E7080, a Multi–Tyrosine Kinase Inhibitor, Suppresses the Progression of Malignant Pleural Mesothelioma with Different Proangiogenic Cytokine Production Profiles

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Abstract

Purpose: Malignant pleural mesothelioma (MPM) is a biologically heterogeneous malignant disease with a poor prognosis. We reported previously that the anti–vascular endothelial growth factor (VEGF) antibody, bevacizumab, effectively inhibited the progression of VEGF-high-producing (but not VEGF-low-producing) MPM cells in orthotopic implantation models, indicating the need for novel therapeutic strategies to improve the poor prognosis of this disease. Therefore, we focused on the multi–tyrosine kinase inhibitor E7080 and assessed its therapeutic efficacy against MPM cells with different proangiogenic cytokine production profiles.

Experimental Design: The efficacy of E7080 was assayed in orthotopic implantation of severe combined immunodeficient mouse models with three human MPM cell lines (MSTO-211H, NCI-H290, and Y-MESO-14).

Results: With regard to proangiogenic cytokine production profiles, MSTO-211H and Y-MESO-14 cells were MPM cells producing high levels of fibroblast growth factor-2 and VEGF, respectively. NCI-H290 cells produced low levels of fibroblast growth factor-2 and VEGF compared with the other two cell lines. E7080 potently suppressed the phosphorylation of VEGF receptor-2 and FGF receptor 1 and, thus, inhibited proliferation of endothelial cells, but not that of the MPM cell lines, *in vitro*. Orthotopically inoculated MSTO-211H cells produced only thoracic tumors, whereas NCI-H290 and Y-MESO-14 cells also developed pleural effusions. Treatment with E7080 potently inhibited the progression of these three MPM cell lines and markedly prolonged mouse survival, which was associated with decreased numbers of tumor-associated vessels and proliferating MPM cells in the tumor.

Conclusions: These results strongly suggest broad-spectrum activity of E7080 against MPM with different proangiogenic cytokine production profiles in humans. (Clin Cancer Res 2009;15(23):7229–37)

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Malignant pleural mesothelioma (MPM) is an aggressively growing tumor, which disseminates into the thoracic cavity and frequently produces a malignant pleural effusion (1). More than 60% of patients with MPM present with a pleural effusion associated with breathlessness, often accompanied by chest wall pain, which compromises their quality of life (2). This type of tumor was once considered rare, but its incidence is increasing worldwide, due primarily to exposure to asbestos and possibly to the SV40 tumor virus. MPM is refractory to conventional chemotherapy and radiotherapy, and also has a poor prognosis, with a median survival time from onset of ~1 year. Although the multitargeted antifolate agent, pemetrexed, in combination with cisplatin, was recently approved as first-line treatment of MPM (3), the overall prognosis of patients with MPM remains very poor, indicating the need for effective novel therapies.

Angiogenesis, the formation of new blood vessels to deliver oxygen and nutrients to the expanding tumor mass, is crucial for the growth and metastasis of solid tumors (4). In many types of cancer, including MPM, there is an inverse correlation

Translational Relevance

Malignant pleural mesothelioma (MPM) is a biologically heterogeneous malignant disease with different susceptibility to antiangiogenic therapy by bevacizumab, an anti–vascular endothelial growth factor antibody. E7080 is the multi–tyrosine kinase inhibitor, which is currently being evaluated in clinical trials. Thus, we focused on E7080 and assessed its therapeutic efficacy against three MPM cell lines with different proangiogenic cytokine production profiles. The three MPM cell lines showed different progression patterns after orthotopic implantation in severe combined immunodeficient mice. Treatment with E7080 potently inhibited the progression of these three MPM cell lines and markedly prolonged mouse survival, which was associated with decreased numbers of tumor-associated vessels and proliferating MPM cells in the tumor. These results of our translational research strongly suggest broad-spectrum activity of E7080 against MPM with different proangiogenic cytokine production profiles in humans.

between microvessel density in tumors and patient survival (5–7). The positive regulators of angiogenesis include vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), platelet-derived growth factor (PDGF), hepatocyte growth factor, and interleukin-8 (8). Of these, VEGF is the most potent endothelial cell–specific mitogen associated with tumor neovascularization. VEGF also has significant effects on vascular permeability, thus mediating the development of pleural effusion and ascites (9, 10). Several inhibitors of VEGF/VEGFR, including monoclonal antibodies directed against VEGF or its receptors, VEGFR-1 and VEGFR-2, and small-molecule tyrosine kinase inhibitors, have recently been shown to have promising antitumor effects (11). For example, we found that bevacizumab, an anti-human VEGF monoclonal antibody (Ab), effectively inhibited thoracic tumors and pleural effusion induced by the VEGF-high-producing MPM cell line, EHMES-10. In contrast, bevacizumab had no effect on thoracic tumors induced by the VEGF-low-producing MPM cell line, MSTO-211H (12), suggesting that MPMs are heterogeneous and that novel therapeutic modalities are necessary for controlling VEGF-low-producing MPMs as well as VEGF-high-producing MPMs.

E7080 is an orally active inhibitor of VEGFR-2, with additional activity against other receptor tyrosine kinases, including FGFRs, PDGFRs, and c-Kit (13). E7080 shows potent antitumor effects in xenograft models of various types of tumors by inhibiting angiogenesis, especially through VEGFR suppression (14). Moreover, E7080 can cause regression of tumors induced by human lung cancer H146 cells, which produce stem cell factor, due to its antiangiogenic activity mediated by inhibition of both c-KIT and VEGF receptor signaling (13). These findings suggest that E7080 may inhibit VEGF-dependent and VEGF-independent angiogenesis and have antitumor activity against a broad spectrum of human solid tumors by suppressing not only VEGFR2, but also other receptor tyrosine kinases. Therefore, we postulated that E7080 may be therapeutically useful for both VEGF-high-producing and VEGF-low-producing MPMs. Here, we examined whether E7080 could inhibit the progression of three MPM cell lines with different potentials to produce proangiogenic cytokines in orthotopically implanted severe combined immunodeficient (SCID) mouse models. Our results indicated that E7080 had potent antitumor activity against both VEGF-high-producing and VEGF-low-producing MPM cells mediated by inhibition of angiogenesis, and therefore prolonged the survival of MPM cell–bearing mice.

Materials and Methods

Reagents. E7080 was provided by Tsukuba Research Laboratories, Eisai Co., Ltd. Imatinib was from Novartis Pharma. Anti-human VEGF neutralizing Ab, bevacizumab, was purchased from Genentech. Recombinant human VEGF165 and FGF-2, anti–FGF-2 neutralizing Ab, anti-FGFR1 neutralizing Ab, anti-PDGF neutralizing Ab (active against PDGF-AA, AB, and BB), and control human IgG were purchased from R&D Systems.

Cell lines. The human MPM cell lines, MSTO-211H and NCI-H290, were purchased from the American Type Culture Collection. Y-MESO-14 cells were established as described previously (15). Cells were cultured in RPMI1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (50 μg/mL) at 37°C in a humidified atmosphere of 5% CO_2 in air. Human microvascular endothelial cells (HMVEC) were maintained in HuMedia-MvG with growth supplements (Kurabo) and used for *in vitro* assay at passages 2 to 5.

Determination of protein levels of VEGF, FGF-2, and PDGF-AA. Culture supernatants, cell lysates, and lysates of thoracic tumors were evaluated. For culture supernatants, tumor cells (2×10^5) were cultured in 2 mL RPMI1640 with 10% FBS for 48 h, the supernatants were harvested, and the concentrations of human VEGF, mouse VEGF, human FGF-2, and human PDGF-AA were determined using ELISA kits (R&D Systems) according to the manufacturer's instructions. Lower detection limits were 31.2 pg/mL for human VEGF, 2.45 pg/mL for mouse VEGF, 10 pg/mL for human FGF-2, and 31.2 pg/mL for human PDGF-AA.

Proliferation of tumor cells and endothelial cells. Cell proliferation was measured by the MTT dye reduction method (16). Briefly, tumor cells (2×10^3 per well), plated in triplicate in 96-well plates, were incubated in RPMI1640 containing 5% to 10% FBS for 24 h. HMVECs (5×10^3 per well), plated in triplicate in 96-well plates precoated with 1.5% gelatin, were incubated in a 5% FBS supplemented MEM medium for 24 h. The cells were incubated for 48 to 72 h in the presence or absence of E7080, VEGF, FGF-2, control human IgG, anti–FGF-2 neutralizing Ab, anti-FGFR1 neutralizing Ab, or bevacizumab. An aliquot of 50 μL of stock MTT solution (2 mg/mL; Sigma) was added to each well, and the cells were incubated for 2 h at 37°C. The media containing the MTT solution were removed and the dark blue crystals were dissolved by adding 100 μL of DMSO. Absorbance was measured with a microplate reader (Dainippon Seiyaku) at test and reference wavelengths of 550 and 630 nm, respectively.

Western blotting and immunoprecipitation. HMVECs or tumor cells were incubated in serum-free MEM for 16 h, followed by incubation with E7080 (1-1000 nmol/L) for 2 h and incubation with VEGF or FGF-2 (20 ng/mL) for 10 min. The cells were washed twice with PBS, harvested in cell lysis buffer [20 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na_3VO_4, 1 μg/mL leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride], and flash frozen on dry ice. After allowing the cells to thaw, the lysates were collected with a rubber scraper, sonicated, and centrifuged at 14,000× *g* (4°C for 10 min). Total protein concentrations were measured using a BCA Protein Assay kit (Pierce). Cell lysates were subjected to SDS-PAGE, and the proteins were transferred onto polyvinylidene difluoride membranes (Atto). The membranes were

blocked with blocking buffer (Nacalai Tesque, Inc.) for 1 h at room temperature, and incubated at 4°C overnight with anti-VEGFR-2 (1:500 dilution; R&D), anti-FGFR1 (1:500 dilution; R&D), or anti–β-actin Ab (1:5,000 dilution; Sigma), followed by incubation for 2 h at room temperature with secondary Ab (horseradish peroxidase–conjugated species-specific Ab). Immunoreactive bands were visualized using an enhanced chemiluminescent substrate (Pierce).

For FGFR1 immunoprecipitation studies, aliquots of 200 μg of total protein extract were precleared with immobilized protein G Sepharose beads (Invitrogen) and incubated with 4 μg/mL polyclonal Ab against FGFR1 (Santa Cruz Biotechnology) in 500 μL of lysis buffer overnight at 4°C. The immune complexes were precipitated for 2 h with 50 μL of a 50% slurry of Protein G Sepharose at 4°C. Immunoprecipitated complexes were washed five times with lysis buffer, resuspended in 2× SDS sample buffer, boiled for 5 min, and resolved by SDS-PAGE. Subsequent immunoblotting was done using anti-phosphotyrosine mouse monoclonal Ab (Upstate), with an Ab to FGFR1 as a control.

Animals. Male SCID mice, ages 5 to 6 wk, were obtained from CLEA Japan and maintained under specific pathogen-free conditions throughout this study. All experiments were done in accordance with the guidelines established by the Tokushima University Committee on Animal Care and Use.

Orthotopic implantation model. Cultured MPM cells were harvested, washed twice, resuspended in PBS, and injected (1×10^6 MSTO-211H and Y-MESO-14 or 3×10^5 NCI-H290 cells in 100 μL of PBS) into the thoracic cavity of the SCID mice (17). After 7 d, the mice were treated with or without oral E7080 daily. The mice were sacrificed 3 wk (MSTO-211H and NCI-H290) or 4 wk (Y-MESO-14) after tumor cell inoculation. The thoracic tumors were carefully removed and weighed; the pleural effusion was harvested using a 1-mL syringe and its volume was measured.

Immunohistochemical determination of proliferating cells and endothelial cells. For bromodeoxyuridine (BrdUrd) staining, the mice were injected i.p. with BrdUrd solution (200 μL; Zymed Labs). Two hours later, the mice were killed and the thoracic tumors were collected. The tumors were cut into 5-mm fragments and placed into either buffered 10% formalin solution or optimum cutting temperature compound (Miles Laboratories) and snap frozen in liquid nitrogen for immunohistochemical analysis. *In vivo* cell proliferation was quantified in paraffin-embedded tissues (4 μm thick) using a BrdUrd staining kit (Zymed Labs). Endothelial cells in frozen tissue sections (8 μm thick) were identified using rat anti-mouse CD31/platelet/endothelial cell adhesion molecule-1 monoclonal Ab (1:100 dilution; BD Pharmingen). The sections were also stained with H&E for routine histologic examination.

Quantification of immunohistochemistry. The five areas containing the highest levels of staining intensity within a section were selected for histologic quantification under light microscopy with 200- to 400-fold magnification. All results were independently evaluated by two authors (K. I. and H. O.).

Statistical analysis. The statistical significance of difference between the *in vitro* and *in vivo* data were analyzed by Student's *t* test, Mann-Whitney *U* test, and one-way ANOVA with Dunnett's *post hoc* test, where applicable. Survival was analyzed by the Kaplan-Meier method. Differences between treatment and control groups were compared with the log-rank test. Differences at $P < 0.05$ were deemed significant.

Results

E7080 inhibited the phosphorylation of VEGFR2 and FGFR1 in HMVEC. We first examined the effects of E7080, a multi–tyrosine kinase inhibitor, on proliferation of human endothelial cells, HMVEC. VEGF and FGF-2 markedly stimulated the proliferation of HMVEC (Fig. 1A), but PDGF failed to do so (data not shown) under our experimental conditions. E7080

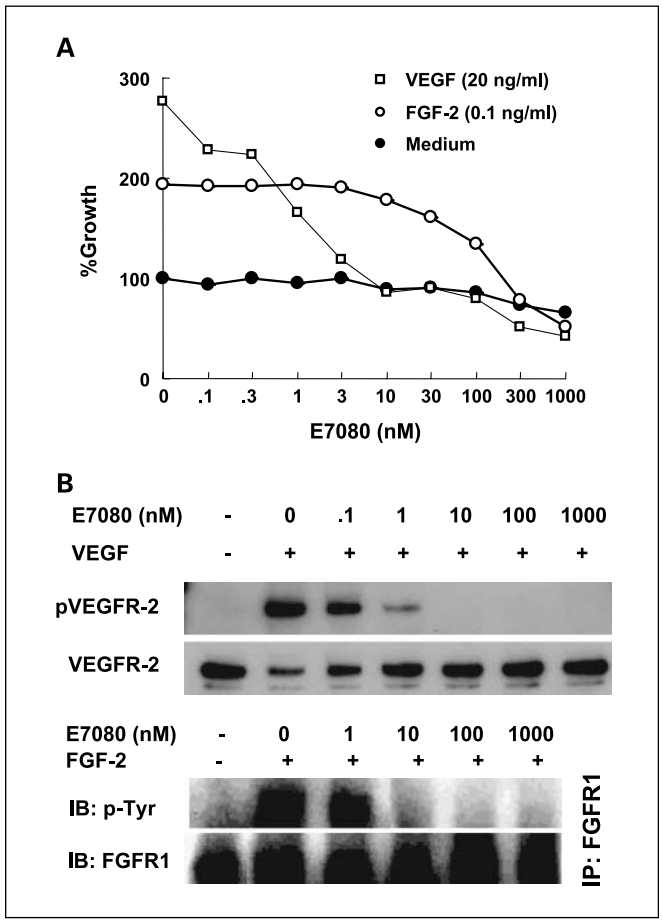

Fig. 1. Effect of E7080 on proliferation and phosphorylation of VEGFR2 and FGFR1 in HMVEC. *A*, HMVECs (5×10^3/well) were incubated for 48 h with E7080 (10 or 200 nmol/L) in the presence or absence of human VEGF or FGF-2. *B*, serum-starved HMVECs were grown for 2 h in the presence or absence of E7080 and stimulated for 10 min with human VEGF (20 ng/mL) or FGF-2 (20 ng/mL). Total cell lysates were immunoprecipitated (*IP*) for detecting FGFR1 but not VEGFR2. Immunoblotting (*IB*) was done with the indicated antibodies. *, $P < 0.05$ compared with medium alone as a control.

inhibited VEGF-induced HMVEC proliferation in a dose-dependent manner, and showed complete blockade at 10 nmol/L. In addition, E7080 at a high concentration (300 nmol/L) completely blocked FGF-2–stimulated HMVEC proliferation (Fig. 1A). We next examined the effects of E7080 on phosphorylation of VEGFR2 and FGFR1 in HMVEC. Whereas stimulation with VEGF and FGF-2 induced phosphorylation of VEGFR2 and FGFR1, respectively, E7080 inhibited this activity in a dose-dependent manner, with complete inhibition at 10 and 100 nmol/L of E7080 for VEGFR2 and FGFR1, respectively (Fig. 1B). These results suggest that, although E7080 is a powerful VEGFR2 inhibitor, it also inhibits FGFR1.

***Three MPM cell lines had different proangiogenic cytokine production profiles* in vitro.** In the next set of experiments, we measured the expression of proangiogenic cytokines (VEGF, FGF-2, and PDGFs) in the three MPM cell lines by ELISA. VEGF was detected predominantly in culture supernatants and Y-MESO-14 cells produced higher levels of VEGF than MSTO-211H or NCI-H290 cells (Fig. 2). In contrast, FGF-2 was detected predominantly in the cell lysate. MSTO-211H cells

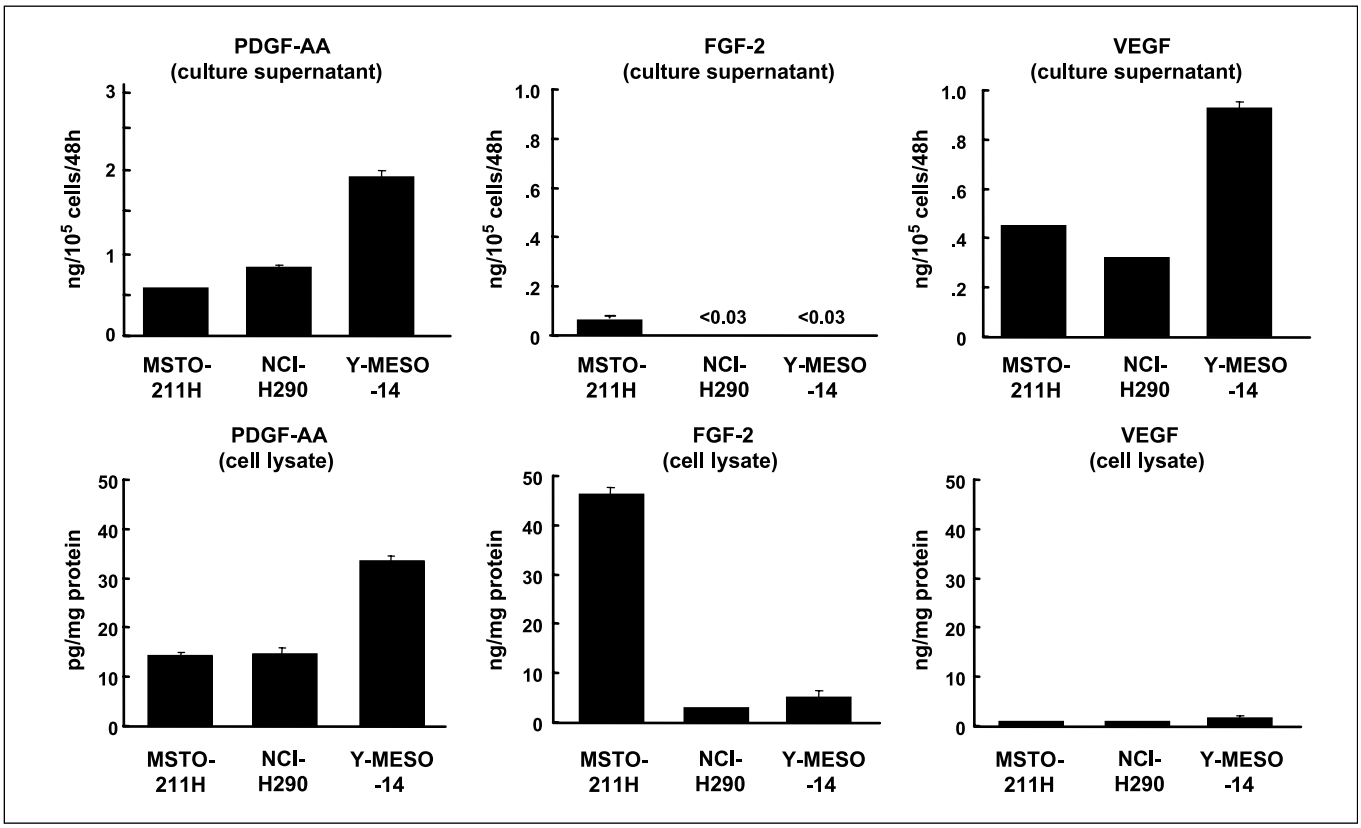

Fig. 2. Proangiogenic cytokine production profiles of three human MPM cell lines *in vitro*. Production of proangiogenic proteins was measured by ELISA. Columns, mean of representative of three independent experiments with similar results; bars, SD.

produced high concentrations of FGF-2 protein in cell lysate, with discernible concentrations of FGF-2 in the culture supernatant, whereas neither NCI-H290 nor Y-MESO-14 cells produced detectable levels of FGF-2 in the cell lysate or supernatants. PDGF-AA, but not PDGF-AB or PDGF-BB (data not shown), was detected in both the culture supernatant and cell lysate of all three cell lines.

***E7080 has no effect on MPM cell proliferation* in vitro.** Several proangiogenic cytokines, including VEGF and FGF-2, have been reported to act as growth factors for MPM cells (18–21). However, we found that neither VEGF nor FGF-2 affected the proliferation of MSTO-211H, NCI-H290, or Y-MESO-14 cells (data not shown), nor did anti-VEGF Ab, anti–FGF-2 Ab, or anti-PDGFs Ab (data not shown), indicating that these proangiogenic cytokines are not growth factors for these three MPM cell lines, at least under our experimental conditions.

Clinically, twice daily administration of 13 mg of E7080 results in a C_{max} of 302 ng/mL (577 nmol/L; ref. 22). At concentrations <1,000 nmol/L, E7080 did not affect the proliferation of MSTO-211H, NCI-H290, or Y-MESO-14 cells, and its IC_{50}s against these three MPM cell lines were 1.5, 12.7, and 3.2 μmol/L, respectively, indicating that E7080 has little effect on the proliferation of these cells under *in vitro* conditions. In parallel experiments, the PDGFR inhibitor imatinib (23, 24), at <1,000 nmol/L, also did not affect the proliferation of these three MPM cell lines (data not shown).

***Three MPM cell lines showed different progression patterns and proangiogenic cytokine production profiles* in vivo.** MPM cells were implanted orthotopically into the thoracic cavities of SCID mice. MSTO-211H cells developed numerous small (<2 mm in diameter) thoracic tumors, but not pleural effusion, by day 21. On the other hand, NCI-H290 cells developed large (>2 mm in diameter) thoracic tumors and pleural effusion by day 21. Y-MESO-14 cells also developed large (>2 mm in diameter) thoracic tumors and pleural effusion by day 28 (Table 1), indicating different progression patterns of the three MPM cell lines in SCID mice.

We further examined the levels of proangiogenic cytokines in thoracic tumors by ELISA using tumor lysate. Whereas ELISA kits for human FGF-2 and human PDGF-AA detected not only human but also mouse cytokines, ELISA kits for human and mouse VEGF were species specific. Consistent with the *in vitro* cytokine production profiles, high levels of VEGF and FGF-2 were detected in tumors produced by Y-MESO-14 and MSTO-211H cells, respectively. Interestingly, higher concentrations of PDGF-AA were detected in tumors produced by H290 and Y-MESO-14 cells compared with tumors produced by MSTO-211H cells (Fig. 3B). These results indicated that tumors produced by MPM cells expressed various proangiogenic cytokines at different levels *in vivo*.

E7080 inhibits the production of thoracic tumors and pleural effusion by MPM cells in orthotopic implantation model. To assess the therapeutic effects of E7080, beginning 7 days after MPM cell inoculation, the mice were treated with oral E7080, or distilled water as a control, daily for 2 weeks, since our preliminary experiments confirmed that these MPM cells produced thoracic tumors larger than 1 mm in diameter at this time (data not shown). Three to 4 weeks after tumor cell inoculation, the

Table 1. Effect of E7080 on thoracic tumor and pleural effusion produced by MPM cells in SCID mice

Cell line	Treatment	Dose	Thoracic tumor			Pleural effusion		
			Incidence	Weight (mg)		Incidence	Volume (μL)	
				Median	Range		Median	Range
MSTO-211H	D.W.		9/9	285	140-420	0/9	0	0
	E7080	1 mg/kg	10/10	90	30-140*	0/10	0	0
		10 mg/kg	10/10	50	45-110*	0/10	0	0
NCI-H290	D.W.		15/15	220	160-290	11/15	100	0-550
	E7080	1 mg/kg	9/9	120	80-160*	0/9	0	0*
		10 mg/kg	15/15	70	30-90*	0/15	0	0*
Y-MESO-14	D.W.		14/15	270	160-315	13/15	320	0-800
	E7080	10 mg/kg	15/15	90	30-127*	0/15	0	0

NOTE: MSTO-211H (1×10^6), NCI-H290 (3×10^5), or Y-MESO-14 (1×10^6) were inoculated into thoracic cavity of SCID mice on day 0. The MPM cell–inoculated mice were treated daily with or without distilled water (D.W.) or E7080 from day 7 to day 20. The mice were sacrificed on day 21 (MSTO-211H and NCI-H290) or day 28 (Y-MESO-14). The thoracic tumor and pleural effusions were evaluated as described in Materials and Methods. Values are the median (minimum-maximum).
*$P < 0.05$ compared with control group.

mice were sacrificed and we evaluated the formation of thoracic tumors and pleural effusion. Our observations indicated that, in MSTO-211H–inoculated mice, treatment with E7080 inhibited the production of thoracic tumors in a dose-dependent manner. In NCI-H290–inoculated mice, treatment with E7080 suppressed the production of thoracic tumors as well as pleural effusion. Moreover, in Y-MESO-14–inoculated mice, E7080 also inhibited the production of thoracic tumors and

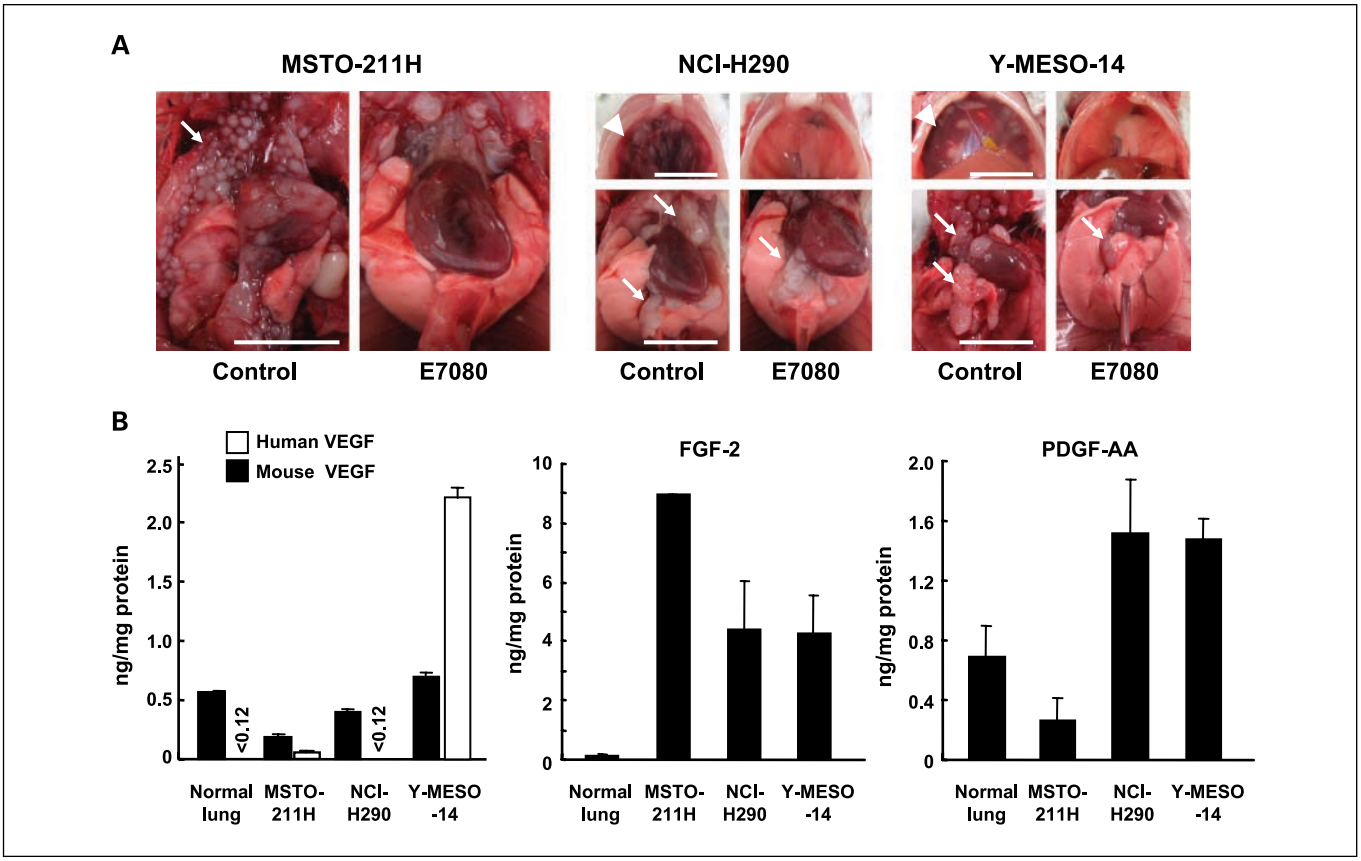

Fig. 3. Effects of E7080 on the production of thoracic tumors and pleural effusion by MPM cells in orthotopic implantation model. *A*, SCID mice inoculated with MSTO-211H (1×10^6), NCI-H290 (3×10^5), or Y-MESO-14 (1×10^6) cells were orally treated with or without E7080 from day 7 to day 20. Three weeks (MSTO-211H and NCI-H290) to 4 wk (Y-MESO-14) after tumor cell inoculation, the MSTO-211H cells produced thoracic tumors, and the NCI-H290 and Y-MESO-14 cells developed thoracic tumors and pleural effusions. Treatment with E7080 inhibited the production of the thoracic tumors and pleural effusions. Arrowheads, pleural effusions. Arrows, thoracic tumors. Bars, 10 mm. *B*, concentrations of mouse and human VEGF in the thoracic tumors and pleural effusion. Tumor cell lysates (25 μg protein/100 μL) were assayed by ELISA. Columns, mean of three independent samples; bars, SD.

pleural effusion (Table 1; Fig. 3A). The dose and schedule of E7080 was well tolerated in all three models, as determined by the absence of weight loss or other signs of acute or delayed toxicity (data not shown).

Immunohistochemical analysis of the tumors in mice treated with or without E7080. Using H&E staining, we observed necrotic lesions in the tumors of mice treated with E7080 (Fig. 4A). Although treatment with E7080 did not affect the production of FGF-2 or VEGF, the number of BrdUrd-positive proliferating tumor cells was decreased in the MSTO-211H–, NCI-H290–, and Y-MESO-14–induced tumors of mice treated with E7080, compared with controls (Fig. 4). To determine the effects of E7080 on tumor angiogenesis, microvessel density was quantified by staining tumor sections for CD31/platelet/endothelial cell adhesion molecule 1. Tumors (all of MSTO-211H, NCI-H290, and Y-MESO-14) from the mice treated with E7080 had significantly lower microvessel density values than controls ($P < 0.01$; Fig. 4B).

Survival benefit of E7080 in MPM cell–bearing SCID mice. Beginning 7 days after inoculation of SCID mice with MPM cells, the mice were treated with or without E7080 for 2 weeks. In the MSTO-211H– and NCI-H290–inoculated model, although there was no significant difference in survival between the control and 1 mg/kg E7080 groups, treatment with 10 mg/kg E7080 significantly prolonged the median survival time compared with the control group (Fig. 5A). In parallel experiments with Y-MESO-14 cells, E7080 treatment (10 mg/kg) also significantly prolonged the median survival time, compared with the control group (Fig. 5A).

As the survival benefit of E7080 was less apparent with a 14-day treatment schedule, we finally examined whether continuous treatment with E7080 further prolonged mouse survival using MSTO-211H cells and Y-MESO-14 cells. The results indicated that in both models, continuous treatment with E7080 from day 7 to the end of the experiment markedly prolonged the survival of mice compared with controls (Fig. 5B). There were no macroscopic changes or body weight loss during short-term or long-term treatment by E7080 in this study, indicating the feasibility of this compound.

Discussion

We have evaluated the therapeutic efficacy of the multiple tyrosine kinase inhibitor, E7080, in orthotopic implantation models, using three human MPM cell lines with different potential to produce proangiogenic cytokines. We found that

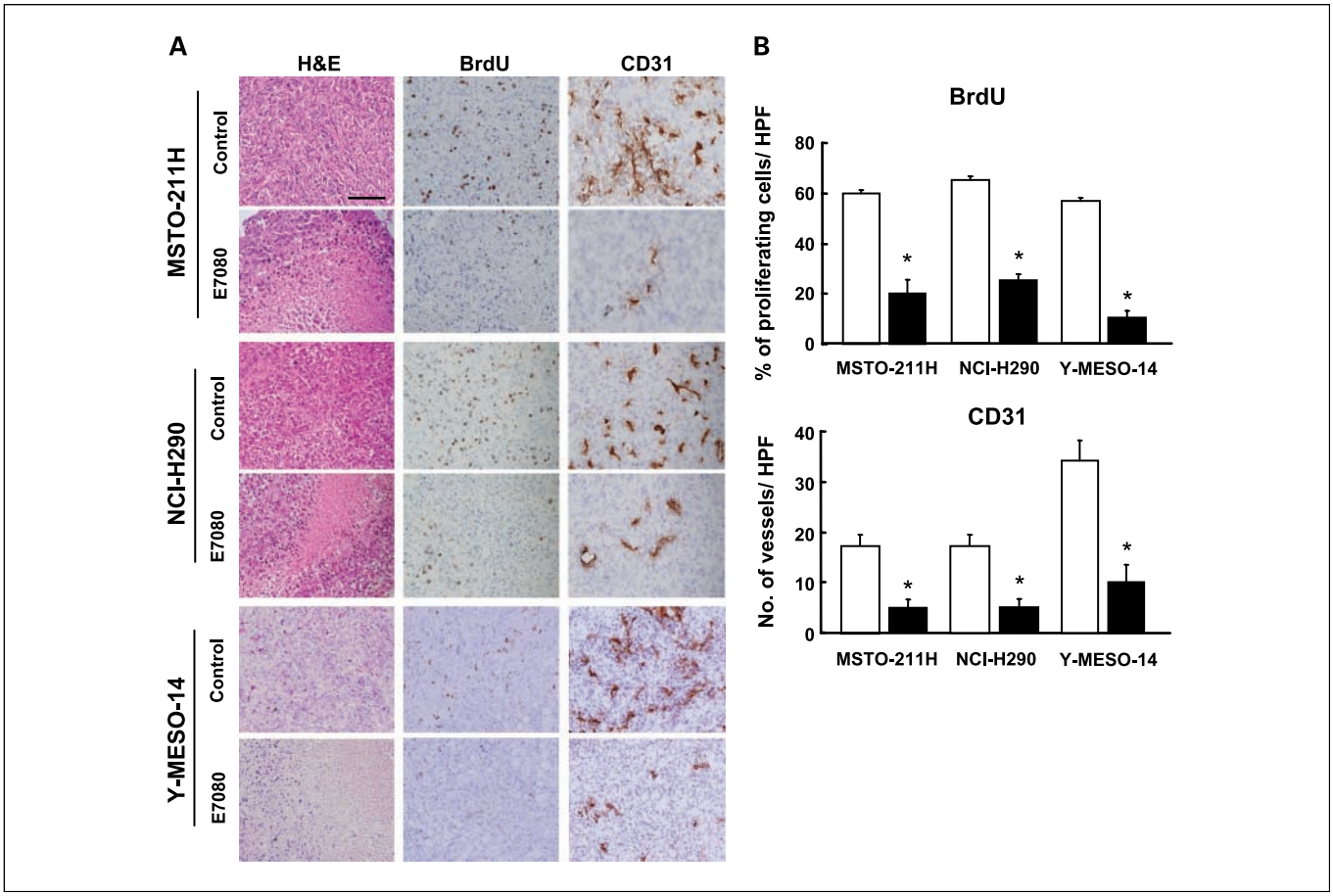

Fig. 4. Histologic analysis of thoracic tumor produced by MPM cells. The MPM cell–bearing SCID mice were treated with or without E7080 for 2 wk and sacrificed on day 21 (MSTO-211H and NCI-H290) or day 28 (Y-MESO-14). The thoracic tumors were analyzed by H&E and immunohistochemistry for BrdUrd, and CD31. *A,* necrotic lesions were found in the tumors of the E7080 treatment group. E7080 inhibited the number of proliferating tumor cells (BrdUrd) and tumor-associated endothelial cells (CD31). Magnification, ×200. Bars, 100 μm. *B,* quantitative immunohistochemical analyses. Columns, mean numbers from five independent areas (*open,* control; *filled,* E7080 10 mg/kg); bars, SD. *, $P < 0.01$ compared with the control. HPF, high-power field.

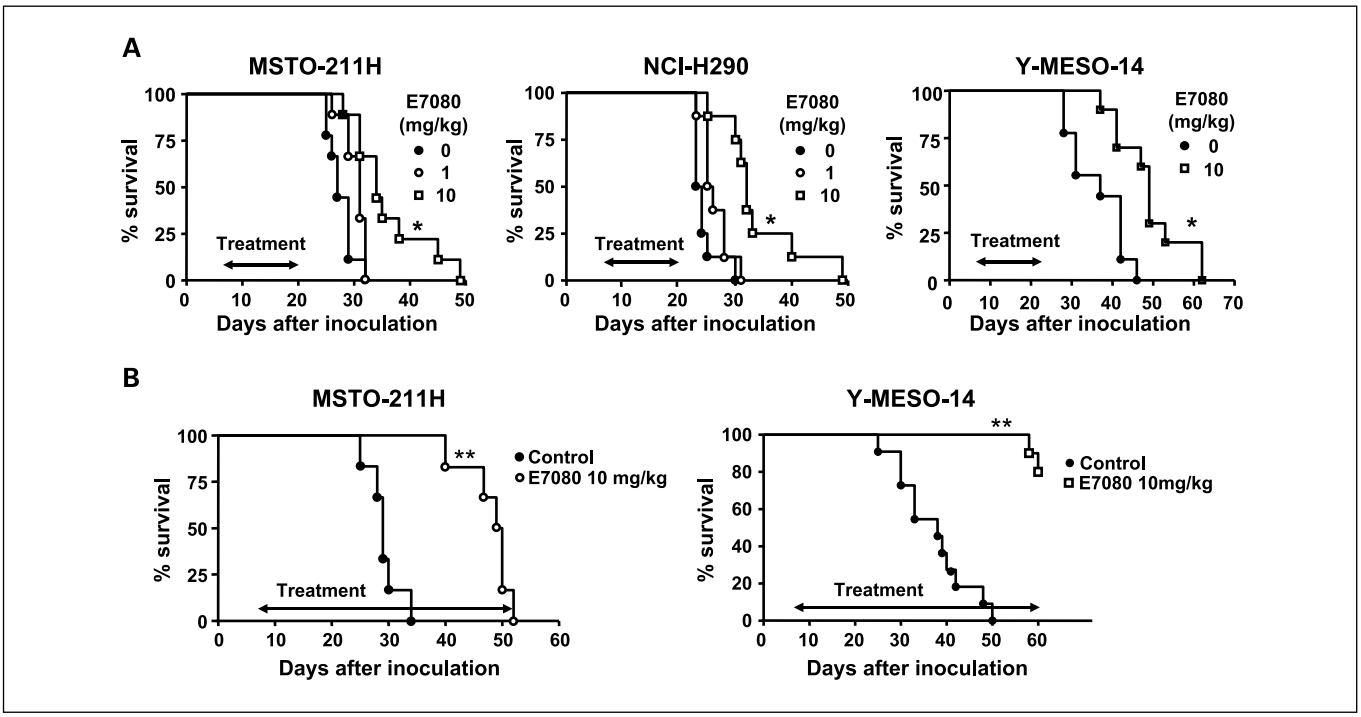

Fig. 5. Effects of E7080 on the survival of MPM cell–bearing SCID mice. *A,* SCID mice inoculated with MSTO-211H (1×10^6), NCI-H290 (3×10^5), or Y-MESO-14 (1×10^6) cells were orally treated with or without E7080 (1 or 10 mg/kg, daily) from day 7 to day 20. *B,* Mice inoculated with MSTO-211H (1×10^6) or Y-MESO-14 (1×10^6) cells were orally treated with or without E7080 (10 mg/kg, daily) from day 7 to the end of the experiment. *, $P < 0.01$; **, $P < 0.001$, compared with control group (log-rank test).

treatment with E7080 markedly inhibited tumor progression and prolonged the survival of SCID mice in all three models, and these findings were associated with reduced numbers of tumor-associated endothelial cells and proliferating MPM cells in the tumors. Our data strongly suggest a broad spectrum of activity of E7080 against MPM with different proangiogenic cytokine production profiles.

In the present study, three MPM cell lines showed different progression patterns after orthotopic implantation into SCID mice. MSTO-211H cells produced low levels of VEGF and high levels of FGF-2, and developed only thoracic tumors in SCID mice, in accordance with our previous report (12). In contrast, Y-MESO-14 cells produced relatively high levels of VEGF and low levels of FGF-2, and developed thoracic tumors and pleural effusions. Pleural effusion developed by Y-MESO-14 cells had high levels of human VEGF (8.2 ± 0.5 ng/mL) presumably produced by the tumor cells. These two models support our previous findings that VEGF is a causal factor in the development of pleural effusions by vascular hyperpermeability (9). Interestingly, VEGF-low-producing NCI-H290 cells induced not only thoracic tumors but also pleural effusion (human VEGF concentration in pleural effusion was 0.7 ± 0.1 ng/mL) in the late stages after orthotopic implantation into SCID mice, although the volume of pleural effusion was smaller than that produced by implantation of VEGF-high-producing Y-MESO-14 cells. This was not unexpected, however, because malignant tumors can develop pleural effusion in several ways, and fluid formation may not be due to VEGF alone (25). Although NCI-H290 cells produce PDGF-AA, it was unlikely to facilitate the production of pleural effusion *because* imatinib (STI571; Glivec), a selective inhibitor of the bcr-abl, c-kit, c-fms, and PDGFR tyrosine kinases (23), did not inhibit the production of thoracic tumors or pleural effusion in this orthotopic implantation model (data not shown). Further experiments to clarify the mechanism(s) underlying these observations are currently under way in our laboratory.

E7080 was originally generated as an angiogenesis inhibitor, which primarily suppresses VEGFR-2 phosphorylation. However, it has been shown to have additional activity against other receptor tyrosine kinases, including FGFRs, PDGFRs, and c-Kit (13, 14). In our three MPM models, E7080 markedly reduced the microvessel density in tumors. At clinically available concentrations (<1,000 nmol/L), however, E7080 did not directly affect the proliferation of MPM cells *in vitro* (14, 22). It is likely that the inhibition of angiogenesis by E7080 may indirectly lead to a decreased proliferation of tumor cells *in vivo*. Collectively, these results suggest that the therapeutic potential of E7080 against MPM may be due to inhibition of angiogenesis.

Multikinase inhibitors, including E7080, are thought to have activities against various types of solid tumor. A broad spectrum of antitumor activity is one of the advantages of multikinase inhibitors over specific inhibitors, such as the anti-VEGF Ab bevacizumab. In fact, E7080 successfully inhibited tumor progression of MSTO-211H cells, which expressed high levels of FGF-2 and low levels of VEGF, whereas bevacizumab did not (12). VEGF was reported to be a factor contributing to the poor prognosis of MPM. However, the level of VEGF expression varied among MPM (7, 26, 27). In the case of

VEGF-low-expressing tumors, other proangiogenic cytokines (PDECGF or FGF-2, etc.) will show compensatory expression and alternatively facilitate angiogenesis (28). As E7080 has inhibitory activity against multiple tyrosine kinases, including VEGFRs and FGFR1, it may be a promising candidate for controlling MPM with various proangiogenic cytokine production profiles.

In our orthotopic implantation model with MPM cells, survival prolongation is one of the most valuable parameters to evaluate therapeutic efficiency. Two weeks of treatment with E7080 at 10 mg/kg significantly inhibited the production of pleural effusion and/or thoracic tumors of three MPM cell lines. However, the same treatment showed only marginal prolongation of survival. These findings strongly suggest that growth of MPM cells was suppressed during E7080 treatment but that they rapidly re grew after cessation of E7080 treatment. A similar phenomenon was also observed in a previous study showing that an anti-VEGF agent markedly inhibited tumor angiogenesis during treatment, but that tumor angiogenesis recovered within one week after withdrawal of treatment (29). Therefore, continuous treatment may be important to maintain the antiangiogenic effect that keeps tumor cells from growing and spreading. In accordance with this concept, we observed that continuous treatment with E7080 markedly prolonged survival of mice in all three MPM models.

E7080 is currently being evaluated in clinical trials. In a phase I clinical study, 27 patients with advanced solid tumors were treated with E7080 (0.5-20 mg, twice daily; ref. 22). The maximum tolerance dose was determined to be 13 mg twice daily, and C_{max} was 302 ng/mL (577 nmol/L). Preliminary results showed that E7080 had durable disease control activity in various types of tumor, including one colorectal cancer patient with a confirmed partial response. In another phase I trial, 52 patients with various tumor types were treated with 0.2 to 32 mg E7080 once daily, which was safe and well tolerated at doses up to 25 mg daily (30). Although the number of patients was limited, tumor regression was observed in patients with melanoma ($n = 2$), renal cell carcinoma ($n = 2$), and sarcoma ($n = 1$). Based on these promising results, phase II clinical trials are currently being planned.

In summary, we have shown here that E7080, a multi–tyrosine kinase inhibitor, could suppress angiogenesis and tumor progression, and hence prolong survival of SCID mice inoculated with three MPM cell lines with different proangiogenic cytokine production profiles. These findings suggest the therapeutic potential of E7080 for treatment of heterogeneous MPM in humans. In addition, our findings further illustrate the value of studying novel strategies using E7080 in combination with chemotherapeutic agents, such as pemetrexed and gemcitabine, to enhance the efficacy, and preclinical experiments on these combinations are currently under way in our laboratory.

Disclosure of Potential Conflicts of Interest

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