggesting a necrotic cell death mechanism under these conditions. These data suggest that anti-HGF/SF–neutralizing antibody therapy has the potential to be effectively combined with either temozolomide or docetaxel in the clinic.

Materials and Methods

Cell lines and reagents. Recombinant human d5-HGF/SF and AMG 102 expressed by Chinese hamster ovary cells were prepared as described (24). U-87 MG cells were obtained from the American Type Culture Collection. Docetaxel was obtained from Aventis Pharmaceuticals and resuspended in DMSO for in vitro cell assays. For the in vivo assays, docetaxel was resuspended in the manufacturer-provided diluent and adjusted to the final concentration used before injection with PBS. Temozolomide was purified in house from capsule form and diluted and adjusted to the final concentration used before injection with PBS. Temozolomide was purified in house from capsule form and adjusted to the final concentration used before injection with PBS.

U-87 MG growth/survival. The U-87 MG cell proliferation assay was done as described (24) with modifications. Two thousand cells per well in MEM medium + 1% fetal bovine serum was used for the docetaxel studies, and 3,000 cells per well in MEM medium + 1% fetal bovine serum was used for the temozolomide studies. The absorbance at 540 nm was read on a microtiter plate reader (SpectraMax PLUS, Molecular Devices). To calculate the percentage of control, the value of treated cells was compared with cells treated with an isotype control antibody or no antibody treatment. Data were generated as either triplicate or quadruplicate points and averaged. Growth data were evaluated with repeated-measures ANOVA using StatView followed by Bonferroni/Dunn to calculate P values.

Caspase-3/7 activity. U-87 MG cells were plated at 10,000 per well in Costar 96-well black assay plates into MEM + 1% fetal bovine serum and treated 24 h later. Twenty-four hours later, the Apo-ONE reagent was prepared according to the manufacturer’s instructions, added to the plate, and read using the Victor2 fluorescent microtiter plate reader at excitation of 485 nm and emission at 530 nm. Data points were generated in triplicate and averaged.

Western blot analysis. Cells were treated with huIgG, control antibody or AMG 102 alone or in combination with docetaxel at 100 nmol/L for 24 h, spun down, and resuspended in 2× Tris-glycine SDS sample buffer (Invitrogen) with 5% β-mercaptoethanol (Sigma). Lysates were sheared through an 18.5-gauge needle four times and stored at -80°C. For Western blots, lysate was loaded on either 4% to 20% or 10% Tris-glycine gels (Invitrogen) and transferred to nitrocellulose membranes (Schleicher and Schnell). Blots were probed for 20 h at 4°C with a 1:500 dilution of a rabbit polyclonal antipoly(ADP)ribose polymerase (PARP) antibody (Cell Signaling). Denaturing analysis was done using Labworks software from UVP, Inc. Values of cleaved PARP were normalized to actin and expressed as fold increase compared with huIgG, control antibody.

Fluorescence-activated cell sorting analysis. Cells were treated for 24 h with the indicated agents and harvested in 1 mL of dissociation buffer (Invitrogen). Cells were then suspended in cytofix/cytoperm solution (BD Biosciences) for 30 min. Cells were washed once in perm/wash buffer (BD Biosciences), transferred to a 96-well U-bottomed plate, and stained with a 1:100 dilution of cleaved caspase-7 antibody or FITC-conjugated cleaved PARP polyclonal antibody (Cell Signaling). After washing once with perm/wash buffer, Alexa 568–conjugated goat anti-rabbit secondary antibody (Invitrogen) was used for the caspase-7 antibody–stained samples. All samples were washed once with perm/wash buffer before transfer to fluorescence-activated cell sorting (FACS) tubes and run on the FACScan instrument (BD Biosciences). Cells that stained positive in the FL-2 channel for cleaved caspase-7 or the FL-1 channel for cleaved PARP were identified and counted to determine the percentage of positive cells compared with total number of cells analyzed.

Tumor growth and measurements. Xenograft studies were done as described (24) with modifications. Eight-week-old female CD1 nude mice (Charles River Laboratories) were injected s.c. with 5 × 10⁶ U-87 MG cells. Tumors were allowed to grow to ~200 mm³ and then the mice were randomized into individual treatment groups (n = 10/group). Tumor measurements and body weights were recorded twice per week. Tumor volume was calculated as length × width × height in mm³. Results are expressed as mean ± SE. All animal experiments were done in a blinded fashion where the person measuring the tumor volumes was unaware of the treatment groups. These studies were conducted in accordance with federal animal care guidelines and were pre-approved by the Amgen Institutional Animal Care and Use Committee. Statistical analysis was performed using repeated-measures ANOVA using StatView followed by Scheffe’s post hoc test to determine P values.

Results

Temozolomide can effectively inhibit U-87 MG growth in vitro. U-87 MG cells were tested for a single-agent dose response to temozolomide. Temozolomide undergoes spontaneous hydrolysis at physiologic pH into its active metabolite 5-(3-methyl triazen-1-yl)imidazole-4-carboxamide (27), which is then thought to mediate cytotoxicity through DNA methylation
(27, 28). Cell response was analyzed using sulforhodamine B (SRB), a total protein dye stain that assesses the total number of surviving cells (29). This method has a linear correlation to cell number and provides a measure of cell growth and survival. Duplicate data points were used to generate a four-parameter curve fit to determine a single agent IC50 value. After 8 days of exposure in vitro, temozolomide was determined to have an IC50 of 21.2 ± 12.5 μmol/L (n = 5) for the U-87 MG cell line. Data from a representative experiment are shown in Fig. 1A.

**Addition of HGF/SF can protect U-87 MG cells against temozolomide-induced cell death.** Previous studies have shown that the HGF/SF/SF:c-Met axis drives U-87 MG proliferation (19, 24). We confirmed the presence of c-Met on the cell surface by FACS analysis using a fluorescently labeled polyclonal anti-c-Met antibody (data not shown). An HGF/SF ELISA was done on cell culture medium supernatants to quantify the levels of HGF/SF secreted in the growth medium 5 days after plating 1 x 10^6 cells in a 10-cm dish. Approximately 340 ± 30 pg/ml of HGF/SF was detected in the medium of U-87 MG cells grown for 5 days postplating.

To simulate a paracrine model of c-Met stimulation, exogenous HGF/SF was added to the medium to supplement the HGF/SF:c-Met autocrine loop in the presence of a suboptimal concentration of temozolomide. HGF/SF was added for 24 h to the medium in low serum conditions (1% fetal bovine serum) before addition of temozolomide. Addition of 75 ng/ml of HGF/SF to the medium was able to completely protect against the effects of a 27.8 μmol/L dose of temozolomide when assayed by the SRB method (Fig. 1B). This suggests that HGF/SF signaling can provide some measure of resistance to temozolomide treatment, and despite the secreted HGF/SF from U-87 MG cells, functional c-Met receptor signaling is not saturated in these cells under in vitro culture conditions. At higher concentrations of temozolomide (>100 μmol/L), however, additional HGF/SF was not able to protect the cells (data not shown).

**AMG 102 enhances the antigrowth effects of temozolomide in vitro.** Because U-87 MG cells are sensitive to both temozolomide and AMG 102 as single agents, we measured the effects of the combination of temozolomide and AMG 102 to assess any increased activity. In a preliminary set of experiments, a dose range of temozolomide was combined with a dose range of AMG 102 and cell growth was measured using the SRB method (data not shown). Each single agent alone was able to inhibit U-87 MG cells effectively at high concentrations >333 nmol/L of temozolomide (Fig. 1C). Cell growth/survival after treatment with the combination was significantly impaired when compared with control cells or either single agent alone (P < 0.0001 for all groups).

**AMG 102 enhances the antigrowth effects of temozolomide in vivo.** U-87 MG tumors are sensitive to HGF/SF-neutralizing antibodies when grown s.c. in athymic nude mice, demonstrating that the growth of this model is driven by the presence of an HGF/SF/SF:c-Met autocrine loop (23, 24). AMG 102 (30 μg) dosed twice per week can completely inhibit tumor growth in this model as a single agent. This tumor model is also sensitive to temozolomide treatment, as >7 mg/kg of temozolomide dosed once per week completely inhibits tumor growth (data not shown). To assess the combination effects of AMG 102 and temozolomide, suboptimal doses of 3 μg twice per week of AMG 102 and 5 mg/kg once per week of temozolomide...
were selected and tumor growth was evaluated. Each drug was dosed alone and in combination in an established disease model, when tumors reached an average size of 200 mm³ (day 14; Fig. 2). Treatment with 3 μg of AMG 102 did not result in statistically significant tumor inhibition in this experiment (33.7% inhibition; \(P > 0.05\)). Treatment with 5 mg/kg temozolomide resulted in 76.2% inhibition of tumor growth on day 29 that was statistically significant when compared with control \((P < 0.0001)\). However, the combination of temozolomide and AMG 102 resulted in a 93% inhibition of tumor growth that was statistically significant compared with treatment with either single agent alone \((#, P < 0.0001)\). Points, mean; bars, SE.

**AMG 102 enhances docetaxel-induced apoptosis.** To investigate whether loss of cell viability was due to increased apoptosis, cells were examined for activation of caspase-3/7 activity. Caspase-3 and caspase-7 are key executioners in the apoptotic process, cleaving downstream enzymes such as PARP, as well as other caspase enzymes. Increased caspase-3/7 activity was observed in a dose-dependent manner in response to docetaxel, with an IC₅₀ of 11.0 nmol/L (Fig. 4A) similar to the growth/survival IC₅₀ as assessed by SRB (see Fig. 3A). The addition of 83 nmol/L (12.5 μg/mL) AMG 102 increased the extent of caspase-3/7 activity across a dose range of docetaxel (Fig. 4B), and slightly reduced the IC₅₀ of docetaxel. Similar effects were observed at 166 nmol/L (25 μg/mL) of AMG 102 across a dose range of docetaxel (data not shown). AMG 102 alone did not induce measurable caspase-3/7 activity when

**Fig. 2.** Combination of AMG 102 with temozolomide results in enhanced tumor inhibition in vivo. U-87 MG cells were injected s.c. into female athymic nude mice \((n = 10/group)\). Treatment with 3 μg AMG 102 twice per week, 5 mg/kg temozolomide once per week, or the combination was initiated after tumors reached an average size of 200 mm³. The combination of AMG 102 and temozolomide significantly increased inhibition of tumor growth compared with treatment with either single agent alone \((#, P < 0.0001)\). Points, mean; bars, SE.
Treatments. Cells were stained with antibodies specific for FACS analysis was used to quantify the number of cells or to the generation of additional cells entering apoptosis. Either increased caspase activity in the same subpopulation of combination-treated samples (Fig. 4B) could be attributed to increased caspase-3/7 activity in response to a wide concentration range of docetaxel alone. Increasing caspase-3/7 activity is observed in response to increasing concentrations of docetaxel. AMG 102 is able to increase the total caspase-3/7 activity generated by docetaxel. Caspase-3/7 activity was measured using an enzymatic activity kit as described in Materials and Methods. Each curve was generated using triplicate data points. FU, fluorescence unit.

Fig. 4. HGF-neutralizing antibody increases docetaxel-induced caspase activity in U-87 MG cells in vitro. A. U-87 MG cells were tested for caspase-3/7 activity in response to a wide concentration range of docetaxel alone. Increasing caspase-3/7 activity is observed in response to increasing concentrations of docetaxel. AMG 102 is able to increase the total caspase-3/7 activity generated by docetaxel. Caspase-3/7 activity was measured using an enzymatic activity kit as described in Materials and Methods. Each curve was generated using triplicate data points. FU, fluorescence unit.

Addition of AMG 102 in Combination with Temozolomide or Docetaxel

HGF-neutralizing antibody increases DOC32-induced caspase activity in U-87 MG cells in vitro. A. U-87 MG cells were tested for caspase-3/7 activity in response to a wide concentration range of docetaxel alone. Increasing caspase-3/7 activity is observed in response to increasing concentrations of docetaxel. AMG 102 is able to increase the total caspase-3/7 activity generated by docetaxel. Caspase-3/7 activity was measured using an enzymatic activity kit as described in Materials and Methods. Each curve was generated using triplicate data points. FU, fluorescence unit.

B. U-87 MG cells were tested for caspase-3/7 activity in response to a wide concentration of docetaxel in combination with 83 nmol/L (12.5 μg/mL) AMG 102. AMG 102 is able to increase the total caspase-3/7 activity generated by docetaxel. Caspase-3/7 activity was measured using an enzymatic activity kit as described in Materials and Methods. Each curve was generated using triplicate data points. FU, fluorescence unit.

Discussion

In this study, we show that AMG 102, a fully human anti-HGF/SF-neutralizing antibody, can enhance the activity of either of the chemotherapeutic agents temozolomide or docetaxel in vitro and in vivo. Combined treatment at suboptimal doses led to greater efficacy compared with treatment with either single agent alone as shown in Fig. 6 (84.7% inhibition of control, P < 0.015 day 30). Furthermore, the combination of drugs showed no evidence of interference in therapeutic efficacy or overt toxicity, as might be indicated by body weight loss, of any of the test groups in the study (data not shown).

In an attempt to confirm that apoptosis was the in vivo mechanism of action of the combination effect of docetaxel and AMG 102, a short-term study was done to test for evidence of apoptosis. Unfortunately, no consistent trend supporting our hypothesis of increased apoptosis could be observed (data not shown). The failure to translate the in vitro results to the in vivo setting could be due to both the transient characteristics of the apoptotic cells as well as the extensive manipulation required to dissociate the tumor into a single-cell suspension for analysis. Further studies, although challenging, may be warranted.
The IC_{50} of temozolomide in our study for the U-87 MG cell line averaged 21.2 μmol/L, and we observed statistically significant combined effects with AMG 102 at a lower concentration of 12.5 μmol/L. These levels are within the observed plasma concentration range for temozolomide in patients; in a study assessing plasma concentrations with continuous dosing at 75 to 200 mg/m²/d, calculated plasma concentrations ranged from 0.10 to 13.99 μg/mL (0.52-72.1 μmol/L; ref. 30). However, the experimental concentrations of temozolomide used in this study exceed the observed clinical cerebrospinal fluid concentrations reported of 0.16 to 9.93 μg/mL (0.82-9.94 μmol/L; ref. 30). Although it is possible that longer-term exposure at lower concentrations would cause some effect in vitro, overall, we do not know how U-87 MG sensitivity to temozolomide translates to the response observed in patients.

A significant finding of this study was that exogenous HGF/SF added to the medium was able to protect cells from temozolomide-induced growth inhibition. HGF/SF, through its receptor c-Met, can activate a number of downstream signaling molecules including the Ras/Erk and phosphatidylinositol 3-kinase/Akt pathways. Previous reports using other glioma cell lines, such as U373, have also shown limited cytoprotective action of HGF/SF against agents such as cisplatin, camptothecin, Adriamycin, and Taxol as well as γ irradiation (31, 32). This protection was abrogated by phosphatidylinositol 3-kinase inhibitors but not by mitogen-activated protein kinase or protein kinase C pathway inhibitors, suggesting a phosphatidylinositol 3-kinase/Akt–dependent mechanism (33). That additional HGF/SF in our study can increase this protective effect suggests that the c-Met receptors in U-87 MG cells are not saturated by the autocrine loop expressed by these cells in vitro; indeed, increased phosphorylation of c-Met is observed with HGF/SF addition to the medium in culture (data not shown). We hypothesize that this increased signaling is responsible for the increased cytoprotective effects versus temozolomide treatment.

In contrast to temozolomide, exogenous HGF/SF was not able to protect against docetaxel in U-87 MG cells (data not shown). However, there was clearly an additive effect in growth inhibition in vitro and in vivo when combined with anti-HGF/SF–neutralizing antibodies. These data may emphasize a fundamental difference in mechanism between these two cancers.
cytotoxics; docetaxel triggers apoptosis after mitotic arrest (reviewed in ref. 33), and additional HGF/SF may not be able to overcome this block before drug-induced apoptosis. However, apoptosis caused by docetaxel was enhanced with anti-HGF/SF antibody, suggesting that the autocrine HGF/SF produced by U-87 MG cells does have measurable cytoprotective effects against docetaxel. It is interesting that additive effects are observed in a growth inhibition assay, despite the action of these two drugs on distinct phases of the cell cycle. This may be due to the ability of each agent to affect a distinct population of the cells resulting in an overall additive effect upon combination. Alternatively, signaling pathways such as the phosphatidylinositol 3-kinase/Akt pathway that is activated by the active HGF/SF–c-Met autocrine loop may offer some cytoprotective effect that is blocked upon addition of AMG 102. Taken together, these data imply that there may be utility for an anti-HGF/SF–neutralizing antibodies. Stromal effects from the host cannot be assessed because the mouse HGF/SF does not activate the human receptor and AMG102 does not recognize or neutralize mouse HGF. However, in human disease, HGF/SF derived from the stroma could be playing a significant role in disease progression via paracrine activation of c-Met in tumors and/or the vasculature (37). Studies have been published suggesting a paracrine role for HGF/SF in multiple cancer types, including cancers of the breast (37), lung (38), prostate (39), etc. (1, 2). In many of these cases, HGF/SF expression correlates with poor prognosis for disease outcome. In these patient populations, there may exist an opportunity for an anti-HGF/SF therapy to show efficacy. Therefore, the application of AMG 102 in the clinic may be more widely applied to cancers that express the c-Met receptor. This report suggests that in cases where docetaxel, temozolomide, or similar mechanistic agents are used, combination therapy may further enhance efficacy.

**References**

12. Tsa0 AS, Garden AS, Kies MS, et al. Phase I/II study of docetaxel, cisplatin, and concomitant boost radiation for locally advanced squamous cell


AMG 102, A Fully Human Anti-Hepatocyte Growth Factor/Scatter Factor Neutralizing Antibody, Enhances the Efficacy of Temozolomide or Docetaxel in U-87 MG Cells and Xenografts

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