A Novel Small-Molecule Inhibitor of Transforming Growth Factor β Type I Receptor Kinase (SM16) Inhibits Murine Mesothelioma Tumor Growth In vivo and Prevents Tumor Recurrence after Surgical Resection

Eiji Suzuki,° Samuel Kim,° H.-Kam Cheung, Michael J. Corbley, Ximei Zhang, Lihong Sun, Feng Shan, Juswinder Singh, Wen-Cherng Lee, Steven M. Albelda, and Leona E. Ling

1Thoracic Oncology Research Laboratory, University of Pennsylvania, Philadelphia, Pennsylvania and 2Oncology Cell Signaling, Biogen Idec, Cambridge, Massachusetts

Abstract

Malignant mesothelioma is an aggressive and lethal pleural cancer that overexpresses transforming growth factor β (TGFβ). We investigated the efficacy of a novel small-molecule TGFβ type I receptor (ALK5) kinase inhibitor, SM16, in the AB12 syngeneic model of malignant mesothelioma. SM16 inhibited TGFβ signaling seen as decreased phosphorylated Smad2/3 levels for at least 3 h following treatment of tumor-bearing mice with a single i.p. bolus of 20 mg/kg SM16. The growth of established AB12 tumors was significantly inhibited by 5 mg/kg/d SM16 (P < 0.001) delivered via s.c. miniosmotic pumps over 28 days. The efficacy of SM16 was a result of a CD8+ antitumor response because (a) the antitumor effects were markedly diminished in severe combined immunodeficient mice and (b) CD8+ T cells isolated from spleens of mice treated with SM16 showed strong antitumor cytolytic effects whereas CD8+ T cells isolated from spleens of tumor-bearing mice treated with control vehicle showed minimal activity. Treatment of mice bearing large tumors with 5 mg/kg/d SM16 after debulking surgery reduced the extent of tumor recurrence from 80% to <20% (P < 0.05). SM16 was also highly effective in blocking and regressing tumors when given p.o. at doses of 0.45 or 0.65 g/kg in mouse chow. Thus, SM16 shows potent activity against established AB12 malignant mesothelioma tumors using an immune-mediated mechanism and can significantly prevent tumor recurrence after resection of bulky AB12 malignant mesothelioma tumors. These data suggest that ALK5 inhibitors, such as SM16, offer significant potential for the treatment of malignant mesothelioma and possibly other cancers. [Cancer Res 2007;67(5):2351–9]

Introduction

Transforming growth factor β (TGFβ) is a multifunctional cytokine overexpressed by a variety of tumors (1–3). Many human cancers show a correlation between overexpression of TGFβ and advanced disease or poor prognosis (1–3). Neutralization of TGFβ has shown potential clinical utility in a variety of murine models of cancer, including breast cancer (4–8), thymoma (9), hepatocellular carcinoma (10), glioma (11, 12), head and neck carcinoma (13), and malignant mesothelioma (14, 15). Depending on the model, the antitumor activity of TGFβ antagonists is mediated by one or more autocrine or paracrine mechanisms, including increased immune surveillance, inhibition of angiogenesis, inhibition of invasion, metastases and epithelial to mesenchymal transition, as well as the inhibition of collagen deposition and tumor interstitial pressure (1, 2, 16–18).

Malignant mesothelioma is an aggressive pulmonary cancer of the pleura and serosa, which has been shown to overexpress TGFβ (reviewed in ref. 15). Pleural fluids from mesothelioma patients contain TGFβ levels that are 3- to 6-fold elevated compared with primary lung cancers (19, 20). Several mesothelioma cell lines also overexpress TGFβ (reviewed in ref. 15). We showed previously that TGFβ seems to contribute significantly to the growth of TGFβ-overexpressing malignant mesothelioma cells in murine malignant mesothelioma tumor models (15). Treatment of tumor-bearing mice with the soluble TGFβ type II receptor fusion protein s(TGFβRII:Fc) inhibited the growth of tumors in two established syngeneic malignant mesothelioma tumor models, AB12 and AC29. These effects seemed to be primarily through the induction of CD8+ T-cell antitumor activity. These preclinical results in malignant mesothelioma and other cancer models show the potential for TGFβ blocking agents in anticancer therapy.

Several TGFβ antagonist agents have been developed for preclinical and clinical investigation. In addition to s(TGFβRII:Fc), an agent that binds TGFβ1 and TGFβ3 with high affinity, several monoclonal antibodies (mAb) and antisense oligonucleotide agents that bind various isoforms of TGFβ have been developed. Of these, one TGFβ1 antisense oligonucleotide is in clinical development in oncology and has shown promising results in a phase I glioma trial (21). Additional TGFβ antagonist agents are in preclinical and clinical development (1).

Small-molecule inhibitors of TGFβ type I receptor (ALK5) kinase have also been developed recently (16, 22). Therapeutically, small-molecule kinase inhibitors provide increased flexibility in dosing regimen and route of administration (i.e., p.o. delivery) compared with both protein- and oligonucleotide-based TGFβ antagonist agents. They also lack the potential for inducing neutralizing antibodies inherent in the protein-based TGFβ antagonist agents. Several of these ALK5 kinase inhibitors have shown efficacy in

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Steven M. Albelda, Thoracic Oncology Research Laboratory, University of Pennsylvania, BB1 II/III, 421 Curie Boulevard, Philadelphia, PA 19104. Phone: 215-573-9969; Fax: 215-573-4469; E-mail: albelda@mail.med.upenn.edu.

www.aacrjournals.org 2351 Cancer Res 2007; 67: (5). March 1, 2007
models of fibrosis (23–25). However, the efficacy of ALK5 inhibitors in preventing tumor growth was only recently shown in an orthotopic model of glioma (12), and several recent reports suggest activity in breast, head and neck, and lung cancer tumor models (13, 26, 27).

To determine if ALK5 kinase inhibitors are effective in treating malignant mesothelioma, we used a novel ALK5 inhibitor, SM16 (13). This small-molecule kinase inhibitor, like those previously described (28), was shown to be a potent selective inhibitor of ALK5 and ALK4, the activin type IB receptor. Our results using SM16 delivered via osmotic pumps or by p.o. administration show that this small, p.o. available inhibitor of TGFβ2 activity can inhibit the growth of and even eradicate established non–TGFβ2-responsive tumors without overt toxicity by inhibiting the immunosuppressive activities of TGFβ2. Agents, such as SM16, show promise for difficult to treat tumors, such as malignant mesothelioma.

Materials and Methods

Animals
Pathogen-free female BALB/c mice (6–8 weeks old, 19–24 g) were purchased from Taconic Laboratories (Germantown, NY). CB-17 severe combined immunodeficient (SCID) mice (6–8 weeks old, 19–24 g) were bred at the Wistar Institute (Philadelphia, PA). All mice were maintained in a pathogen-free animal facility for at least 1 week before each experiment. The animal use committees of the Wistar Institute and University of Pennsylvania approved all animal study protocols described in this publication, and experiments were conducted in compliance with the Guide for the Care and Use of Laboratory Animals.

Cell Lines

A murine malignant mesothelioma cell line, AB12, was obtained from Dr. Bruce Robinson (University of Western Australia, Nedlands, Western Australia, Australia). This line was derived in BALB/c mice and grows well as flank tumors (29). AB12 cells secrete large amounts (462 pmol/10⁶ cells/24 h) of TGFβ2, with most of this TGFβ2 in latent form (30). L1C2 cells are a mouse lung cancer cell line obtained from the American Type Culture Collection (Rockville, MD). Both cell lines were cultured and maintained in high-glucose DMEM (Mediatech, Washington, DC) supplemented with 10% fetal bovine serum (FBS; Georgia Biotechnology, Atlanta, GA), 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L glutamine. The cell line was regularly tested and maintained negative for Mycoplasma spp.

ALK5 Inhibitor (SM16)

SM16, an ALK5/ALK4 kinase inhibitor with a molecular weight of 430, was described previously (13,28). Briefly, SM16 binds ALK5 (K1/2, 10 nmol/L) and ALK4 (K1/2, 1.5 nmol/L) with high affinity at the ATP-binding site. In HepG2 cells, SM16 inhibits TGFβ2-induced plasminogen activator inhibitor-luciferase activity (IC50, 64 nmol/L) and TGFβ2- or activin-induced Smad2 phosphorylation at concentrations between 100 and 620 nmol/L. SM16 was tested against >60 related and unrelated kinases and showed moderate off-target activity only against Raf (IC50, 1 mmol/L) and p38/SAPKα (IC50, 0.8 mmol/L). SM16 exhibited no inhibitory activity against ALK family members ALK1 and ALK3. ALK7 blocking activity has not been determined.

Inhibition of TGFβ2-Induced Smad2/3 Phosphorylation in AB12 Cells

Confluent AB12 cells were preincubated for 1 h at 37°C in 2 mL DMEM plus 0.5% FBS supplemented with a 1:3 dilution series of SM16. Final DMSO concentration was 1%, TGFβ2 (R&D Systems, Minneapolis, MN) was added and cells were incubated for 1.5 h at 37°C. The cells were rinsed with cold PBS and lysed in 150 μL SDS-PAGE loading buffer containing 20 mmol/L NaF and a protease inhibitor cocktail (Complete; Roche, Nutley, NJ). The samples were separated on a 10% SDS-PAGE gel and transferred to nitrocellulose membranes for Western blot analysis. The membranes were blocked for 1 h at room temperature in 5% milk/PBS-Tween 20. Total Smad2 was analyzed with a rabbit polyclonal anti-Smad2/3 primary antibody (Cell Signaling, Beverly, MA), 1:2,000 in 5% milk, overnight at 4°C followed by a horseradish peroxidase (HRP)-linked goat anti-rabbit secondary antibody (Bio-Rad, Hercules, CA), 1:4,000, for 1 h at room temperature. The blot was stripped with Restore (Pierce Biotechnology, Rockford, IL). Phosphorylation of Smad2 was analyzed with a rabbit polyclonal anti–phosphorylated Smad2 primary antibody (Cell Signaling), 1:2,000 in 5% milk, overnight at 4°C followed by the same secondary antibody. Blots were developed with SuperSignal West Pico (Pierce Biotechnology) and visualized by exposure to film.

Inhibition of TGFβ3 Signaling in AB12 Tumors In vivo by SM16

BALB/c mice were injected on the right flank with 1 × 10⁶ AB12 tumor cells. When tumors reached 200 to 250 mm³ in size, the mice were injected with a single i.p. bolus of 40 mg/kg SM16 in 20% Captisol (CyDex, Inc., Lenexa, KS). The mice were sacrificed and the AB12 tumors were dissected and snap frozen in liquid nitrogen. To measure phosphorylated Smad2 and total Smad2 in tumors, pulverized frozen AB12 tumor samples were extracted for 1 h at 4°C in T-PER buffer (Pierce Biotechnology) containing protease inhibitors (Roche). A total of 5 μg of each tumor extract was separated on SDS-PAGE and transferred to nitrocellulose membrane for Western blot analysis. Phosphorylated Smad2/3 levels were detected as above. Total Smad2 protein levels were detected by stripping the blots or by probing duplicate blots with an anti-Smad2 mouse primary antibody (Cell Signaling). 1:1,000,000 dilution (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), and the blot was then developed as above.

AB12 Tumor Efficacy Models

Antitumor efficacy with SM16 delivered via osmotic pump. BALB/c mice were injected on the right flank with 1 × 10⁶ AB12 tumor cells. For s.c. pump delivery, SM16 was formulated in vehicle, 20% Captisol, at various concentrations (0, 5, 10, and 20 mg/mL) and loaded into Alzet model 2004 miniosmotic pumps (Alzet Corp., Cupertino, CA). The pumps were allowed to prime in sterile saline for 18 to 20 h before implantation to reach the expected dispensing rate of 0.25 μL/h. At this rate, the above concentrations of SM16 would provide doses of 0, 1.25, 2.5, and 5 mg/kg/d per 23 g mouse. When the tumors reached a minimal volume of 300 mm³ (12 days after tumor cell inoculation), mice were anesthetized with i.p. injection of 70 mg/kg ketamine and 7 mg/kg xylazine and the pumps were implanted s.c. on the left flank of the mice. The SCID mice were studied using the same experimental design as the BALB/c mice. Tumor volumes were estimated using the formula (π × long axis × short axis × short axis) / 6. We did measurements of tumors twice weekly. At sacrifice, plasma was obtained under anesthesia and analyzed for SM16 plasma levels.

Debulking surgery models. BALB/c mice were injected on the right flank with 1 × 10⁶ AB12 tumor cells. When the tumors reached a minimal volume of 850 mm³ (25 days after tumor cell inoculation), mice were anesthetized and a complete resection of the tumors was attempted. All macroscopically visible tumor was removed and the wound was closed using silk suture. At the time of debulking, mice were randomly divided into two groups and one group was implanted with minipumps loaded with 20% Captisol (control) on the left flank and the other group was implanted with minipumps loaded with 20 mg/mL SM16. Tumor recurrence was defined as the first day when a tumor was unambiguously visible or palpable (approximately 2 × 2 mm). Unless otherwise mentioned, each control or experimental group had a minimum of five mice. Plasma was obtained under anesthesia and analyzed for SM16.

P.o. delivery model. AB12 cells were injected into the right flanks of BALB/c mice as described above. Mice were fed standard chow ad libitum. When tumors grew to ~100 mm³, the mice were randomized into...
In vivo Tumor Neutralization Assay (Winn Assay)

Winn assays were done as described previously (31). Splenocytes were isolated and CD8+ T lymphocytes were purified using the MACS system (Miltenyi Biotec, Auburn, CA). This cell population contained >90% CD8+ cells by flow cytometry (data not shown). The CD8+ T lymphocyte–enriched population from normal, tumor-sensitized, or tumor-sensitized and treated mice was admixed with viable AB12 tumor cells at a ratio of three purified CD8+ splenocytes for each tumor cell, and the mixture was inoculated s.c. into the flanks of naive BALB/c mice. Each mixture thus contained 0.5 × 106 tumor cells and 1.5 × 107 CD8+ T cells. This ratio has previously been determined to be optimal for detecting positive and negative effects (32). To show specificity, Winn assays were also done as above but using L1C2 lung cancer cells (which grow in BALB/c mice) as targets. Tumor growth was measured after 1 week and expressed as the mean ± SE of at least five mice per group.

Bioanalytic Analysis of SM16 in Plasma

The concentration of SM16 in plasma was analyzed by high-performance liquid chromatography on a Zorbax SB-C8 3.5-μm (2.1 × 50 mm) column (Agilent, Palo Alto, CA) followed by mass spectrometry (MS) on a triple quadrupole mass spectrometer (SCIEX API 4000, Applied Biosystems, Foster City, CA) equipped with a Turbo Ion Spray probe operated in positive ion mode. Plasma SM16 was first subjected to solid-phase extraction on an Oasis HLB μElution SPE plate (Waters, Milford, MA) preconditioned with methanol and water (SPE), washed with 5% methanol, and eluted with acetonitrile/isopropanol (40:60). Samples were further diluted with 50:50:0.1 acetonitrile/water/formic acid (v/v/v) before analysis by liquid chromatography-mass spectrometry using multiple reaction monitoring. Data were collected and processed using Analyst version 1.4.1 (Applied Biosystems).

Statistical Analyses

Data comparing differences between two groups were assessed using unpaired Student’s t test. ANOVA with post hoc testing was used for multiple comparisons. Differences in survival were analyzed using the log-rank test. Differences were considered significant when P was <0.05. Statistical analysis was conducted using the StatView 5.0 for Windows program.

Results

Inhibition of TGFβ signaling in cultured AB12 cells and AB12 tumors by SM16. The ability of SM16 to inhibit TGFβ signaling was evaluated in AB12 cells in vitro. As previously reported (15), AB12 cells express a low basal level of phosphorylated Smad2, which is further increased by incubation with exogeneous TGFβ (Fig. 1A, left). However, addition of TGFβ (10 ng/mL) along with SM16 at concentrations ranging from 8 to 2,000 nmol/L prevented the TGFβ-dependent elevation of phosphorylated Smad2 in a dose-dependent manner. The total amount of Smad2 protein was unchanged under these conditions. The approximate IC50 for inhibition of phosphorylated Smad2 in these cells was 200 nmol/L.

AB12 tumors, formed after s.c. implantation of cells in syngeneic BALB/c mice, showed constitutive activation of the TGFβ pathway as indicated by the presence of phosphorylated Smad2 in these tumors (Fig. 1B, vehicle treated). Administration of SM16 significantly decreased the phosphorylated Smad2 level in these tumors for up to 12 h, with the phosphorylated Smad2 signal returning at 24 h after administration (Fig. 1B). These results suggest that SM16 is able to distribute to s.c. AB12 tumors and inhibit TGFβ signaling in these tumors for at least 12 h after a single dose.

SM16 is an effective inhibitor of AB12 tumor growth. To investigate the potential therapeutic utility of SM16 and to determine the circulating level of SM16 required for efficacy, we gave various concentrations of SM16 using miniosmotic pumps to mice bearing established AB12 tumors. AB12 tumors in BALB/c mice were allowed to reach 300 mm3 in size, at which time treatment with SM16 was initiated. No differences were observed in tumor growth in mice that did not undergo surgical pump implantation compared with those surgically implanted with pumps containing the vehicle alone (data not shown). However, SM16 significantly inhibited tumor growth at a dose of 5 mg/kg/d compared with the vehicle control (P < 0.001; Fig. 2A). At day 28, tumors treated with 5 mg/kg/d SM16 were 599 ± 59 mm3 versus 1,358 ± 127 mm3 in control groups, a more than 2.4-fold reduction in size. Treatment with the two lower doses, 1.25 and 2.5 mg/kg/d, showed a nonstatistical trend of tumor growth inhibition compared with vehicle control, suggesting dose-dependent inhibition by SM16.
SM16 levels in plasma at day 28 were measured to identify the circulating SM16 concentration required to achieve efficacy. Figure 2B shows that the average plasma concentration of SM16 was 0.77 ± 0.08 μmol/L at 1.25 mg/kg/d, 0.70 ± 0.07 μmol/L at 2.5 mg/kg/d, and 1.52 ± 0.33 μmol/L at 5 mg/kg/d. Statistically significant efficacy was achieved at the 5 mg/kg/d dose, which corresponded to a plasma concentration of 1.5 μmol/L. Interestingly, a similar concentration (2 μmol/L) of SM16 was able to significantly inhibit phosphorylated Smad2 in cultured AB12 cells to unstimulated levels (Fig. 1A). These data show the efficacy of SM16 in inhibiting the growth of AB12 malignant mesothelioma tumors in this syngeneic model.

SM16 efficacy is primarily immune cell mediated: loss of efficacy in SCID mice. We next tested whether the efficacy of SM16 was primarily due to increased antitumor immune responses as seen previously with sTGFβRII:Fc in this model (15). SCID mice bearing AB12 tumors (300 mm³ in size) were treated with either vehicle alone (control) or 5 mg/kg/d SM16. As shown in Fig. 3, there was no significant difference in tumor volume between control-treated mice and SM16-treated mice at any time during the 12 days of treatment. AB12 tumors grew significantly faster in SCID compared with BALB/c mice so that treatment began at day 7 and sacrifice of the SM16- and vehicle-treated groups was required 12 days after the initiation of treatment. These results confirm the importance of lymphocytes for SM16 activity in the AB12 tumor model.

SM16 prevents loss of CTL activity. In our previous study, we showed that (a) BALB/c mice bearing small AB12 tumors generate endogenous CTL activity in the spleen; (b) when tumors became larger in size, the endogenous CTL lost their ability to lyse target AB12 tumors; and (c) blockade of TGFβ using sTGFβRII:Fc in mice bearing small AB12 tumors prevented the loss of CTL activity (15). To evaluate if SM16 treatment also prevented the loss of CTL activity in the AB12 tumor model, we assayed for splenic CTLs using an in vivo tumor neutralization assay (Winn assay; Fig. 4).

We prepared two control groups of CD8⁺ T-cell preparations as follows: group 1, naïve CD8⁺ T cells from the spleens of nontumor-bearing animals (negative control), and group 2, CD8⁺ T cells from animals bearing “small” (≤ 100 mm³, 7 days after tumor cell inoculation) AB12 tumors (positive control). We also prepared two experimental groups: group 3, CD8⁺ T cells from tumor-bearing mice treated with vehicle alone, and group 4, CD8⁺ T cells from tumor-bearing mice treated with 5 mg/kg/d SM16. In groups 3 and 4, animals with established AB12 tumors (300 mm³) were treated with vehicle- or SM16-loaded pumps (SM16) for 10 days. After the treatment for 10 days, the tumors grew to a size of ~ 500 mm³ in the control animals, whereas they were reduced to ~ 200 mm³ in treated animals. Splenocytes were isolated and CD8⁺ T cells were purified from these two groups of animals.

CD8⁺ T cells from all groups were mixed with fresh AB12 tumor cells in a ratio of three CD8⁺ T cells to one tumor cell, and the mixtures were injected into the flanks of naive BALB/c mice. This ratio was established from preliminary dose titration studies, establishing that both positive and negative effects could be seen at this ratio. Tumors were allowed to grow for 1 week. As shown in Fig. 4, control AB12 tumor cells grew to a size of ~ 300 mm³ in 1 week. Addition of CD8⁺ T cells from nontumor-bearing animals (group 1) did not significantly slow the tumor growth. In contrast, tumor cells mixed with CD8⁺ T cells from animals bearing “small” tumors (group 2) at a lymphocyte to tumor ratio of 3:1 grew to only 99 mm³. Compared with tumors mixed with naïve CD8⁺ T cells (group 1), this represented a 67% decrease of the growth (P < 0.01). Thus, in animals bearing small tumors, some spontaneous antitumor CD8⁺ T-cell activity is induced by the AB12 tumor cells.

As previously reported (15), as the tumor increases in size, this CD8⁺ T-cell activity is lost. This can be seen in the T cells from group 3, where CD8⁺ T cells from animals treated with vehicle lost all antitumor activity. However, the mice treated with SM16...
Groups were as follows: no CD8 cells added (AB12 cells only), naive CD8+ injected into the flanks of naive mice. Tumor size was measured after 1 wk.

Addition of CD8+ T cells from nontumor-bearing animals (naive, group 1) did not show antitumor cytolytic activity against AB12 cells. Extent of tumor growth in mice 1 wk after implantation with L1C2 cells alone (group 2), splenic CD8+ T cells from AB12-bearing mice treated with vehicle (control; group 3), and splenic CD8+ T cells from AB12-bearing mice treated with SM16 (group 4) showed persistent antitumor cytolytic activity. The extent of tumor recurrence was significantly different between the naive, control, and SM16-treated groups. The data above confirmed that SM16 could be useful for treatment of large established AB12 tumors and that CD8+ T cells were generated (or maintained).

**Treatment with SM16 after surgery markedly reduces the extent of tumor recurrence.** The data above confirmed that SM16 could be useful for treatment of large established AB12 tumors and that CD8+ T cells were generated (or maintained). Next, we evaluated the potential therapeutic utility of SM16 in another clinically relevant animal model: postsurgical adjuvant therapy.

BALB/c mice were injected on the right flank with 1 x 10^6 AB12 tumor cells. When the tumors reached a minimal volume of 850 mm^3 a complete resection of the tumors was done and mice were randomly divided into two groups. One group was implanted with vehicle-loaded pumps (control) and the other group was implanted with pumps loaded with 5 mg/kg/d SM16. After resection of large AB12 tumors, only 14% of control-treated mice remained recurrence-free at 19 days after debulking surgery. In contrast, 83% of SM16-treated mice remained recurrence-free (P < 0.01; Fig. 5). These data show that adjuvant therapy with SM16 markedly reduces the extent of tumor recurrence after surgery.

**P.o. administration of SM16 induces tumor regression.** The data above clearly show the efficacy of SM16 when delivered via a s.c. pump. For clinical use, however, it would be much more desirable to give drug via the p.o. route. To further explore efficacy and drug exposure obtainable with SM16 via a p.o. route, SM16 was formulated at various doses (0.25, 0.45, 0.65, and 1.7 g/kg chow) into standard mouse chow. Given that the mice consumed ~5 g chow per day, the dose of SM16 at the 0.65 g/kg dose was estimated to be 3.25 mg/d.

**Figure 4.** SM16 prevents loss of CTL activity. A, splenic CTL activity was measured using an in vivo tumor neutralization assay (Winn assay). CD8+ T cells from the spleens of following groups were mixed with AB12 tumor cells and injected into the flanks of naive mice. Tumor size was measured after 1 wk. Groups were as follows: no CD8 cells added (AB12 cells only), naive CD8+ T cells from nontumor-bearing mice (group 1), CD8+ T cells from animals bearing “small” tumors (i.e., ~ 100 mm^3) 7 d after tumor cell inoculation (group 2), CD8+ T cells from tumor-bearing mice that had been treated with vehicle alone (group 3), and CD8+ T cells from tumor-bearing mice treated with SM16 (group 4). Control AB12 tumor cells grew to a size of ~300 mm^3 in 1 wk. Addition of CD8+ T cells from nontumor-bearing animals (naive, group 1) did not significantly slow the tumor growth. In contrast, tumor cells mixed with CD8+ T cells from animals bearing “small” tumors (group 2) at a lymphocyte to tumor ratio of 3:1 grew to only 99 mm^3. *, P < 0.01. However, CD8+ T cells from animals bearing “large” tumors similar to group 1, but treated with vehicle (group 3), lost all antitumor activity. **, significantly different from small AB12 tumors and SM16-treated tumors. In contrast, mice treated with SM16 (SM16 CD8+ T cells, group 4) showed persistent antitumor cytolytic activity. A, *, P < 0.01, compared with AB12 and control CD8+ tumors. B, CD8+ T cells from mice fed with SM16 chow had strong cytotoxic activity against AB12 cells. C, CD8+ T cells from AB12 tumor-bearing mice treated with SM16 (SM16 CD8+ T cells, group 4) showed antitumor cytolytic activity against large L1C2 cells. Extent of tumor growth in mice 1 wk after implantation with AB12 cells alone (AB12 alone), AB12 cells mixed with splenic CD8+ T cells from nontumor-bearing mice (CD8/AB12 bearing), and AB12 cells mixed with splenic CD8+ T cells from AB12-bearing mice that had been treated with SM16 (CD8/SM16-treated AB12 bearing) showed no cytotoxic activity against L1C2 cells.

**Figure 5.** Treatment with SM16 after surgery markedly reduces the extent of tumor recurrence. BALB/c mice were injected on the right flank with AB12 tumor cells. When the tumors reached a minimal volume of 850 mm^3 (~25 d after tumor cell inoculation), a complete resection of the tumors was attempted. One group was implanted with pumps loaded with vehicle (control; ▲) and the other group was implanted with pumps loaded with SM16 (●). After resection of large AB12 tumors, only 14% of control-treated mice remained recurrence-free at 19 d after debulking surgery. In contrast, 83% of SM16-treated mice remained recurrence-free. *, P < 0.01.
Sampling of the plasma drug levels was done at two time points in the day, early morning (a.m.) and afternoon (p.m.), corresponding to periods shortly after peak activity and feed consumption (a.m.) and during the nadir of activity and feed consumption (p.m.) for this nocturnal species. The plasma levels of SM16 achieved for each dose did not vary more than 1.5- to 2-fold between the a.m. and p.m. time points (Fig. 6B). Levels of 4.3 ± 2.6 μmol/L, 6.7 ± 2.4 μmol/L, and 9.8 ± 2.8 μmol/L, respectively, were measured for the 0.25, 0.45, and 0.65 g/kg doses. As expected, the vehicle group showed no detectable SM16 above background. These plasma levels achieved through p.o. administration were approximately 3-, 4.5-, and 6.5-fold higher than those obtained with the s.c. miniosmotic pump administration (level of 1.5 μmol/L), which is consistent with the increased efficacy noted in these p.o. studies. In spite of these higher plasma levels, no differences were observed in weight gain, feed consumption, or appearance and behavior of the 0.25, 0.45, and 0.65 g/kg SM16 chow-fed versus control chow-fed mice (Fig. 6B). In contrast, animals fed the highest dose, 1.7 g/kg, showed clear evidence of toxicity manifested by an ill-looking appearance and a loss of weight (Fig. 6C). No changes apparent in consumption of feed were noted between the control and SM16 containing chow-fed groups (data not shown).

Discussion

Malignant mesothelioma is an aggressive cancer with a median survival of 6 to 9 months and no effective therapies (33). We and others have shown previously that both mouse models of malignant mesothelioma and human malignant mesothelioma exhibit immunosuppressive activity preventing effective antitumor responses (15, 30, 32). Given that malignant mesothelioma cell lines and human tumors express high levels of TGFβ in tumor tissue and in pleural effusion fluid, it is likely that this highly immunosuppressive, protumor cytokine plays an important role in promoting immunosuppression of antitumor responses. The inhibition of TGFβ by the sTGFβRII-Fc fusion protein and anti-TGFβ1 antisense results in growth inhibition in mouse models of malignant mesothelioma (14, 15). These results suggest that blockade of TGFβ signaling may be of therapeutic benefit in malignant mesothelioma. Although TGFβ has a complex role in the establishment and progression of epithelial tumors (3, 7, 17, 34), there are now many studies, including our own, showing that blocking TGFβ in established tumors can limit growth and metastasis (see Introduction). Accordingly, several approaches, including antibodies, soluble receptors, and antisense oligonucleotides, are being evaluated (reviewed in refs. 1, 16, 22).

The studies presented here characterize the antitumor activity of an ALK5 kinase inhibitor, SM16. The ability of SM16 to inhibit ALK5-dependent TGFβ signaling was shown in AB12 mouse mesothelioma cells where SM16 inhibited TGFβ-induced phosphorylated Smad2. SM16 exhibits the ability to penetrate AB12 s.c. tumors because it is able to cause sustained inhibition of AB12 tumor phosphorylated Smad2 for at least 3 h following an i.p. administration. To determine the effect of SM16 on tumor growth, SM16 was initially given via a s.c. miniosmotic pump to AB12 tumor-bearing mice. This mode of delivery maintains consistent plasma levels of SM16 over the course of the study (data not shown). The increase in plasma levels of SM16 between the low doses (1.25 and 2.5 mg/kg/d) and the high dose (5 mg/kg/d) correlated well with the significant efficacy achieved at 5 mg/kg/d.

Mice bearing AB12 tumors of ~110 mm³ were treated with various doses of SM16-formulated chow. The animals were observed for tumor size, gross toxicity, and weight loss. As shown in Fig. 6, there was a dramatic reduction in tumor size induced by all doses of SM16 tested. Tumors were actually completely eradicated at the 0.65 and 0.45 g/kg doses; however, even at the 0.25 g/kg, there was a marked and statistically significant (P < 0.01) decrease in tumor volume over the 2 weeks of treatment such that the final mean tumor size in this group was 97 mm³ or 11% of the mean tumor size in the control chow-treated animals.

Figure 6. P.o. administration of SM16 induces tumor regression. A, mice bearing AB12 tumors were treated with various doses of SM16 formulated into mouse chow beginning when the tumors reached a size of 100 mm³ (day 6) and tumor sizes were measured. Tumor size was significantly smaller (P < 0.01) in all doses of SM16 tested. B, bioanalytic measurement of SM16 levels in plasma. Sampling of the plasma drug levels was done at two time points in the day: early morning (a.m.) and afternoon (p.m.). A dose response was observed. The plasma levels of SM16 achieved for each dose did not vary more than 1.5- to 2-fold between the a.m. and p.m. time points and were dose proportional. These plasma levels achieved through p.o. administration were approximately 3-, 4.5-, and 6.5-fold higher than those obtained with the s.c. miniosmotic pump administration (level of 1.5 μmol/L), which is consistent with the increased efficacy noted in these p.o. studies. In spite of these higher plasma levels, no differences were observed in weight gain, feed consumption, or appearance and behavior of the 0.25, 0.45, and 0.65 g/kg SM16 chow-fed versus control chow-fed mice (Fig. 6B). In contrast, animals fed the highest dose, 1.7 g/kg, showed clear evidence of toxicity manifested by an ill-looking appearance and a loss of weight (Fig. 6C). No changes apparent in consumption of feed were noted between the control and SM16 containing chow-fed groups (data not shown).
compared with the marginal effect at the low doses. These results suggest that the antitumor effect of SM16 is dependent on exposure and can be achieved with consistent inhibition of TGFβ signaling over time. The plasma levels of SM16 achieved in these studies suggested that efficacy required concentrations of 1.5 μmol/L SM16. At this concentration, SM16 also has significant ALK4 and some p38 and Raf inhibition activity in in vitro biochemical assays. It is theoretically possible that the in vivo activity of SM16 is derived from ALK4 inhibition or in part from p38 and/or Raf inhibition. However, the extent of inhibition of tumor growth and stimulation of antitumor CD8+ T cells of SM16 is very similar to that seen with the soluble TGFβRII:Fc fusion protein, a TGFβ-specific antagonist, indicating that the efficacy of SM16 maybe solely due to TGFβ signaling antagonism.

The p.o. activity of SM16 was shown by the inhibition of AB12 tumor growth when SM16 was given through feed. This method of delivery, like that achieved with the s.c. miniosmotic pump, also resulted in constant circulating levels of SM16 and sustained suppression of tumor phosphorylated Smad2 (data not shown). The plasma concentration of SM16 was not significantly different between the a.m. and p.m. bleeds within each dose group, whereas there was a linear dose-dependent increase in plasma SM16 when the compound was given at 0.25 to 0.65 g/kg SM16 in feed (Fig. 6B). The highest dose group showed a much greater variability in plasma levels at both time points, which was likely due to the effect of the toxicity observed in this dose group on drug metabolism (Fig. 6B and C). Significant efficacy was obtained at all dose levels (Fig. 6A), and the plasma levels of SM16 for all dose groups in this p.o. study exceeded 2 μmol/L, with the lowest group (0.25 g/kg) showing ~5 μmol/L plasma levels. These findings are also very consistent with target plasma level of 1.5 μmol/L required for efficacy identified in the s.c. miniosmotic pump studies.

The efficacy of SM16 against AB12 malignant mesothelioma tumors, like that of the sTGFβRII:Fc fusion protein, seems to be primarily immune mediated, specifically through CD8+ antitumor T cells. The efficacy seen in BALB/c mice was lost in SCID mice, which lack T and B cells (Fig. 3). In addition, antitumor CTL activity was maintained in SM16-treated mice bearing large AB12 tumors, whereas the antitumor CTL activity found in all small AB12 tumor-bearing mice was lost in vehicle-treated mice when their tumors grew large (Fig. 4). In addition, tumors from control mice and animals treated with SM16 for 5 days were stained for apoptosis, proliferation, and angiogenesis using the same methods and reagents as described by Ge et al. (26). Although we could see increased areas of necrosis and infiltration in the SM16-treated tumors, staining for apoptosis, proliferation, and angiogenesis was all quite low and unchanged between treatment groups (data not shown).

The blockade of antitumor CTL activity by TGFβ and the enhancement of CTL activity by TGFβ antagonists have been documented in glioma (12), breast cancer (26), thymoma, and prostate models (35–37). Indeed, the lack of clinical benefit following immunotherapy-induced, systemic antitumor CTLs may be due to the expression of immunosuppressive cytokines, such as TGFβ, in certain cancers (38–40). TGFβ is a potent immunosuppressing cytokine and is found at high levels in pleural effusion fluid from human mesothelioma patients (20). In mesothelioma and other human cancers, antitumor CTLs are elicited but often lack antitumor killing activity, remain at the periphery of tumors, and exhibit markers of inactivation (39–43). It has been postulated that these antitumor CTLs are inhibited by cytokines, such as TGFβ or interleukin-10, expressed by tumors or T regulatory cells resident in tumors (35, 39, 44). Indeed, recent studies show that the TGFβ may inhibit CTL activity through inhibition of granzyme activity (36). Given these findings, TGFβ antagonists, such as SM16, may prove to be useful immunotherapy agents for the treatment of malignant mesothelioma and other tumors dependent on TGFβ for immunosuppression.

Most forms of immunotherapy are maximally effective with minimal tumor burdens. This results in a more optimal ratio of CTL to tumor cells, allows better access of immunocytes to tumor cells, and minimizes the local and systemic immunosuppression induced by most tumors (45, 46). We hypothesized that one way to achieve this more optimal situation would be to combine SM16 therapy with surgical debulking. In most human and animal tumors, surgical debulking, by itself, is largely ineffective; there is usually rapid regrowth of tumor. In the AB12 malignant mesothelioma model, we also observed high recurrence rates (86%) after debulking large flank tumors (Fig. 5; ref. 47). However, when debulking was accompanied by SM16 therapy, the rate of recurrence was markedly reduced to 17%. We hypothesize that TGFβ blockade prevented tumor-induced immunosuppression and allowed effective antitumor immune responses to occur.

TGFβ antagonists, including ALK5 inhibitors, have also shown antimetastatic activity in mouse models of tumor metastases (6–8, 12, 27, 48, 49). This activity may be associated with the proinvasive, angiogenic, prosurvival, and stromal-inducing activities of TGFβ (1). Indeed, TGFβ is associated with tumor recurrence, metastases, and poor prognosis in a significant number of human cancers (1–3). Together, the immune-enhancing, anti-invasive, antiangiogenic, and antimetastatic activity of TGFβ antagonists suggests that agents, such as SM16, are particularly well suited for treatment of cancer recurrence after debulking.

ALK5 inhibitors differ from the TGFβ ligand-binding agents in their ability to block both the TGFβ and activin/nodal pathways at the level of TGFβ and activin type I receptors, ALK5 and ALK4 respectively. The ability to inhibit signaling by all isoforms of TGFβ differs from the selective TGFβ1 and TGFβ3 blockade afforded by the sTGFβRII:Fc or selective TGFβ1 or TGFβ2 mAbs. There is also some evidence that the activin signaling may function similarly to TGFβ in preventing epithelial hyperplasia while increasing the malignancy of advanced cancers (50–52). This suggests that activin, like TGFβ, may play a protumorigenic role especially in later stage cancers (53–57). Although this additional activity of SM16 may be important in tumors where activin/nodal signaling contributes to tumor growth, the efficacy and mechanism of action obtained with SM16 and the sTGFβRII:Fc were very similar, suggesting that TGFβ may be the primary target in the AB12 model of mesothelioma.

Our results with SM16 in mesothelioma are very consistent with the findings in a recent publication of Ge et al. (26) who used a different p.o. bioavailable TGFβ type 1 receptor kinase inhibitor (SD-208) to treat murine breast cancer. Like SM16, SD-208 was able to (a) inhibit Smad2 phosphorylation in vitro and in vivo at similar concentrations, (b) inhibit the growth of 4T1 breast cancer cells in immunocompetent animal but not in nude mice, and (c) stimulate the generation of CTLs. Of note, treatment with SD-208 was only attempted in animals with minimal disease; drug was given only 1 day after tumor cell injection. In contrast, we used SM16 to treat relatively large established tumors as well as minimal disease (in the surgical adjuvant setting).

In summary, the studies presented here show the potential utility of ALK5 inhibitor SM16 in treating malignant tumors,
especially those expressing high levels of TGFβ, such as malignant mesothelioma. Although this article focused in detail on a mesothelioma cell line, we have data that SM16 has similar activity in mouse splenic natural killer cell activity: implications for a possible role of tumor cell/ host TGFβ interactions in human breast cancer progression. J Clin Invest 1993;92:2569–76.


Acknowledgments

Received 7/5/2006; revised 11/21/2006; accepted 12/14/2006.

Grant support: National Cancer Institute grant P01 CA 66726.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Cheryl Black, Tracy Kruger, Van Phan, Liyu Yang, Ellen Rohde, and Zhanyong Li for their expertise in providing bioanalytical mass spectrometry analysis for these studies.

References


TGF-β Inhibitor Blocks Malignant Mesothelioma Growth


A Novel Small-Molecule Inhibitor of Transforming Growth Factor β Type I Receptor Kinase (SM16) Inhibits Murine Mesothelioma Tumor Growth \textit{In vivo} and Prevents Tumor Recurrence after Surgical Resection
