Marked Antiangiogenic and Antitumor Efficacy of AG3340 in Chemoresistant Human Non-Small Cell Lung Cancer Tumors: Single Agent and Combination Chemotherapy Studies

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ABSTRACT

Effective therapy is needed to improve the survival of patients with advanced lung cancers. We studied the effects of a selective metalloprotease inhibitor, AG3340, on chemoresistant human non-small cell lung cancer tumors (line MV522) in vivo. Mice bearing s.c. tumors were given twice-daily oral doses of AG3340. As a single agent, AG3340 inhibited angiogenesis (up to 77%) and tumor growth (up to 65%) in a dose-dependent manner at well-tolerated daily doses up to 400 mg/kg/day and induced significant tumor necrosis. In contrast, tumors were relatively insensitive to carboplatin with ~25% growth inhibition observed at a maximum tolerated dose of approximately 30 mg/kg/week (given i.p., twice weekly). Carboplatin inhibited tumor growth markedly only at toxic doses, demonstrating a superior therapeutic index of AG3340 to carboplatin in this tumor model. A suboptimal dose of AG3340, when used in combination with an ineffective maximum tolerated dose of carboplatin, resulted in greater tumor growth inhibitions than those produced by either agent alone. Similarly, growth inhibition was enhanced when AG3340 was used in combination with paclitaxel. Cotreatment with carboplatin did not alter AG3340 plasma concentrations achieved acutely after oral dosing. These data demonstrate an antiangiogenic and antitumor effect of AG3340 when used as a single agent and enhanced growth inhibitions when AG3340 is used in combination with cytotoxic agents. These data suggest that treatment with this novel matrix metalloprotease inhibitor may be beneficial in advanced lung cancers and other chemoresistant malignancies.

INTRODUCTION

Carcinoma of the lung results in more morbidity and mortality than any other cancer in the United States (1, 2). Five-year survival of patients with newly diagnosed NSCLC is only 10–15% because most patients present with advanced, metastatic disease that does not respond to therapy (1–3). New treatment strategies are needed to improve the therapy of patients with lung cancer. Many new agents, including taxanes and gemcitabine, have been developed to treat lung cancer (3, 4). Novel antiangiogenic agents are also being developed in an attempt to circumvent resistance that often develops to chemotherapy (5, 6). New therapies that have progressed to the clinic have produced higher response rates and survival times compared with established treatment regimens containing platinum analogues. Additionally, adverse effects associated with new therapies have been generally decreased (3, 4). New treatments with even greater therapeutic indices are still needed to improve the therapy of lung cancer.

Inhibition of MMP activity represents a promising approach toward improving the therapy of aggressive, metastatic disease (7–11). Prominent antitumor activity has been reported in animal models after treatment with both broad-spectrum (12, 13) and selective (14–16) MMP inhibitors. It would be desirable to specifically target the inhibition of gelatinases, key MMPs that promote tumor invasion, metastasis, and angiogenesis (17–22).

Toward this goal, we have created a noncytotoxic, selectively selective MMP inhibitor, AG3340, using a protein structure-based drug design. AG3340 inhibits gelatinases [MMP-2 (Gelatinase A) and MMP-9 (Gelatinase B)], stromelysin-1 (MMP-3), collagenase-3 (MMP-13), and membrane type MMP-1 (MMP-14) potently (with Ki values of 50–260, 30, 300, and 300 pm, respectively) but rather weakly inhibits collagenase-1 (MMP-1) and matrilysin (MMP-7; with Ki values of 8.1 and 54 nm, respectively; Ref. 23).

AG3340 has a broad spectrum of antitumor activity in rodent tumor models after p.o. and i.p. dosing (16). Antitumor activity has been associated with prominent inhibition of metastasis in rodent tumor models (16, 24), with inhibition of angiogenesis (25), cellular proliferation (25, 26), and increased apoptosis (27) in human tumor models in vivo. AG3340 crosses both the blood-brain barrier and blood-retinal barrier in monkeys and rodents (28) and is orally bioavailable in rodents and humans.

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The abbreviations used are: NSCLC, non-small cell lung cancer; Ki, concentration that inhibits enzyme activity by 50%; MMP, matrix metalloprotease; MTD, maximum tolerated dose; HPLC, high-pressure liquid chromatography.

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2 The abbreviations used are: NSCLC, non-small cell lung cancer; Ki, concentration that inhibits enzyme activity by 50%; MMP, matrix metalloprotease; MTD, maximum tolerated dose; HPLC, high-pressure liquid chromatography.

3 B. Shetty, K. Zhang, and D. Shalinsky, unpublished data.
(16, 25, 29), which suggests that AG3340 may have clinical utility as an orally active MMP inhibitor for diseases in which angiogenesis contributes to the pathology, including cancers and ophthalmic diseases.

AG3340 has not been studied previously in human lung cancer models. Two human lung cancer models have now been used to study AG3340. These models mimic the aggressiveness and chemoresistance of clinical NSCLC. In the first model, reported here, human NSCLC tumor cells (line MV522) were used. MV522 tumors induce morbidity, are chemoresistant, and reportedly metastasize to the lung after s.c. implantation in nude mice (30). In the second model, large cell lung cancer tumors were implanted orthotopically intrabronchially in nude rats (31). Preliminary results indicate that parenterally administered AG3340 inhibited the growth and metastasis of primary orthotopic lung cancers in that model but did not increase animal survival (32). However, combination therapy with orally administered AG3340 and a tolerated dose of carboplatin produced a significant increase in animal survival in this aggressive tumor model (33).

We report here that AG3340 has marked antitumor efficacy against chemoresistant MV522 NSCLC tumors, associated with prominent antiangiogenic activity. Importantly, the therapeutic index of AG3340 was positive and superior to that of cytotoxic agents in this tumor model. Additionally, AG3340 enhanced the antitumor efficacy of cytotoxic agents in combination treatment, supporting further study of AG3340 clinically in patients with advanced NSCLC.

MATERIALS AND METHODS

Cell line. Human MV522 tumor cells were isolated as metastatic variants of human UCP3 lung cancer cells that had metastasized to the rodent lung after s.c. implantation (30) and were kindly provided by Dr. Michael Kelner (University of California-San Diego, La Jolla, CA). Cells were cultured in RPMI 1640 (Mediatech, Inc., Herndon, VA) at 37°C in 95% air/5% CO2. Exponentially growing cells were harvested from tissue culture to initiate tumor biology studies.

Reagents. AG3340, 3(S)-2,2-dimethyl-4-[4-pyridin-4-yloxy]-benzenesulfonyl]-thiomorpholine-3-carboxylic acid hydroxyamide (Mw, 423.5) was synthesized at Agouron Pharmaceuticals, Inc. as described previously (25) and was stored desiccated in amber vials at 4°C. AG3340 was solubilized in sterile water (pH 2.3). The solution was filtered under sterile conditions, stored at 4°C, and made fresh every 2 weeks. Carboplatin was purchased from Sigma Chemical Corp. (St. Louis, MO) and was solubilized in sterile water. Paclitaxel was purchased from ICN Biologicals, Inc. (Aurora, OH) and was solubilized in ethanol:Tween 80:saline (25:25:50). Fresh drug solutions were prepared every 2 weeks and stored in amber vials at 4°C.

Animals. Female athymic BALB/c nu/nu nude mice (ages, 6–8 weeks) were obtained from Bantin & Kingman Universal Limited (Fremont, CA). Animals were housed in sterile conditions in class II hoods, as described previously (25), in accordance with procedures approved by the Institutional Animal Care and Use Committee at Agouron Pharmaceuticals, Inc. Mice were generally kept in quarantine for 1 week after arrival with access to sterilized food and water ad libitum. General health was assessed daily.

Tumor Biology Studies. Studies were initiated by implanting 5 × 106 tumor cells s.c. bilaterally (two sites/mouse) using a sterile 25-gauge × 1.5-μm needle (Butler, Union City, CA). Mice were randomized, ear-punched for identification, and housed in groups of three/cage after tumor implantation; each study consisted of control and AG3340-treated groups containing 10–12 animals/group. Tumors were allowed to establish for 5 days before beginning dosing with AG3340, carboplatin, paclitaxel, or their respective vehicles.

Tumor growth was assessed by measuring the length and width of s.c. tumors with electronic calipers. Volumes were calculated using the formula

\[
\text{Volume} = \frac{(\text{length})(\text{width})^2}{2}
\]

Additionally, the effects of dosing regimens, vehicles, and AG3340 on the body weights and the general health of the mice were assessed throughout the experiments. Animal weights were recorded beginning 5 days after tumor implantation before beginning drug treatment. Experiments were conducted for up to 64 days.

Dosing Route and Schedule for AG3340, Carboplatin, and Paclitaxel. AG3340 was administered p.o. (5 ml/kg) using sterile 18-gauge × 2-inch intragastric feeding needles (Polyplur and Sons, Inc., New Hyde Park, NY). The animals were dosed daily, 7 days per week, b.i.d. at approximately 9 a.m. and 4 p.m., at doses up to 400 mg/kg/day (2800 mg/kg/week). The fractionated dose for a group given 100 mg/kg/day was 50 mg/kg per dose (given b.i.d.).

Carboplatin and paclitaxel were administered i.p. (10 ml/kg) using 25-gauge × 5/8-inch needles. Carboplatin was given twice weekly on Monday and Thursday at up to 120 mg/kg/week; one-half of the weekly dose was given each time. Carboplatin was given ~1 min after the administration of AG3340 or its vehicle.

Paclitaxel was administered i.p., three times weekly on Monday, Wednesday, and Friday at up to 22.5 mg/kg/week; one-third of the weekly dose was given each time. Paclitaxel was given ~1 min after the administration of AG3340 or its vehicle. In combination chemotherapy studies, animals received AG3340 or its vehicle p.o. and carboplatin or paclitaxel, or their respective vehicles, i.p.

Tumor Collection: Histology. Tumors were collected after 39–52 days of study as indicated in the “Results” section. After the mice were killed by cervical dislocation and exsanguination, tumors were surgically removed using sterile instruments, fixed in 10% buffered formalin and embedded in paraffin. Five-μm sections of tumors were cut using a Leica RM2025 microtome and fixed on slides using a Leica Tissue Embedding Console System (Leica EG1160), deparaffinized, and stained with H&E. Tumor morphologies were analyzed in a masked fashion by a medical histopathologist (N. M. V.) with extensive experience in preclinical tumor biology models. A minimum of five tumors per group were analyzed in each study.

Tumor CD-31 Staining. To assess angiogenesis in tumors, blood vessels were stained with an antibody to the endo-
thelial cell marker, CD-31 (34). Frozen tumor sections were cut and allowed to air dry. Sections were fixed in acetone, blocked in 10% goat serum with 1% BSA, and exposed to the primary rat antimouse antibody to CD-31 for an hour followed by biotinylated antirat IgG2a. Alkaline phosphatase-conjugated streptavidin was then added. After adding the alkaline phosphatase substrate, nuclei were counterstained with Nuclear Fast Red. Rabbit antihuman Factor VIII was used as a positive control.

CD-31 staining was quantified manually in a masked fashion by quantifying the number of stained blood vessels in at least five randomly chosen 200 × fields/tumor from 6–8 tumors/group. Vessels containing stained lumens were counted, and each lumen was scored as one vessel regardless of size. Longitudinally staining vessels were also counted and each longitudinal area was scored as one vessel regardless of length. Punctate areas of staining were also counted, with care not to score stained areas that were deemed to clearly represent non-specific background staining. By including punctate stainings, we erred on the side of not discarding data that may have represented angiogenesis at the earliest-stage of endothelial cell proliferation. Additionally, these punctate stainings were included so as not to overestimate the degree of inhibition of angiogenesis by the drug treatments. The quantitation of CD-31 staining represents an estimate of vessel number that is subject to investigator interpretation and variation.

Calculation of Therapeutic Index. The therapeutic index was defined as the ratio of the MTD4 to a dose that inhibited tumor growth by 50%:

\[
\text{Therapeutic index} = \frac{\text{MTD}}{\text{IC}_{50}}
\]

Plasma Concentrations of AG3340 after Cotreatment with Carboplatin. Mice pretreated for over 5 weeks with AG3340 alone on a b.i.d. regimen were given a single dose of AG3340 just before being sacrificed. Mice were dosed 17 h after completion of the previous day’s dosing, with either 50 or 200 mg/kg per dose AG3340 as part of their normal normal daily dosing regimen. In addition, mice that had been pretreated with AG3340 and carboplatin for over 5 weeks were given a single dose of AG3340 immediately followed by a dose of carboplatin. The final dose of carboplatin was 10, 15, or 20 mg/kg, respectively, depending on the weekly regimen.

In all of the groups, dosing was begun 17 h after the previous day’s dose, when plasma concentrations of AG3340 would have been at a trough of 1–3 ng/ml (25). Blood was collected 1 h after dosing by cardiac puncture after mice were anesthetized with metafane.

Plasma samples were obtained by centrifugation (10 min at 2400 rpm, (Sorvall RT7, Newtown, CT). Plasma was stored at −70°C until analyzed by HPLC.

Extraction and HPLC Analysis of AG3340. AG3340 in mouse plasma was quantified using an acetonitrile extraction (2 ml acetonitrile:100 μl plasma) followed by an isocratic HPLC-UV analysis method. Extracts were evaporated to dryness and reconstituted with 125 μl of mobile phase [32:68, acetonitrile:25 mM NH₄H₂PO₄ containing 2.5 ml/liter triethylammonium (pH 7.0)]. Ninety five μl of reconstituted sample containing AG3340 were injected into a Hewlett Packard 1100 Chem Station HPLC system and analyzed with a variable wavelength detector. Chromatography was performed using a reverse phase HPLC column (Metachem metasil basic C₁₈ column (3 mm × 150 mm)) at a flow rate of 0.5 ml/min equipped with an autoinjector. The retention time for AG3340 was approximately 7.3 min. The limit of quantitation was 250 ng/ml, and quality control values ranged from ≤15 to 30% of expected values.

Data Analyses. Efficacy data are reported as mean ± SE unless indicated otherwise. Body weight data are reported as mean ± SD. Statistical analyses of data were performed using unpaired Student’s t test with two-tailed comparison. Differences of P < 0.05 were considered to be significantly different from control.

Analyses of the Interaction between AG3340 and Carboplatin on Tumor Growth. The AG3340-carboplatin combination study was retrospectively analyzed by using the proportional hazards model of Carter et al. (35). Data were analyzed from all of the groups receiving the following doses: 100 or 400 mg/kg/day AG3340, 20 or 40 mg/kg/week carboplatin, 100 mg/kg/day AG3340 + 20 mg/kg/week carboplatin, or 100 mg/kg/day AG3340 + 40 mg/kg/week carboplatin (Table 1). The hazards function used to describe the instantaneous risk of treatment failure at any time is

\[
\lambda(t) = \lambda_0(t) \exp(\beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4)
\]

where \(x_1\) is the concentration of AG3340 and \(x_2\) is the concentration of carboplatin. Treatment failure was considered to have occurred on the day when tumor volume reached 1000 mm³ or the animal died. Optimal single-agent and combination treatments were calculated from the derivatives of the hazards function using the fitted parameter values as described by Carter et al. (36). Therapeutic synergism is asserted for a drug combination if the optimized combination improved the response (i.e., growth inhibition) over the optimized dose of either drug alone.

RESULTS

Antitumor Efficacy of AG3340. MV522 tumors became palpable 5 days after implantation (Fig. 1). Significant decreases in tumor growth became evident after 10–15 days with 100 and 400 mg/kg/day AG3340. Thereafter, AG3340 decreased tumor growth dose-dependently. After 45 days,5 tumor volumes in mice treated with 100 and 400 mg/kg/day AG3340 were significantly decreased to 31.8 ± 2.7% and 65.1 ± 7.1%, respectively, of control tumor volumes (P < 0.05; Fig. 1A). Growth inhibitions appeared nearly complete as evident by a flattening of the growth curve over days 24–42 at the high dose of AG3340. Interestingly, when dosing with 400 mg/kg/day AG3340 was stopped, tumors regrew, which sug-

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4 Defined as a dose that resulted in body weight decreases of ≥7.5% relative to vehicle-treated controls and no more than moderate signs of ill health.

5 Day 45 was chosen arbitrarily. The magnitude of inhibition was similar after day 24 of the study.
Antitumor Efficacy of AG3340 in NSCLC

Table 1  Effects of AG3340, carboplatin, or a combination of AG3340 and carboplatin on tumor growth and animal body weights across studies

<table>
<thead>
<tr>
<th>Agent</th>
<th>Dose</th>
<th>Percent tumor growth inhibition(^a)</th>
<th>Percent body weight change(^b)</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control vehicle</td>
<td>(mg/kg/day)</td>
<td>0</td>
<td>-8.8</td>
<td>3</td>
</tr>
<tr>
<td>AG3340</td>
<td>100</td>
<td>32.1</td>
<td>-3.9</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>39.4</td>
<td>+0.3</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>60.3</td>
<td>-6.0</td>
<td>2</td>
</tr>
<tr>
<td>Carboplatin</td>
<td>(mg/kg/wk)</td>
<td>20</td>
<td>13.4</td>
<td>-9.6</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>29.4</td>
<td>-24.5</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>55.6</td>
<td>-9.0(^c)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>64.8</td>
<td>-21.9</td>
<td>1</td>
</tr>
<tr>
<td>AG3340 + Carboplat</td>
<td>100</td>
<td></td>
<td>39.1</td>
<td>-14.6(^c)</td>
</tr>
<tr>
<td>AG3340</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>40</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AG3340 + Carboplat</td>
<td>400</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Mean value on day 38 relative to controls.
\(^b\) Mean value on day 38 relative to starting weight on day 3–5 before treatment began.
\(^c\) \(n\) number of studies (with 10–12 animals/group and two tumors/animal).
\(^d\) Control animals corresponding to this group lost 14.9\% body weight in this study (shown in Fig. 6).

Table 1 indicates that AG3340 produced cytostatic effects on tumor growth (Fig. 1A).

In separate studies, AG3340 was administered at 200 mg/kg/day and tumor growth was decreased by 39.4 ± 2.3\% (mean ± SD over two studies; \(P < 0.005\)). The average growth inhibition produced by AG3340 across studies is shown in the inset in Fig. 1A. Data in the inset were compared after 38 days across 2–3 studies and show the percent inhibition of tumor volumes relative to vehicle-treated controls. These data indicate that AG3340 produced a shallow dose-response curve for tumor growth inhibition.

Dose Tolerance to AG3340. AG3340 was well tolerated in tumor-bearing mice (Fig. 1B). Over 38 days, control animals bearing MV522 tumors lost 14.4 ± 1.5\% (SD) body weight (\(P < 0.002\)). At the same time, mice treated with 100 and 400 mg/kg/day AG3340 had body weight decreases of 10.5 ± 1.1\% and 12.5 ± 2.4\%, respectively. Weight decreases plateaued thereafter, and no changes in weights or health were noted between groups. These observations were repeated in a second study. However, in a third study, control animals lost 11.0\% body weight after 38 days (\(P < 0.05\) versus mean initial weight) and AG3340 (200 mg/kg/day) attenuated these decreases to -3.5\% (\(P > 0.05\) versus mean initial weight), which suggests a beneficial effect of AG3340 in the latter study.

The MTD for AG3340 was not definitively reached in this experiment and was assumed to be ≥400 mg/kg/day. In nude rats, the MTD for AG3340 was between 600 and 2000 mg/kg/day with b.i.d. dosing.\(^7\)

 Morphologies of Tumors Treated with AG3340. Tumors were collected 39–52 days into the study when growth inhibitions produced by AG3340 were evident as shown in Fig. 1. Fig. 2A shows control tumors that were collected on day 39. Control tumors were comprised primarily of poorly to moderately differentiated carcinoma cells interspersed with a moderate amount of stromal cells as shown by H&E staining. Pockets of tumor necrosis were also observed (not shown). In comparison, AG3340-treated tumors also contained moderate stromal cell infiltration but tended to be less differentiated than control tumors. Tumors in mice treated with 100 mg/kg/day AG3340 were collected on day 52, whereas tumors treated with 400 mg/kg/day AG3340 were collected on day 39. The different collection days were the result of harvesting tumors at different times because of the development of carboplatin toxicity (Fig. 2B); control and AG3340-treated groups had to be collected at times matching the carboplatin-containing regimens (in combination studies) to make proper comparisons. Significant increases in the amount of necrosis (areas containing cellular debris) were observed, and these increases were dose-dependent (Fig. 2, B and C).

An approximate 2-fold increase in necrosis over control (\(P < 0.05\)) was determined by masked analysis (by N. M. V.) of tumors in animals treated with 400 mg/kg/day AG3340 (4–6 tumors/group). Thus, physical caliper measurements of gross tumor volumes underestimated the actual percent inhibition because gross measurements included both viable and necrotic tumor areas.

Antiangiogenic Effects of AG3340. Angiogenesis was assessed by CD-31 staining (34) of tumors along with morphological assessments. Control tumors were highly vascularized as shown by prominent CD-31 staining of blood vessels (Fig. 2D). Cross-sectional staining of vessel lumens was evident as well as longitudinal staining and punctate staining of developing or incomplete vessels. Control tumors had 98 ± 25 vessels/200× field (\(n = 7\)). AG3340 dose-dependently decreased the number of CD-31-positive vessels by 45 ± 16\% and 77 ± 16\% at 100 and 400 mg/kg/day, respectively (\(P < 0.002\); Fig. 2, E and F, and Fig. 3). Importantly, vessels in AG3340-treated tumors had incomplete CD-31 staining (arrows in Fig. 2) and had a large percentage of vessels that appeared qualitatively different from control vessels; the percentage of vessels with cross-sectional lumens was greatly decreased. At 400 mg/kg/day, only vessel pieces or remnants stained positively for CD-31 (Fig. 2F), demonstrating a profoundly qualitative and quantitative inhibition of angiogenesis in these tumors. Inhibition of tumor angiogenesis was associated with increased tumor necrosis (Fig. 2 and 3).

\(^6\) Relative to the mean initial weight recorded on day 5 before dosing began.

\(^7\) D. R. Shalinsky and J. Brekken, unpublished data.
Antitumor Efficacy of Carboplatin. Carboplatin was relatively ineffective in decreasing tumor growth at doses approximating its MTD of \( \geq 30 \) mg/kg/week (Fig. 4). A statistically significant inhibition in tumor growth of 29.4 \( \pm \) 2.0\% \( (P, 0.02) \) was observed after dosing with 40 mg/kg/week. Similar inhibitions were achieved after dosing with 20 mg/kg/week, but these decreases were not statistically significant. Carboplatin inhibited tumor growth markedly only at higher, toxic doses (Table 1). Maximum inhibitions of 65 \( \pm \) 17\% were observed after dosing with 120 mg/kg/week.

Dose Tolerance to Carboplatin. Tolerance was assessed in tumor growth studies. Carboplatin was tolerated at 20 mg/kg/week through 45 days of study (Fig. 4B). Weights and health decreased dramatically thereafter necessitating euthanasia. After dosing with 40 mg/kg/week, carboplatin was relatively poorly tolerated. Body weights decreased significantly compared with controls after 2–3 weeks. Mice treated with 40 mg/kg/week carboplatin suffered weight decreases of approximately 10\% more than controls after 38 days (Fig. 4B). Thereafter, precipitous decreases (\( \geq 20\% \)) in body weights were recorded, necessitating euthanasia of most animals on day 39.

The 120 mg/kg/week-dose clearly exceeded the MTD for carboplatin as shown by body weight decreases recorded on day 38 (Table 1). This dose also had a pronounced effect on morbidity.

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Fig. 1  Dose-dependent inhibition of MV522 tumor growth by AG3340 (A) and tolerance to AG3340 as shown by body weight changes (B). A, inset, the percent inhibition of tumor growth relative to control tumors on day 39 across three studies. Values for efficacy are the mean \( \pm \) SE of 10–12 mice bearing two tumors/mouse. SD values for weights were \( \leq 15\% \) (omitted for clarity). BID, b.i.d.; PO, p.o.

Fig. 2  Morphological and antiangiogenic effects of AG3340 as assessed by H&E (A–C) and CD-31 staining (D–F). Representative staining in control (A and D), 100 mg/kg/day AG3340 (B and E), and 400 mg/kg/day AG3340 (C and F) is shown. \( \times200. \)
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Differentiated compared with control tumors (Fig. 5, controls). Representative histologies are shown in Fig. 5. Pockets mg/kg/week had inconsistent increases in necrosis relative to the 60 mg/kg/week-dose was very poorly tolerated and produced by CD-31 staining of MV522 tumors. Values are mean \( \pm SD \) of the number of CD-31 positive vessels obtained by counting at least five separate fields per tumor from six to eight tumors per group. * \( P < 0.002 \), different from control.

**Morphological Effects of Carboplatin in Tumors.** Tumors in mice dosed with 20–40 mg/kg/week were analyzed. Tumors in mice treated with regimens containing 20 mg/kg/week carboplatin were collected on the 52nd day of study. Because of the toxicity produced by a 40 mg/kg/week-dose, most mice treated with this dose of carboplatin (single agent and in combination) were euthanized, and their tumors were collected on day 39.

Tumors in mice treated with carboplatin at doses \( \leqslant 40 \) mg/kg/week had inconsistent increases in necrosis relative to controls. Representative histologies are shown in Fig. 5. Pockets of necrosis were observed in tumors that were generally poorly differentiated compared with control tumors (Fig. 5, A–C). Necrosis produced by carboplatin was dose-dependent, but the prevalence of necrotic tissues was relatively less than that produced by AG3340 (Fig. 2C versus Fig. 5C).

**Effects of Carboplatin on Angiogenesis in Tumors.** At a dose of 20 mg/kg/week, widespread CD-31-positive staining was evident on blood vessels (Fig. 5E). Overall, the extent of staining appeared decreased because vessels were generally smaller than in control tumors (Fig. 2D and 5D versus 5E and 5F). However, the number of vessels did not appear to be altered, and the cross-sectional morphologies of carboplatin-treated vessels were generally shaped similarly to those of controls. Punctate CD-31 staining was also observed as was seen in control tumors (Fig. 5, D–F). The pattern of CD-31 staining was similar at doses of 20–40 mg/kg/week carboplatin, with tumors in both of the groups showing cross-sectional areas of small and large vessels (Fig. 5, E and F). In summary, carboplatin decreased the size of blood vessels equivalently at doses of 20–40 mg/kg/week relative to vehicle-treated controls but did not appear to decrease the number of vessels. CD-31 staining was not assessed in tumors after treatment with higher, more toxic doses.

**Combination Chemotherapy with AG3340 and Carboplatin.** To model a clinical situation, doses of carboplatin that approximated its ineffective MTD (Fig. 4) were studied in combination with AG3340. Because AG3340 significantly inhibited tumor growth alone (Fig. 1), a suboptimal dose of AG3340 that modestly decreased tumor growth was chosen to allow for the potential of enhanced efficacy in the combination.

Fig. 6A shows the growth of MV522 tumors treated with AG3340 (100 mg/kg/day), carboplatin (40 mg/kg/week), and their combination. This combination significantly increased growth inhibitions over that produced by either agent alone \( (P < 0.01, \text{ combination versus single agent treatment}) \). The enhancement became evident after 24 days and was additive based on quantitative decreases in tumor growth recorded by caliper measurements. After 38 days, the magnitude of growth inhibition was 49.4 \pm 4.0% (Table 1). Inhibitions were deemed to have been synergistic based on histological assessments (discussion to follow).

In the combination study, dosing with 40 mg/kg/week carboplatin was stopped on day 38 after carboplatin had induced severe toxicity (Fig. 6B). Most of the animals in both of the carboplatin-containing regimens (single agent and in combination) were euthanized. A subset of three animals/group from the carboplatin and AG3340 + carboplatin groups were left on study, whereas the control and AG3340-treated groups were left intact except to collect three animals/group for comparative histological assessments. AG3340 dosing was continued without interruption in the remaining AG3340-treated animals.

The growth of tumors exposed to the combination regimen had plateaued on day 38 relative to tumors in mice treated with single agents (Fig. 6A). This plateau was continued for 7 days after the cessation of carboplatin dosing. Tumors then began to grow, but their volumes stayed below those of the single-agent groups over the remainder of the study. At a lower dose of carboplatin (20 mg/kg/week), the combination of AG3340 + carboplatin also produced greater tumor growth inhibitions as compared with either agent alone (Table 1).

Additionally, a combination of 400 mg/kg/day AG3340 + 40 mg/kg/week carboplatin was studied. Growth inhibitions of 62.2 \pm 4.6% \( (P < 0.001) \) were produced by the combination of these agents (Table 1), which was similar to inhibitions produced by AG3340 alone (60.3%). On the basis of assessment of tumor histologies, it seemed that the inhibition produced by AG3340 alone may have been at a maximum because of a large degree of tumor necrosis (data not shown). Greater inhibitions at this higher dose of AG3340 in combination therapy may, therefore, not have been possible.

**Tolerance to AG3340 and Carboplatin in Combination Chemotherapy.** Tolerance to carboplatin was not significantly changed by coadministration of AG3340 (Fig. 6B). Body weight changes across studies have been summarized for these agents in Table 1. In combination, carboplatin was studied at doses up to 40 mg/kg/week, and AG3340 was studied at doses...
of up to 400 mg/kg/day. Because AG3340 did not affect animal health or weights in these studies (Fig. 1B and Fig. 6B), carboplatin was deemed primarily responsible for the toxicities that were observed in combination regimens. Murine weights and health began to recover after the cessation of carboplatin dosing in single agent and combination regimens (Fig. 6B).

Proportional Hazards Analysis of the Interaction between AG3340 and Carboplatin. The data were retrospectively fit to a proportional hazards model to investigate whether the drugs inhibited tumor cell growth synergistically in combination (Table 2). The majority of animals receiving 40 mg/kg/week carboplatin were removed from the study on day 38 because the average weight loss in those groups was more than 20%. No criteria for treatment failure were applied to the individual animals that were euthanized on day 39. For the purposes of the analysis, censored animals could not be considered to have failed treatment by day 38, i.e., all that could be inferred about their survival was that they were healthy on day 38. The few animals from these groups that were not euthanized on day 39 did not give enough detailed information to characterize carboplatin toxicity nor drug interaction.

Thus, the data also were analyzed under two different operating assumptions in an attempt to bound results. Both assumptions concern the fate of the animals receiving 40 mg/
kg/week carboplatin that were removed from the study because of carboplatin toxicity. The first assumption was that all of those animals died the next day because of carboplatin toxicity. The second assumption was that one-half of those animals died the next day because of carboplatin toxicity; no assumption was made about the life span of the other half.

The optimal combination of AG3340 and carboplatin was calculated from the fitted model (Table 2). For all of the assumptions made about the censored animals, the predicted optimal doses of AG3340 in combination with carboplatin were similar. The predicted optimal doses of carboplatin, both in combination with AG3340 and as monotherapy, vary more. These differences between the predicted optimum doses of carboplatin clearly are tied to the different assumptions about the degree of carboplatin toxicity, in that the optimal dose of carboplatin decreases as the degree of assumed carboplatin toxicity increases.

The hazard (i.e., risk of treatment failure) for the optimal combination was computed and compared with the predicted optimal doses of the two drugs as monotherapy (Table 2). Regardless of the assumption made about the censored animals, the optimized monotherapies and combination therapy lowered the risk of treatment failure relative to that of the control group. The risk of treatment failure at any time was lowered by the optimized combination to 13–16% of that of the control group. In all of the cases, the hazard associated with the optimal dose of AG3340 and carboplatin in combination was lower than the hazards associated with the optimal doses of AG3340 and carboplatin as single agents.

These results suggest that the combination of AG3340 and carboplatin had a better-than-additive effect on tumor growth inhibition over that produced by monotherapy with carboplatin or AG3340. However, because of extensive censoring of animals because of carboplatin toxicity, a definitive conclusion of synergy could not be made. Thus, the hazards analysis indicated that the nature of the interaction between AG3340 and carboplatin was investigated.

![Figure 6](image_url)

**Fig. 6** Inhibition of tumor growth by 100 mg/kg/day AG3340 and 40 mg/kg/week carboplatin (A) and tolerance to this regimen as shown by body weight changes (B). Values are mean of 10–12 mice bearing two tumors/mouse. SEs were ±20% (omitted for clarity). Significant inhibitions for tumor growth were observed for each single agent, and for their combination versus single agents, as indicated in Fig. 1A.

**Table 2** Predicted optimal treatments for AG3340 and carboplatin

<table>
<thead>
<tr>
<th></th>
<th>Optimized combination</th>
<th>Optimized single-agents</th>
</tr>
</thead>
<tbody>
<tr>
<td>AG3340</td>
<td>Carboxplatin</td>
<td>AG3340</td>
</tr>
<tr>
<td>Dose (mg/kg/day)</td>
<td>274</td>
<td>21.2</td>
</tr>
<tr>
<td>Relative hazard</td>
<td>13%</td>
<td>19%</td>
</tr>
<tr>
<td>Dose (mg/kg/week)</td>
<td>271</td>
<td>16.1</td>
</tr>
<tr>
<td>Relative hazard</td>
<td>15%</td>
<td>22%</td>
</tr>
<tr>
<td>All failed</td>
<td>272</td>
<td>15.1</td>
</tr>
<tr>
<td>Relative hazard</td>
<td>16%</td>
<td>23%</td>
</tr>
</tbody>
</table>

* All of the animals receiving 40 mg/kg/week carboplatin that were removed from the study on day 38 were regarded as being healthy on day 38.

* Includes additional assumption about censored animals.
Platin was consistent with, but did not prove, that there had been a synergistic interaction.

**Morphological and Antiangiogenic Effects of AG3340 and Carboplatin in Tumors.** Marked necrosis was noted in tumors in mice treated with 100 mg/kg/day AG3340 and 20 mg/kg/week carboplatin (Fig. 7A). Generally, the extent of necrosis was greater in tumors after receiving this combination as compared with that observed after single agent treatment, but this increase was not always seen (data not shown). Patterns of CD-31 staining were disrupted relative to the corresponding single agent treatments (Fig. 2E and 5E), with both a decrease in the number and size of vessels observed (Fig. 7B).

The combination of 100 mg/kg/day AG3340 and 40 mg/kg/week carboplatin consistently produced necrosis (Fig. 7C) and inhibited or disrupted CD-31 staining (Fig. 7D) to a greater extent than that produced by the same respective single agents (Fig. 2E and 5F). The extent of the necrosis and inhibition of CD-31 staining approximated that of a high dose of 400 mg/kg/day AG3340 alone (Fig. 2, C and F). In this combination, in which the antitumor efficacy is shown in Fig. 6A, CD-31 staining was generally limited to blood vessel pieces or remnants. When cross-sectional lumens were evident, CD-31 staining was incomplete around the perimeter of the vessels (arrows in Fig. 7, B and D). These data suggest that tumor growth and angiogenesis were inhibited in an enhanced or synergistic manner by a combination of suboptimal doses of AG3340 and carboplatin.

**Combination Treatment of Tumors with AG3340 and Paclitaxel.** The effects of AG3340 and paclitaxel on tumor growth were also studied. Paclitaxel inhibited tumor growth by 61.2 ± 10.0% (P < 0.03) and produced significant morbidity at a weekly dose of 22.5 mg/kg (data not shown). To avoid the toxicity, a suboptimal dose of paclitaxel (11 mg/kg/week) was tested together with AG3340 (100 mg/kg/day). Paclitaxel did not affect tumor growth at this dose, but AG3340 decreased growth significantly by 37.4 ± 3.7% (P < 0.02; Fig. 8A). The combination regimen inhibited the growth of MV522 tumors by 54.2 ± 4.8%, with the additional decrease achieving statistical significance relative to AG3340 alone (P < 0.02). Thus, paclitaxel potentiated the growth inhibitory effects of AG3340 in this model. Tolerance to paclitaxel was not affected by cotreatment with AG3340 (Fig. 8B).

**Superior Therapeutic Index of AG3340 to Carboplatin.** A superior therapeutic index of AG3340 was demonstrated by its ability to markedly inhibit tumor growth at well-tolerated doses (Fig. 1) in contrast to carboplatin and paclitaxel, which did so only at toxic doses (Figs. 6 and 8; Table 1). Two approaches were used to calculate a therapeutic index. In the first, the window of achievable inhibition for tumor growth was assumed to be 100%. In this case, the therapeutic index for AG3340 was 1.4, based on a MTD of 400 mg/kg/day and an IC50 for growth inhibition of 296 mg/kg/day. In contrast, the therapeutic index for carboplatin was 0.5 using a MTD of 30 mg/kg/week and an IC50 value of 60 mg/kg/week.

In the second approach, it was assumed that tumor volume measurements, upon which the index was derived, were confounded by drug-induced necrosis. In other words, growth inhibitions were assumed to have been underestimated because of the necrosis included within volume measurements; it was assumed that tumors could not be inhibited by more than 65%, as

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9 To prevent the development of paclitaxel-induced toxicity, paclitaxel dosing was stopped between days 20 and 27 (in single agent and in combination).
was observed with mono or combination therapy. An IC\textsubscript{50} for tumor growth inhibition under this assumption would, therefore, equal a 33% decrease (65%/2) in tumor volumes. In this case, the therapeutic index for AG3340 was >4 based on a MTD of 2800 mg/kg/week (400 mg/kg/day) and an IC\textsubscript{50} for tumor growth inhibition of 700 mg/kg/week (100 mg/kg/day). In comparison, the therapeutic index for carboplatin was <0.75 based on a MTD of 30 mg/kg/week and an IC\textsubscript{50} for tumor growth inhibition of 40 mg/kg/week (Table 1).

In either scenario, AG3340 produced a therapeutic index superior to that of carboplatin. These values are only estimates in an attempt to quantify the differences in effectiveness of these agents in this tumor model. The index values are conservative because efficacy values were based on gross tumor volume measurements without regard to drug-induced tumor necrosis. If tumor volumes could have been pruned of nonviable tissues, increased therapeutic indices would have resulted, and the increase would have been greater for AG3340 than for carboplatin because of the more prominent effects of AG3340 on inducing necrosis. Additionally, the computed value for the therapeutic index of AG3340 is doubly conservative because the MTD for AG3340 was underestimated.

Plasma Concentrations of AG3340 after Dosing with Carboplatin. Plasma concentrations of AG3340 were quantified 1 h after dosing with AG3340 in animals that had been pretreated either with AG3340 alone or with a combination of AG3340 and carboplatin (Fig. 9). After receiving a single dose of 50 mg/kg AG3340, plasma concentrations of AG3340 ranged from 300–710 ng/ml after 1 h; concentrations were 2800 ng/ml after dosing with 200 mg/kg AG3340. Pretreatment with carboplatin had no discernible effect on the plasma concentrations of AG3340 when AG3340 and carboplatin were administered concurrently (n = three experiments with two to three animals/time point; Fig. 9).

**DISCUSSION**

We determined the antitumor efficacy of AG3340, a novel metalloprotease inhibitor, in an aggressive preclinical lung cancer tumor model that mimics key features of clinical NSCLC (3, 4). Initially, we confirmed the findings of Kelner et al. (30) that MV522 tumors are chemoresistant and induce significant morbidity in nude mice. Thus, this was a challenging paradigm to test the anticancer effects of AG3340.

AG3340 produced prominent tumor growth inhibitions by itself (Fig. 1). Inhibitions were dose-dependent and notable because of the chemoresistance of MV522 tumors. Importantly,
AG3340 induced significant tumor necrosis (Fig. 2), indicating that physical caliper measurements underestimated actual growth inhibitions. The 65% inhibition of tumor growth produced by AG3340 (400 mg/kg/day) in this model corresponded to a total or near total growth inhibition when tumor necrosis was taken into account. AG3340 has also produced significant tumor necrosis in androgen-independent prostatic PC-3 tumors in vivo (27).

Importantly, AG3340 had a positive, superior therapeutic index relative to carboplatin in this tumor model. Carboplatin was ineffective as a cytotoxic agent as it often is clinically (37). This observation could also be extended to paclitaxel, which, along with carboplatin, did not inhibit the growth of MV522 tumors to an appreciable extent at doses approximating its MTD.

Tumor growth inhibitions produced by AG3340 were cytostatic (Fig. 1). This is not altogether surprising in light of the noncytotoxic nature of AG3340. However, in another human tumor model that is sensitive to growth inhibitory effects of AG3340, quiescent colon tumor cells did not reenter the cell cycle after the cessation of AG3340 dosing. Rather, increased retention of blood led to increased tumor volumes that created an apparent regrowth.10 The basis for the regrowth has not been investigated in MV522 tumors and warrants further study. Nevertheless, these data indicate that continuous dosing of AG3340 was required to maintain tumor growth inhibitions in this lung cancer model.

CD-31 staining has been reported to reflect newly formed blood vessels and correlate with clinical prognosis and malignancy in cancer patients (38–42) and was, therefore, studied in this tumor model. The high vascular density of these tumors was shown by extensive CD-31 staining, an endothelial cell marker expressed on newly forming blood vessels (34). Because of the semiquantitative nature of manual scoring of CD-31-positive vessels, the pattern of CD-31 staining in tumors was scrutinized for qualitative changes, and photomicrographs were prepared, which allow the readers to judge the effects of the agents studied here on angiogenesis (Fig. 2, 5, and 7).

AG3340 dose-dependently decreased the number of blood vessels in MV522 tumors (Fig. 3). Furthermore, AG3340 altered the pattern of CD-31 staining so that vessels lost their cross-sectional luminal morphologies (Fig. 2). At 400 mg/kg/day, generally only vessel pieces or remnants stained positively for CD-31 in tumors (Fig. 2F). These qualitative changes were not produced by carboplatin even at a dose of 40 mg/kg/week (Fig. 5F), which significantly, albeit modestly, decreased tumor growth. These data suggest that AG3340 inhibited tumor growth by prominently inhibiting angiogenesis, whereas carboplatin did not have its predominant effect on tumor angiogenesis.

Kerbel, Teicher, and others (6, 43, 44) have proposed a novel approach of combining antiangiogenic with cytotoxic agents to improve cancer chemotherapy. This contention is based on the requirement of tumors for blood supply and on the hypothesis that the host vasculature will not become resistant to antiangiogenic therapy (6, 45, 46). Recent reports demonstrating that resistance does not develop to the antiangiogenic agent, angiostatin (46, 47), bolster this hypothesis. Antiangiogenic agents, such as angiostatin and TNP-470, enhance the efficacy of cytotoxic agents or radiation in preclinical tumor models (43, 44, 48). Furthermore, the MMP inhibitor, batimastat, enhances the cytotoxicity of cisplatin in nude mice bearing ovarian carcinomas (49). Inhibition of MMP-associated angiogenesis (18) may have contributed to the enhanced efficacy observed in that study. These reports, along with our own reported here, support further clinical investigation of AG3340 in combination with cytotoxic agents.

Combination therapy with AG3340 and carboplatin seemed to be superior to single agent treatments in this NSCLC tumor model. Analysis of tumor growth inhibitions, the extent of tumor necrosis, and the inhibition of angiogenesis led to this conclusion (Fig. 2–7). Histological assessments suggested that inhibitions may have been synergistic, but this could not be reliably quantified. A proportional hazards approach was, therefore, taken to determine whether inhibitions produced by the combination were synergistic. Hazards analysis also suggested that the drugs in combination may have been better than either drug alone. Unfortunately, the censoring of animals receiving 40 mg/kg/week carboplatin was so extensive that no definitive conclusion of synergy could be drawn based on the hazard analysis of the data generated in these experiments. Nevertheless, a comprehensive analysis of tumor histology, angiogenesis, and growth data suggested that combination therapy was superior to monotherapy in this tumor model.

Enhanced tumor growth inhibitions were also produced by the combination of an ineffective dose of paclitaxel with a suboptimal dose of AG3340 (Fig. 8). However, in the case of paclitaxel, the enhanced response was due to a potentiation of the effects of AG3340, supporting the finding that AG3340 acted as the primary stimulus for inhibiting the growth of MV522 tumors. These data suggest that AG3340 may produce an enhanced antitumor efficacy with a variety of cytotoxic agents.

Notably, AG3340 did not alter tolerance to cytotoxic agents. AG3340 did not augment the toxicities of cytotoxic agents, even when tested in combination with a poorly tolerated dose of carboplatin (Fig. 6B) or with a dose of paclitaxel that was approximately 50% of its MTD (Fig. 8B). In summary, AG3340 was well tolerated as a single agent and in combination regimens.

Suboptimal doses of AG3340 or carboplatin alone affected angiogenesis moderately or modestly, respectively, (Fig. 2E and 5F) compared with effects observed in combination therapy (Fig. 7D). Thus, the enhanced efficacy in combination was associated with an enhanced inhibition of angiogenesis. Interestingly, the magnitude of the enhanced antiangiogenic effect of combination therapy was similar to that produced by AG3340 alone (Fig. 2F), which suggests that AG3340 contributed heavily to the effect. Combination therapy markedly enhanced the antiangiogenic effects of AG3340. However, carboplatin also had an effect on blood vessels, generally decreasing their size, which suggests that carboplatin may also have contributed significantly to the synergistic decrease in angiogenesis observed after combination chemotherapy.

Additionally, other cytostatic or cytotoxic mechanisms of

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10 D. R. Shalinsky, unpublished data.
tumor growth inhibition would likely have been induced by AG3340 and carboplatin. Mechanisms for each agent would naturally have been quite different. For example, AG3340, a noncytotoxic MMP inhibitor, would have likely disrupted signaling and attachment functions of the cellular matrix, on which both host and carcinoma tissues depend for survival (50). In fact, AG3340 has prominent antiproliferative and proapoptotic activities in other preclinical tumor models (23–27), which supports this contention. On the other hand, a cytotoxic agent such as carboplatin would nonspecifically suppress the function of DNA, RNA, and macromolecule proteins to disrupt cell survival (51). Thus, these data support an emerging concept that it will be beneficial to combine antiangiogenic and cytotoxic agents to improve cancer chemotherapy (6, 43, 44, 48, 49).

To determine whether the enhanced efficacy in combination may have been due to altered pharmacokinetics of AG3340, plasma concentrations of AG3340 were examined. Carboplatin did not alter plasma concentrations of AG3340 (Fig. 9) as might have been expected if carboplatin had acted to increase AG3340 concentrations to enhance its activity. Conversely, AG3340 may have enhanced the plasma or tumor concentrations of cytotoxic agents. There is a potential basis for this possibility because it has been reported that the angiogenesis inhibitor, TNP-470, can increase tumor and tissue levels of cis-platinum (52), when given in combination. This possibility was not tested and cannot be ruled out. Theoretically, increased vessel permeabilities could occur after the initial disruption of angiogenesis by novel inhibitors, leading to increased tumor exposure to cytotoxic agents. Over time, vessels may have regressed or stopped developing, which could lead to decreased delivery of cytotoxic agents. Additionally, tumors may change their dependency on angiogenic pathways when faced with antiangiogenic therapy, which may change blood flow or vessel permeabilities over time. Additional studies are needed to better explore potential pharmacokinetic interactions of AG3340 in combination therapy.

In conclusion, AG3340 produced remarkable antitumor efficacy in chemoresistant human NSCLC tumors (line MV522). Efficacy was associated with a prominent inhibition of angiogenesis and induction of marked tumor necrosis. Efficacy was associated with a prominent inhibition of angiogenesis and induction of marked tumor necrosis (51). Thus, these data support an emerging concept that it will be beneficial to combine antiangiogenic and cytotoxic agents to improve cancer chemotherapy (6, 43, 44, 48, 49).

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Marked Antiangiogenic and Antitumor Efficacy of AG3340 in Chemoresistant Human Non-Small Cell Lung Cancer Tumors: Single Agent and Combination Chemotherapy Studies

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