Controlling Tumor Angiogenesis and Metastasis of C26 Murine Colon Adenocarcinoma by a New Matrix Metalloproteinase Inhibitor, KB-R7785, in Two Tumor Models

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ABSTRACT

Experimental evidence has directly implicated matrix metalloproteinases (MMPs) in the remodeling of the stromal tissue surrounding tumors. Thus, MMP inhibitors could limit the expansion of both neoplastic cell compartment and endothelial cell compartment of a tumor. Much of the work on the role of MMP inhibitors has concentrated on their inhibitory effects on tumor cell invasion. We have examined the effects of a new MMP inhibitor, KB-R7785 (acting on MMP-1, MMP-3, and MMP-9), on tumor angiogenesis and metastasis of murine colon adenocarcinoma (C-26) in two tumor models in BALB/c mice (transparent chamber model and lung colonization model). KB-R7785 has not shown inhibitory effects on in vitro growth of either C-26 or KOP2.16 murine endothelial cells. In vivo, KB-R7785 administered twice daily for 15 days (100 mg/kg, i.p.), starting the day of tumor inoculation (5 × 10^6 C26 cells) in transparent chamber, has resulted in 88.2% suppression of tumor growth, compared with that in vehicle-administered mice (controls). Tumors grown in controls have doubled their area in 3.3 days, whereas those treated by KB-R7785 progressed almost four times slower (tumor area doubling time, 12 days).

KB-R7785 rendered centrally avascular tumors with only a rim of peripheral neovascularization, which had significant lower functional vascular density and vascular area than the corresponding parameters in control tumors 10 days after inoculation (79.9 ± 6.7 cm/cm^2 versus 164.1 ± 10.1 cm/cm^2 (P < 0.01) and 19.8 ± 1.5% versus 42.6 ± 2.7% (P < 0.01), respectively). In the lung colonization model (tail vein inoculation of 5 × 10^4 C-26 cells), administration of KB-R7785 (100 mg/kg, i.p.) twice daily for 20 days has reduced the number of surface metastasis by 85.8% and abolished the tumor burden, as compared with controls. The few metastatic colonies found in the lungs of KB-R7785 treated mice appeared to be dormant (i.e., staining with von Willebrand factor antibody revealed few, if any, positive cells within the metastatic foci from MMP inhibitor-treated lungs, whereas terminal deoxynucleotidyl transferase-mediated nick end labeling showed a 4-fold increase in the rate of tumor cell apoptosis compared with controls. The fact that KB-R7785 interferes with early steps of angiogenesis and cancer spread suggests that MMP inhibitors may control both primary and secondary tumor growths by limiting the expansion of endothelial cells, as well as cancer cells, composing the tumors.

INTRODUCTION

The growth of a tumor and its ability to metastasize is angiogenesis-dependent (1). Angiogenesis is one of the crucial steps in the transition of a tumor from a small, harmless cluster of mutated cells to a large, malignant growth, capable of spreading to other organs throughout the body (2, 3). The principal barriers to tumor growth and spread are the extracellular matrix compartments. Experimental evidence has directly implicated MMPs, as well as other proteases, in remodeling of the stromal tissue surrounding the tumor (4–6). Still, as a tumor may be looked on as a two-cell compartment model, tumor cell compartment, and endothelial cell compartment (7), inhibitors of matrix-degrading enzymes could limit the expansion of both compartments, thus, interfering with both tumor angiogenesis and cancer spread. This rationale makes the inhibitors of MMPs an attractive target for anticancer agents. Much of the work on the role of MMP inhibitors has concentrated on their inhibitory effects on tumor cell invasion in vivo and in vitro models. In the present study, we describe the antiangiogenesis and antimetastatic properties of a new MMP inhibitor, KB-R7785 (8), on tumor angiogenesis and metastasis of C-26 colon adenocarcinoma using two tumor models in BALB/c mice (transparent chamber model and lung colonization model).

MATERIALS AND METHODS

Cells. BALB/c-derived, C-26 colon adenocarcinoma cell line was grown in RPMI (Life Technologies, Inc., Grand Island, NY) supplemented with 10% FCS (Sanko Junyaku, Tokyo, Japan), 0.56% NaHCO_3, 2 mM of L-glutamine, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 10 mM of HEPES. The murine endothelial cell line KOP2.16 (9) was kindly provided by Drs. Hideo Yagita and Ko Okumura (Department of Immunology, Juntendo University School of Medicine, Tokyo, Japan). The KOP2.16 cell line was grown in DMEM (Life Technologies, Inc.) containing 20% FCS, 10 mM of HEPES, 2 mM of L-glutamine, 1 mM of sodium pyruvate, 100 units/ml penicillin, and 100 mg/ml streptomycin. Cultures were maintained in a humidified 5% CO_2 atmosphere at 37°C.

Drug Treatment. The hydroxamic acid-based metalloproteinase inhibitor KB-R7785, [4-(N-hydroxyamino)-2R-isobutyl-3S-methylsuccinyl]-L-phenylglycine-N-methylamide (8), was supplied by the New Drug Discovery Research Laboratory (Kanebo LTD, Osaka, Japan). Its enzyme inhibition profile, expressed as IC_{50}, is as follows: 3 nm for MMP-1 (collagenase), 1.9 nm for MMP-3 (stromelysin), and 3.9 nm for the M, 92,000 gelatinase (MMP-9). For in vivo administration, KB-R7785 was suspended in 0.5% CMC up to 10 mg/ml and administered i.p. at a dose of 100 mg/kg twice daily, starting the day of tumor inoculation until day 15 in the transparent chamber model and until day 20 in the lung metastasis model, respectively.

Animal and Tumor Models. Male BALB/c mice (body weight, 19–23 g), purchased from Nippon Clea Co. (Tokyo, Japan), were maintained in the Institute of Experimental Animals (Tohoku University School of Medicine, Sendai, Japan) in a germ-free environment and allowed free access to food and water.

Transparent Chamber Model. The two titanium frames that make the framework of transparent chamber were designed in our laboratory after the previously reported (10) and manufactured in collaboration with Aoba Science Ltd. (Sendai, Japan). The chamber implantation is similar to that previously described models (10–12) and manufactured in collaboration with Aoba Science Ltd. (Sendai, Japan). The chamber implantation is similar to that previously described (13), however, the simplified design of the chamber allows an easier manipulation and reduced implantation time (average, 40 min/
mouse). After implantation, the mice were housed individually and allowed to recover 2–3 days. Then, a 2-μl tumor cell mass (~5 × 10^6 cells, viability 98%) was inoculated onto the upper layer of the skin of the access chamber after removing its coverslip (12). For intravital microscopy, unanesthetized animals were immobilized in polyethylene tubes attached to the stage of a microscope (OPTIPHOTO 66, Nikon, Tokyo, Japan). Observations were made with Nikon X2 (numerical aperture, 0.05), Nikon X4 (numerical aperture, 0.10), Nikon X10 (numerical aperture, 0.25), and Nikon X20 (numerical aperture, 0.40) objectives using transillumination and a filter for converting artificial light into daylight. Vessel contrast enhancement by fluorescence staining of plasma was not required with this tumor (C-26 adenocarcinoma) because it had offered no benefit over the transillumination technique in processing and assessing microvascular parameters in preliminary experiments. Observations were captured with an intensified CCD camera (TEC-470; Optronics Co., Chelmsford, MA) attached to the microscope and serially connected to a TV monitor (BM-1400S; Victor Co., Tokyo, Japan) and recorded on a S-VHS video tape (OPTIPHOTO 66, Nikon, Tokyo, Japan). Observations were made with Nikon X10 (numerical aperture, 0.25), and Nikon X20 (numerical aperture, 0.40) objectives using transillumination and a filter for converting artificial light into daylight. Vessel contrast enhancement by fluorescence staining of plasma was not required with this tumor (C-26 adenocarcinoma) because it had offered no benefit over the transillumination technique in processing and assessing microvascular parameters in preliminary experiments. Observations were captured with an intensified CCD camera (TEC-470; Optronics Co., Chelmsford, MA) attached to the microscope and serially connected to a TV monitor (BM-1400S; Victor Co., Tokyo, Japan) and recorded on a S-VHS video tape recorder (HR-X1; Victor Co.). The off-line analysis of the video tape recordings and calculation of microvascular parameters were performed following those previously described by Borgstrom et al. (14) and Leunig et al. (1). Still video frames (680 × 480 pixels) were captured at a resolution of 72 pixels/inch with Avid Video Shop 3.0 (Avid Technology, Inc., Tewksbury, MA) on a Power Macintosh 7100/80AV computer (Apple Computer, Inc., Cupertino, CA). The vessel outline of each of the frame (recorded with X10 objective) was carefully drawn in a separate transparent layer, using Adobe Photoshop 4.0 (Adobe Systems, Inc., Mountain View, CA), with the pencil tool of one pixel line width after the defective channels of the RGB color images of the video-captured frame were eliminated and the image transformed to the gray scale mode. The values for brightness and contrast in midtones, shadows, and highlights of gray scale images were manually set for optimal vessel delineation. The drawn vessel contours were subsequently filled with black using the paint bucket tool to render a binary (blank and white) copy of the neovascular network from the captured video frame. The binary images were then skeletonized (reduced to one pixel-wide skeletons) with the public domain NIH Image 1.61 software. The binary images and their skeletonized mimics were used to calculate three microvascular parameters: functional vascular density, vascular area, and mean vessel diameter. The functional vascular density (total vascular length/observation area) is proportional with the number of black pixels in a skeletonized image, and the vascular area (occupied by tumor vessels/observation area) is proportional with the squared number of black pixels in a binary image. The unit transformations (pixels to mm or μm) were computed with NIH Image software after calibration for known lengths and areas. Mean vessel diameter was obtained from the ratio of the vascular area and total vascular length. Observations were performed at days 0, 3, 7, and 10 after tumor inoculation, interval dictated by the rapid tumor growth (C-26 T2 was 3.3 days). After day 7, the microvascular parameters in C-26 tumors characterize tumor vessels only, because, at this stage, no underlying preexistent host vessel is observed through the thick tumor mass. The functional vascular density, vascular area, and mean vessel diameter of tumor-free striated muscle (indicated in Figs. 5 and 6 by dashed lines) were calculated in preliminary experiments in BALB/c mice with nontumor-bearing chambers and found to remain constant over the observation time.

**Lung Colonization Model.** C-26 cells were prepared as a single cell suspension in sterile PBS at a concentration of 2.5 × 10^6/ml, and a volume of 200 μl (~5 × 10^5 cells, viability 97%) was injected i.v. into the lateral tail vein of BALB/c mice. Animals were sacrificed by a high dose of pentobarbital sodium (250 mg/kg, i.p.) after 20 days, when control mice became moribund. All animal experiments were performed according to our institutional and NIH guidelines for care and use of research animals.

**Histology.** Specimens were fixed in phospho-lysine-paraformaldehyde solution at 4°C overnight, embedded in Tissue-Tek® O.C.T. Compound (Sakura Finetchnical Co., Ltd., Tokyo, Japan), and stored at -80°C. Sections (5 μm) were cut for immunohistochemical staining. Expression of von Willebrand factor was detected using rabbit polyclonal Abs (8 mg/ml; Dako, Tokyo, Japan) after the standard protocol from Vector Laboratories (Burlingame, CA). TdT labeling was performed with In Situ Apoptosis Detection Kit (Dako), and the apoptotic index was estimated by the percentage of positive stained cells at 3000 cells counted/each lung section (three in each group). **Data Analysis.** Experiments were performed two times, and similar results were obtained. Representative data from each experiment is presented (mean values ± SD or SE, as described in the figure legends). One way ANOVA computed with Microsoft Excel analysis pack (Microsoft Corporation and GrayMatter International, Redmond, WA) was used for statistical analysis. P ≤0.05 was considered significant.

**RESULTS**

*In vitro* proliferation of C-26 colon adenocarcinoma cells, as well as K0P2.16 cells (murine endothelial cells; Ref. 9), in the presence of...
KB-R7785 was assessed by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyl-tetrazolium bromide absorbance assay (15). Both C-26 and KOP2.16 cells were plated in quintuplicate into 96-well plates (10^4 cells/well, concentration determined as the cell number/well that give the maximum absorbance within the range of linearity), and the effect of serial dilutions of KB-R7785 was determined two times at 96 h. KB-R7785 has not shown significant inhibitory effect on in vitro growth of either adenocarcinoma or endothelial cells at concentrations ranging from 5 nm to 80 micromolar (data not shown).

**Transparent Chamber Model.** Administration of MMP inhibitor KB-R7785 (100 mg/kg, i.p.) twice daily for 15 days, starting the day of tumor inoculation, has inhibited growth (Figs. 1 and 2) and neo-vascularization (Figs. 3-6) of C-26 tumors in dorsal skinfold chamber of BALB/c mice (n = 5 in each group). Fig. 1A shows representative specimens from control (top) and treated mice (bottom). Large tumors developed in control mice, whereas no tumor rose onto the skin of mice given MMP inhibitor. Tumor area, as a measure of tumor growth, was by 88.2% smaller in treated mice compared with that in controls at the end of experiment (day 15) and, except day 3, it was significantly lower than that in vehicle-administered mice at all time points: 6.3 ± 1.2 mm^2 versus 11.7 ± 1.4 mm^2 (P < 0.01) at day 7, 7.4 ± 1.1 mm^2 versus 21.8 ± 2.5 mm^2 (P < 0.01) at day 10, and 10.8 ± 1.6 versus 92.6 ± 27.8 mm^2 (P < 0.01) at day 15, respectively (Fig. 2). T_2 was almost 4-fold longer in treated mice (T_2 = 12 days) as compared with that in controls (T_2 = 3.3 days).

An overview of neovascularization of C-26 tumors in treated and control mice is shown in Fig. 3. The top illustrates tumor cell inoculation onto the striated skin muscle within the transparent chamber (day 0). Tumor cell masses were seen at low magnification as shadowed areas (Fig. 3, A and B, arrowheads). At day 3, tumors appeared in both treatment and control group as speckled reddish areas, whereas the underlying host vessels became dilated and tortuous. At this stage, the neovascular changes appear both complex and indistinct, and we found quantification of angiogenesis difficult to accomplish. After 7 days, control tumors developed a functional neovascular network with sprouts and early vessels covering up all tumor area (Fig. 3C and Fig. 4A) and allowing blood to flow. Three days later, these tumors grew almost two times wider (T_2 = 3.3 days), and their neovascular network matured, showing large tortuous vessels with abrupt changes in diameter (Fig. 3E and Fig. 4C). In contrast, MMP inhibitor-treated tumors showed central avascular areas (Fig. 3D) at day 7 and delayed neovascularization with deficient vessels at their periphery (Fig. 4B). At day 10, the tumors remained avascular (Fig. 3F), and their peripheral vessels remained sparse and frail (Fig. 3F and 4D). The vessels developed at the periphery of MMP inhibitor-treated tumors showed significantly lower functional vascular density and vascular area com-
pared with vessels from control tumors at both day 7 [17.3 ± 5.3% versus 39.3 ± 1.7% (P < 0.01) and 96.3 ± 28.0 cm/cm² versus 239.1 ± 18.0 cm/cm² (P < 0.01), respectively] and day 10 [19.8 ± 1.5% versus 42.6 ± 2.7 (P < 0.01) and 79.9 ± 6.7 versus 164.1 ± 10.1 cm/cm² (P < 0.01), respectively; Fig. 5]. In the control group, the functional vascular density decreased with time, from 239.1 ± 18.0 cm/cm² at day 7 to 164.1 ± 10.1 cm/cm² at day 10 (P = 0.010), whereas the mean tumor vessel diameter increased significantly during the same interval [16.8 ± 0.4 μm versus 26.2 ± 0.69 μm (P < 0.01), respectively]. Mean vessel diameter of peripheral vessels in MMP inhibitor-treated tumors did not differ significantly from that of tumor vessels from controls over the same observation period (Fig. 6).

In both groups, mice steadily increased in weight from day 0 until day 15. There was no difference in body weight of animals administered KB-R7785 compared with that of controls at the end of the experiment (26.7 ± 0.9 g versus 27.0 ± 1.1 g; P = 0.58). No other physical or behavioral changes were noticed in the MMP inhibitor-treated mice.

**Lung Colonization Model.** Administration of KB-R7785 (100 mg/kg, i.p.) twice daily for 20 days, starting the day of tumor inoculation, suppressed formation and growth of lung metastasis (n = 5 in

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Fig. 4. Details from the square-marked areas from Fig. 3, illustrating the effect of vehicle (left) and MMP inhibitor (right) on angiogenesis of C-26 tumors. A, early vessels and sprouts (dark spots, arrowheads) can be seen in tumors from vehicle-administered mice at day 7. B, sparse and leaky vessels (arrowheads) that rose at the periphery of tumors in MMP inhibitor-treated mice (day 7). C, typical mature neovasculature with large tortuous vessels and abrupt changes in diameter (arrowheads) at day 10. D, at day 10, vessels at the periphery of tumors from MMP inhibitor-treated mice have not changed much from day 7, as seen in control tumors. Photographs (digitized) were achieved using transillumination and a X10 objective; Bar, 300 μm.

**Fig. 5.** The effect of vehicle and KB-R7785 (100 mg/kg, i.p.) on the functional vascular density [A; vessel length/observation-tumor area (cm/cm²)] and vascular area (B; the area occupied by tumor vessels per tumor area as percentage) in C-26 tumors grown within the dorsal skinfold chamber of BALB/c mice. Functional vascular density, as well as vascular area, are significantly lower in the treatment group than in the control group at both day 7 and day 10. The vascular parameters in KB-R7785-administered mice characterize the vessels that rose at the periphery of the tumor, as their central regions were avascular (see Fig. 3). Functional vascular density decreased in control mice from day 7 to day 10 (P = 0.01), whereas the vascular diameter increased over the same period (Fig. 6.). At each observation time, three tumor areas/mouse were randomly selected and captured from the video recordings with the X10 objective and processed into binary copies and skeletonized images on a Macintosh computer (see “Materials and Methods”). Dashed lines, the vascular density (218.7 ± 11.0 cm/cm²) and vascular area (22.6 ± 1.7%) of the underlying striated muscle in the skinfold chamber onto which tumors develop. Values are plotted as mean ± SE, n = 5; *, P < 0.01; #, P < 0.05.
each group). Fig. 7 shows the large metastatic nodules that formed in the lungs from control mice (A, arrows) and the apparent tumor-free lungs of MMP inhibitor-administered mice (B). The number of surface metastasis was significantly smaller in treated mice than in controls, 15.8 ± 6.6 versus 112 ± 48.5 (P < 0.01; Fig. 8A). Besides, the average lung weight of mice given MMP inhibitor was comparable with that of age- and sex-matched nontumor-bearing animals, 0.20 ± 0.06 g and 0.21 ± 0.04, respectively (Fig. 8B), whereas the mean lung weight in vehicle-administered animals was 2.5 times higher, 0.51 ± 0.18 g (P < 0.01).

Analysis of apoptotic index by TdT labeling of fragmented DNA (Fig. 9) showed a 4-fold increase in apoptosis in tumor cells from metastatic colonies in lungs of KB-R7785-treated mice (6.2 ± 1.2%), as compared with that in controls (1.5 ± 0.4%). Increased DNA fragmentation was also seen at agarose gel electrophoresis of DNA extracted from treated tumors compared with that from control tumors, although with a lower sensitivity than by terminal deoxynucleotidyl transferase-mediated nick end labeling method (data not shown).

Immunostaining with von Willebrand factor Ab revealed numerous positive stained cells defining tumor vessels of various sizes within the lung metastatic nodules in control mice (Fig. 10, arrows). Few, if any, positive stained cells could be seen within the micrometastasis from the KB-R7785-treated mice besides the endothelial cells that outline the central vessels from which the colonies may have been formed. (Fig. 10B, arrows). Similar findings were obtained when staining was performed with murine anti-CD31 Abs.

Mice receiving MMP inhibitor gained weight (22.6 ± 0.8 g at day 0 and 24.4 ± 1.6 g at day 20; P < 0.06) and appeared healthy during the experiment. In contrast, vehicle-administered mice became moribund and presented signs of dispnea, ataxia, and characteristic fur changes 18 days after tumor inoculation. All mice were sacrificed at day 20. Their average weight at day 20 was comparable with that recorded at the beginning of the experiment, but with a 10-fold higher SD (22.7 ± 0.3 g versus 22.0 ± 3.0 g; P = 0.6).
DISCUSSION

Two tumor models, transparent chamber model and lung colonization model, were used to study the effect of a new MMP inhibitor, KB-R7785 (acting on MMP-1, MMP-3, and MMP-9), on tumor angiogenesis and metastasis of C-26 colon adenocarcinoma. KB-R7785 proved to be a potent antiangiogenesis and antimetastatic agent.

Transparent chamber is a model particularly useful to study the temporal sequence of events occurring during early stages of angiogenesis. In the present studies, KB-R7785 suppressed tumor growth apparently by preventing efficient vascularization of C26 inocula within the dorsal skinfold chamber of BALB/c mice. One week after tumor inoculation, functional vascular networks, which consistently covered all tumor areas, developed in animals given 0.5% CMC as a vehicle. Three days later, these tumors grew two times wider and showed significantly larger vessels. The functional vascular density decreased in this group from a value exceeding that of the underlying muscle capillaries at day 7 to a lower one at day 10 (Fig. 5). The high functional vascular density (total vessel length/tumor area) observed in control mice at day 7 may be explained by the prevalence during the very early steps of neovascularization of vessel sprouting over vessel maturation (increase in diameter), the latter being characteristic to the later steps of tumor angiogenesis. However, the possibility of capturing vessels from deeper planes, because of the thinner tumors, at day 7 as compared with day 10 may also explain this difference. Administration of the MMP inhibitor limited the expansion of the vascular areas into the nonvascularized ones by rendering centrally avascular tumors with only a rim of peripheral neovascularization. These tumors failed to thrive, as they were nourished by a peripheral neovascular network with vessels of significant lower functional vascular density and vascular area compared with those of vessels developed in control tumors at both day 7 and day 10. MMP inhibitor did not significantly affect the mean vessel diameter of vessels that rose at the periphery of the tumor. We found the pattern of inhibition of angiogenesis by KB-R7785 different from that of a well-known antiangiogenic agent, TNP-470 (O-choracetyl carbamoyl fumagilol; Takeda, Osaka, Japan), tested in the same tumor model. Avascular tumors could not be seen with administration of TNP-470 (30 mg/kg, s.c.) every 2nd day starting with day 0 after inoculation of 3 × 10⁵ C-26 cells within the dorsal skinfold chambers of BALB/c mice. Although TNP-470 could inhibit tumor growth by 75% compared with vehicle-administered mice, it allowed complete and homogenous vascularization of tumors. As opposed to KB-R7785, TNP-470 did not affect the functional vascular density of tumors, but did affect the tumor vessel diameter at both 1 week as well as 2 weeks after inoculation. These findings suggest that KB-R7785 may primarily affect the early steps of angiogenesis, endothelial cell invasion, and capillary sprouting, whereas TNP-470 may interfere more with the later steps, possibly maturation of newly formed vessels.

In the lung metastatic model, KB-R7785 rendered lungs of normal weight and markedly inhibited the number of surface metastasis. Thus KB-R7785 could possibly have limited both extravasation and subsequent tumor development. Direct evidence on these mechanisms is aimed to be obtained observing the interaction of tumor cells with host microvasculature by intravital microscopy. A couple of studies have shown that MMP regulation may affect metastatic growth mainly at the postextravasation stage (16, 17). Also, new insights into the contributions of MMPs to metastasis, based on evidence from direct in vivo observations of its early steps, have been described recently by Chambers and Matrisian (18).

The micrometastasis in KB-R7785-treated lungs were found avascular and undergoing a 4-fold higher rate of apoptosis than that in growing lung tumors from moribund control animals. It has been previously shown that distinct angiogenesis inhibitors (such as TNP-470, angiotatin, endostatin, integrin αVβ3 antagonists, and others) increase apoptosis in tumors in vivo, whereas few of them do so in vitro. Angiogenesis inhibitors were shown to increase apoptosis among both tumor cell population (19–22) and endothelial cell population (23–25). It is an increased apoptotic rate, rather than a decreased proliferation, that is believed to restrain small tumor colonies from expanding for prolonged periods of time in the presence of natural or synthetic angiogenesis inhibitors and hold them in a dormant state (19).

Taken together, these data show that KB-R7785 interferes with early steps of angiogenesis and cancer spread. This suggests that
MMP inhibitors may control both primary and secondary tumor growths by limiting expansion of endothelial cells as well as cancer cells composing the tumors. It is in our interest to further study the effects of KB-R7785 and several related compounds on established (vascularized) tumors and on a long-term basis, either alone or in association with other drugs acting on complementary angiogenesis inhibitory pathways.

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