[CANCER RESEARCH 61, 2232–2238, March 1, 2001]

Inhibition of Angiogenesis and Tumor Growth by SCH221153, a Dual $\alpha_v\beta_3$ and $\alpha_v\beta_5$ Integrin Receptor Antagonist[1]

C. Chandra Kumar,[2] Michael Malkowski, Zizhang Yin, Elena Tanghetti, Bo Yaremko, Terry Nechuta, Judy Varner, Ming Liu, Elizabeth M. Smith, Bernie Neustadt, Marco Presta, and Lydia Armstrong

Departments of Tumor Biology [C. C. K. , M. M., Z. Y., B. Y., M. L., L. A.] and Medicinal Chemistry [T. N., E. M. S., B. N.], Schering-Plough Research Institute, Kenilworth, New Jersey 07033; Department of Biomedical Sciences and Biotechnology, School of Medicine, University of Brescia, 25123 Brescia, Italy [E. T., M. P.]; University of California, San Diego, California [J. V.]

ABSTRACT

New blood vessel formation is essential for tumor growth and metastatic spread. Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ are arginine-glycine-aspartic acid-dependent adhesion receptors that play a critical role in angiogenesis. Hence, selective dual $\alpha_v\beta_3$ and $\alpha_v\beta_5$ antagonists may represent a novel class of angiogenesis and tumor-growth inhibitors. Here, an arginine-glycine-aspartic acid-based peptidomimetic library was screened to identify $\alpha_v\beta_3$ antagonists. Selected compounds were then modified to generate potent and selective dual inhibitors of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ receptors. One of these compounds, SCH 221153, inhibited the binding of echistatin to $\alpha_v\beta_3$ (IC_{50} = 3.2 nM) and $\alpha_v\beta_5$ (IC_{50} = 1.7 nM) with similar potency. Its IC_{50} values for related $\alpha_{IIb}\beta_3$ and $\alpha_5\beta_1$ receptors were 1294 nM and 421 nM, respectively, indicating that SCH 221153 is highly selective for $\alpha_v\beta_3$ and $\alpha_v\beta_5$ receptors. In cell-based assays, SCH 221153 inhibited the binding of echistatin to $\alpha_v\beta_3$- and $\alpha_v\beta_5$-expressing 293 cells and blocked the adhesion of endothelial cells to immobilized vitronectin and fibroblast growth factor 2 (FGF2). SCH 221153, but not the inactive analogue SCH 216687, was effective in inhibiting FGF2 and vascular endothelial growth factor-induced endothelial cell proliferation *in vitro* with an IC_{50} equal to 3–10 μM. Angiogenesis induced by FGF2 in the chick chorioallantoic membrane assay was also inhibited by SCH 221153. Finally, SCH 221153 exerted a significant inhibition on tumor growth induced by intradermal or s.c. injection of human melanoma LOX cells in severe combined immunodeficient mice.

INTRODUCTION

The concept that tumor growth and its metastatic spread are dependent on the formation of new blood vessels has sparked an interest in identifying protein targets amenable to small-molecule drug discovery (1–3). The angiogenic process depends on vascular endothelial cell proliferation, migration, and invasion (4). A family of adhesion receptors known as integrin receptors regulates these processes. Integrins are composed of noncovalently associated α and β chains and recognize the RGD[3] sequence present in their matrix ligands (5). Nevertheless, they are capable of distinguishing different RGD-containing proteins, thus showing different specificity for various ECM cell-adhesive proteins. Among the various members of the integrin family, $\alpha_v\beta_3$ has been found to play a very significant role in the process of angiogenesis. Integrin $\alpha_v\beta_3$ is a promiscuous receptor inasmuch as it is capable of interacting with a number of ECM proteins, including VN, fibrinogen, FN, and thrombospondin (4, 6), as well as to other proteins with different biological functions including FGF2 (7) and metalloproteinase MMP-2 (8). In addition, $\alpha_v\beta_3$ has been shown to associate with activated platelet-derived growth factor, insulin, and VEGF receptors to facilitate optimal activation of cell proliferative signaling pathways (9, 10) and to prevent apoptosis (11).

Integrin $\alpha_v\beta_3$ is minimally expressed on resting or normal blood vessels, but is significantly up-regulated on vascular cells within human tumors or in response to certain growth factors *in vitro* (6, 12–14). For example, FGF2 markedly increases β3 mRNA and surface expression in cultured human dermal microvascular endothelial cells (15, 16). FGF2 and tumor necrosis factor α stimulate $\alpha_v\beta_3$ expression on developing blood vessels in the chicken CAM (12) and on the rabbit cornea (17). Endothelial cells exposed to growth factors, or those undergoing angiogenesis in tumors, wounds, or inflammatory tissue, express high levels of $\alpha_v\beta_3$ (12). Up-regulation of $\alpha_v\beta_3$ expression is also induced by human tumors cultured on the CAM, by human tumors grown in human skin explants grafted onto SCID mice, and on rabbit cornea (13). In fact, recent studies suggest that $\alpha_v\beta_3$ may serve as a useful diagnostic or prognostic indicator of tumors (18). Furthermore, antagonists of $\alpha_v\beta_3$, including both cyclic RGD peptides and monoclonal antibodies, significantly inhibited angiogenesis induced by cytokines and solid tumor fragments (12). Importantly, recent findings suggest that these antiangiogenic effects may be attributable to the ability of these antagonists to induce apoptosis in proliferating blood vessels (11). Remarkably, $\alpha_v\beta_3$ antagonists had very little effect on preexisting blood vessels, indicating the usefulness of targeting this receptor for therapeutic benefit without adverse side effects. A characteristic feature of $\alpha_v\beta_3$ that makes it an attractive target for therapeutic intervention is its relatively limited cellular distribution. It is not generally expressed on epithelial cells and is expressed only at low levels on a subset of B cells, some cells of macrophage lineage, smooth muscle cells, and activated endothelial cells (15, 16). Integrin $\alpha_v\beta_3$ is also expressed on certain invasive tumors including metastatic melanoma (19, 20) and late-stage glioblastoma (21), where it contributes to their malignant phenotype.

Recent studies have implicated a related integrin, $\alpha_v\beta_5$, in angiogenesis under certain conditions. For example, Friedlander *et al.* (17, 22) have shown that antibody antagonists of $\alpha_v\beta_3$ inhibit FGF2-stimulated angiogenesis and antagonists of integrin $\alpha_v\beta_5$ inhibit VEGF-stimulated angiogenesis in the corneal and CAM models. Recent studies have shown that kinase-deleted mutants of Src block VEGF-induced, but not FGF2-induced, angiogenesis (23). These results suggest that FGF2 and VEGF may activate different angiogenic pathways that require $\alpha_v\beta_3$ and $\alpha_v\beta_5$, respectively. Therefore, dual antagonists of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ may be useful in blocking tumor-induced angiogenesis.

In this study, we describe the pharmacological and biological characterization of a nonpeptide small molecule (SCH 221153) that is a potent inhibitor of both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin receptors. We demonstrate that SCH 221153 inhibits vascular endothelial cell adhesion and proliferation mediated by FGF2 and VEGF. SCH 221153

Received 10/5/00; accepted 12/28/00.

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.

[1] This work was supported in part by grants from the Associazione Italiana per la Ricerca sul Cancro, the Istituto Superiore di Sanità (AIDS Project), the National Research Council (Target Project on Biotechnology), and the Ministero dell'Università e della Ricerca Scientifica e Tecnologicala ("60%") to M. P.

[2] To whom requests for reprints should be addressed, at Department of Tumor Biology, Schering-Plough Research Institute, 2015 Galloping Hill Road, Kenilworth, NJ 07033. Phone: (908) 740-7328; Fax: (908) 740-3918; E-mail: chandra.kumar@spcorp.com.

[3] The abbreviations used are: RGD, arginine-glycine-aspartic acid; cRGD, cyclic RGD; VEGF, vascular endothelial growth factor; ECM, extracellular matrix; VN, vitronectin; FN, fibronectin; FGF2, fibroblast growth factor 2; CAM, chorioallantoic membrane; SCID, severe combined immunodeficient; HUVEC, human umbilical vascular endothelial cell; mpk, mg/kg body weight; TNF-α, tumor necrosis factor α.

inhibited FGF2-induced angiogenesis in chick chorioallantoic membrane and inhibited the growth of human tumor xenografts in SCID mice.

MATERIALS AND METHODS

Materials

DMEM, L-glutamine, nonessential amino acids, gentamicin, and synthetic RGD containing peptides were purchased from Life Technologies, Inc. (Gaithersburg, MD). Fetal bovine serum was from Hazleton Biologicals (Lenox, KS). Microlite-2 plates were obtained from Dynatech Corporation (Chantilly, VA). Flash plates were purchased from NEN Life Science Products, Inc. (Boston, MA). Multiscreen-FB opaque plates (1.0-μm Glass Fiber Type B filter) were from Millipore (Billerica, MA). Falcon Microtest III microtiter plates are from Falcon (Franklin Lakes, NJ). $\alpha_v\beta_3$-specific monoclonal antibodies (LM609), $\alpha_v\beta_5$-specific monoclonal antibodies (P1F6), and LM609 and P1F6-coupled to affigel matrix were purchased from Chemicon International, Inc. (Temecula, CA). ^{125}I-Echistatin labeled by the lactoperoxidase method to a specific activity of 2000 Ci/mmol was from Amersham International (Chicago, IL). ^{125}I-Fibronectin labeled by lactoperoxidase method to a specific activity of 11.6 μCi/μg was purchased from NEN Life Science Products, Inc. Echistatin was purchased from Bachem (Torrence, CA). Human recombinant FGF2 was expressed and purified to homogeneity from transformed *Escherichia coli* cells by Heparin-Sepharose affinity chromatography (24). GRGDSPK and GRADSPK peptides were from Neosystem Laboratoire (Strasbourg, France). Bovine FN and VN were from Sigma (St. Louis, MO). The 165-amino acid isoform of VEGF was from Calbiochem (San Diego, CA).

Protein Purification

$\alpha_v\beta_3$ was purified as described previously (25). Purified $\alpha_{IIb}\beta_3$ receptor was provided by Dr. Leslie Parise of the University of North Carolina (NC). Purified $\alpha_5\beta_1$ receptor was purchased from Chemicon International, Inc.

Solid-Phase Receptor-binding Assay

The receptor-binding assays were performed as described previously (25). Receptors $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_{IIb}\beta_3$ were diluted to 500ng/ml in coating buffer [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM $MnCl_2$], whereas $\alpha_5\beta_1$ was diluted to 1000ng/ml in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.2 mM $CaCl_2$, 10 mM $MgCl_2$, and 1 mM $MnCl_2$. An aliquot of diluted receptors (100 μl/well) was added to 96-well Flash microtiter plates and incubated overnight at 4°C. Coating solution was removed by aspiration and 200 μl of blocking solution [50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 3% BSA) was added to the wells and incubated for 2 h at room temperature. After incubation, the plates were rinsed three times with 200 μl of binding solution (coating buffer containing 0.1% BSA) and incubated with appropriate radiolabeled ligands for 3 h at room temperature. Fifty pM of radiolabeled echistatin was used for $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_{IIb}\beta_3$ receptors. Radiolabeled FN (300 pM) was used for $\alpha_5\beta_1$ receptor-binding assay. After incubation, the plates were sealed and counted in the Top Count (Packard).

Cell-binding Assay

Cell-binding assays were performed as described before (21).

Cell-Adhesion Assay

One hundred-μl aliquots of 100 mM $NaHCO_3$ (pH 9.6; carbonate buffer), containing the adhesive molecule under test were added to polystyrene non-tissue culture microtiter plates at 20 μg/ml. After 16 h of incubation at 4°C the solution was removed and wells were washed three times with cold PBS. For the cell-adhesion assay, confluent cultures of GM 7373 cells were trypsinized, washed, and resuspended with the appropriate medium. Previous observations had indicated that low concentrations of serum were required in some experiments for optimal cell adhesion to FGF2-coated plastic (7). For this reason, 1% FCS was used routinely in cell-adhesion experiments.

Transformed fetal bovine aortic endothelial GM7373 cells were used for the cell-adhesion assays. They were obtained from the N.I.G.M.S. Human Genetic Mutant Cell Repository (Institute for Medical Research, Camden, NJ). They correspond to the BFA-1c 1BPT multilayered clone described by Grinspan *et al.* (26). GM 7373 cells (50,000) were resuspended in 200 μl of medium and immediately seeded onto 96-well plates coated with the molecule under test in the absence or in the presence of the indicated concentrations of SCH 221153, SCH 216687, GRGDSPK, or GRADSPK. Cell adhesion was allowed to occur for 2 h at 37°C. Then, wells were washed once with 2 mM EDTA in PBS and once in MEM without serum. The washing procedure was repeated three times. Adherent cells were fixed in 3.7% paraformaldehyde/0.1 M sucrose in PBS, washed with PBS, and stained with methylene blue/Azur II (1:1, v/v). Plates were read with a microplate reader at 595 nm.

Cell Proliferation Assays

Short-Term Assay. GM 7373 cells were seeded at 75,000 cells/cm^2 in 96-well plates in Eagle's minimal essential medium containing 10% FCS, vitamins, and essential and nonessential amino acids. After overnight incubation, cells were treated with the mitogen under test plus 0.4% FCS in the absence or in the presence of the indicated concentrations of SCH 221153, SCH 216687, GRGDSPK, or GRADSPK. After 24 h, cells were trypsinized and counted. Under these experimental conditions, control cultures incubated in 0.4% FCS with no addition or with 10 ng/ml FGF2 underwent 0.1–0.2 and 0.7–0.8 cell population doublings, respectively. Cells grown in 10% FCS underwent one cell population doubling (27).

Long-Term Assay. HUVECs at passage 3 (Clonetics) were seeded at 2500 cells/well in 96-well plates in complete EGM-2 medium (Clonetics). After 24 h, all cell cultures were incubated in EGM-2 medium devoid of FGF2, VEGF, and heparin. Then wells were divided into three series: one was added with 10 ng/ml FGF2; the second one was added with 10 ng/ml FGF2 plus 30 ng/ml VEGF; and the third one was left untreated. Next, all series were treated with the indicated concentrations of SCH 221153 or SCH 216687. After 6 days, cells were stained with crystal violet and plates were read with a microplate reader at 595 nm.

Chick CAM Assay

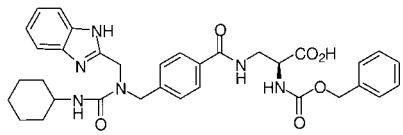
Embryos (10 days of age) were used in this assay, as described (12).

Mouse Xenograft Models

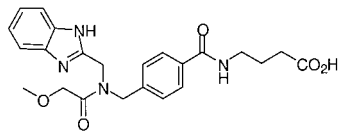
Female SCID mice (Charles River Laboratories, Wilmington, MA), 4–6 weeks of age were used for the tumor xenograft studies. In one study, SCID mice (Charles River Laboratories, Wilmington, MA) were inoculated intradermally on day 0 with human melanoma-derived LOX cells, which express very low levels of $\alpha_v\beta_3$. Starting on day 1, SCH 221153 was administered twice daily through i.p. injection at various doses for 15 days. In another study, SCID mice were inoculated s.c. with LOX tumor cells on day 0 and starting on day 1 were treated with SCH 221153 twice daily through i.p. injection for 15 days. SCH 221153 was dissolved in 20% (w/v) HPβCD, and mice in the vehicle control group received 20% HPβCD. The number of cells number used was 2×10^5 for intradermal inoculation and 5×10^5 for s.c. inoculation. Each tumor was measured in three dimensions on days 8, 11, and 15. Tumor volume was calculated with the formula of $V = 1/6 \times \pi \times L \times W \times T$, where L, W, and T represent length, width, and thickness, respectively (28). The data were expressed as the means ± or the means ± SD. Student's and Mann-Whitney tests were used to assess differences between means or meridians using the InStat software package (GraphPad Software, Inc., San Diego, CA).

RESULTS

Identification of a Potent and Selective RGD Peptidomimetic Inhibitor of Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$. A library of synthetic compounds mimicking the RGD-motif were designed using combinatorial methods to explore a diverse array of structures. These compounds were screened in the solid-phase receptor-binding assay to identify ones that inhibited the binding of radiolabeled echistatin to human integrin $\alpha_v\beta_3$. The general structures of the compounds in the library contained three units linked linearly. The basic unit consists of a 2-(aminoalkyl)benzimidazole, which is acylated on the amine to fur-



SCH 221153



SCH 216687

Fig. 1. Structure of the synthetic RGD mimetics, SCH 221153 and SCH 2116687, used in the present study.

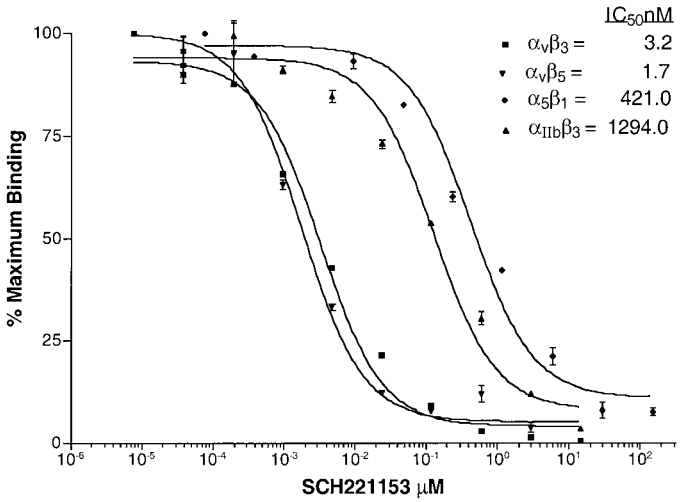


Fig. 2. SCH 221153 is a potent and selective inhibitor of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins. Binding of radiolabeled echistatin to integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_{IIb}\beta_3$ was measured as described in "Materials and Methods." For $\alpha_5\beta_1$ integrin, radiolabeled FN was used as the ligand. Each data point represents the mean results of three independent determinations.

nish an amide or urea. The core unit is a proprietary *meta*- or *para*-methylenebenzoyl bifunctional unit. The acidic unit consists of a variety of amino-carboxylic acids we wished to explore. This format of basic-core-acidic groups is well exemplified in known $\alpha_v\beta_3$ antagonists. An initial set of 10 10-component mixtures used 10 polar natural amino acids. A second set of these mixtures used 10 non-natural amino acids. A third set of 10 single compounds used γ-amino butyric acid as the amino acid. None of these library materials showed activity at 100 μg/ml in the receptor-binding assay. A set of 10 compounds using diaminopropionic acid was then prepared and showed strong activity. Compounds of interest were subsequently modified by medicinal chemistry efforts to enhance potency and selectivity for $\alpha_v\beta_3$. Fig. 1 shows the structure of the active compound, SCH 221153, identified via this process. SCH 216687 was inactive in the integrin receptor-binding assays and was used here as a negative control. Fig. 2 shows that SCH 221153 inhibited the binding of radiolabeled echistatin to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ in a dose-dependent manner with an IC_{50} equal to 3.2 and 1.7 nM, respectively. Selectivity of this compound was demonstrated by its poor antagonist activity for $\alpha_{IIb}\beta_3$ and $\alpha_5\beta_1$ receptors (IC_{50} values equal to 1294 nM and 421 nM, respectively). Consistent with these results, SCH 221153 was found to be ineffective in blocking ADP-induced platelet aggregation *in vitro* (data not shown).

Inhibition of Ligand-binding and Adhesion of 293-$\alpha_v\beta_3$ and 293-$\alpha_v\beta_5$ Cells by SCH 221153. Next we tested the binding of radiolabeled echistatin to HEK-293 cells stably expressing $\alpha_v\beta_3$ and $\alpha_v\beta_5$ receptors. For this purpose, 293-$\alpha_v\beta_3$ and 293-$\alpha_v\beta_5$ cells were harvested from tissue culture flasks, placed in suspension and incubated with ^{125}I-echistatin for 2 h in the presence of diluent or increasing concentrations of SCH 221153 or of cRGD peptide. As shown in Fig. 3*a*, both molecules inhibit the binding of radiolabeled echistatin to 293-$\alpha_v\beta_3$ cells in a dose-dependent manner with IC_{50} values equal to 348 nM and 92 nM for SCH 221153 and cRGD, respectively. SCH 221153 and cRGD were also similarly effective in blocking the binding of echistatin to $\alpha_v\beta_5$-293 cells in a dose dependent manner (Fig. 3*b*). In agreement with these observations, SCH 221153 and cRGD were similarly effective in blocking the adhesion of 293-$\alpha_v\beta_3$ and 293-$\alpha_v\beta_5$ cells to VN-coated plastic (results not shown).

Inhibition of Endothelial Cell Adhesion to VN and FGF2 by SCH 221153. Previous observations have shown that immobilized FGF2 can mediate endothelial cell adhesion and spreading via $\alpha_v\beta_3$ integrin interaction (7). On this basis, we evaluated the capacity of SCH 221153 and SCH 216687 to affect the adhesion of fetal bovine aortic endothelial GM 7373 cells to immobilized FGF2, VN, and FN. In this experiment, GM7373 cells were seeded onto non-tissue culture plates coated with the different substrata and allowed to adhere for 2 h before quantitating the number of adherent cells. Under these conditions, no significant cell adhesion and spreading were observed for BSA-coated plastic plates. As shown in Fig. 4, SCH 221153 exerts an inhibitory activity on the cell adhesive capacity of FGF2 and VN (IC_{50} equal to 3.0 and 1.0 μM, respectively, for the two substrates) that

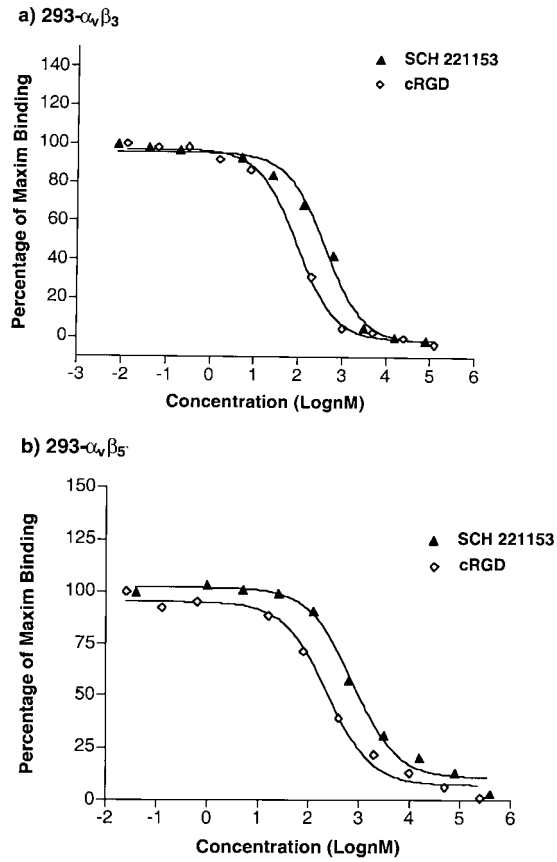


Fig. 3. Dose-dependent inhibition of the binding of echistatin to 293-$\alpha_v\beta_3$ and 293-$\alpha_v\beta_5$ cells. HEK-293 cells expressing $\alpha_v\beta_3$ and $\alpha_v\beta_5$ were cultured and suspended in binding buffer and allowed to bind to radiolabeled echistatin as described before (21). Values shown are the means of at least two determinations.

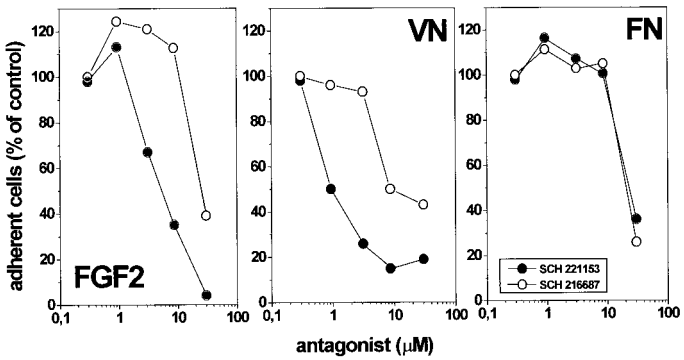


Fig. 4. Effect of SCH 221153 on endothelial GM 7373 cell adhesion. Non-tissue culture plastic 96-well plates were incubated with carbonate buffer containing 20 μg/ml VN (●, ○), FGF2 (▲, △), or FN (■, □). Then, GM 7373 cells were seeded onto coated plates in the absence or in the presence of increasing concentrations of SCH 221153 (*closed symbols*) or SCH 216687 (*open symbols*) and allowed to adhere for 2 h at 37°C. Then, adherent cells were fixed, stained, and the plates read with a microplate reader at 595 nm. Data are expressed as the percentage of cell adhesion observed in the absence of antagonist.

is 10 times more potent than that exerted by SCH 216687. Both molecules poorly affected GM 7373 adhesion to immobilized FN with an IC_{50} equal to ~20 μM.

SCH 221153 Inhibits Endothelial Cell Proliferation Induced by FGF2 and VEGF. SCH 221153 was evaluated for the capacity to affect the mitogenic activity exerted by FGF2 in a short-term proliferation assay. For this purpose, subconfluent endothelial GM 7373 cells were incubated with 10 ng/ml FGF2 or 30 ng/ml VEGF in the presence of increasing concentrations of SCH 221153 or SCH 216687, and cells were counted 24 h thereafter. As shown in Fig. 5*A*, SCH 221153 inhibits the mitogenic activity of FGF2 and VEGF in a dose-dependent manner with an IC_{50} equal to 3–10 μM, whereas SCH 216687 was ineffective. The linear integrin-binding peptide GRGDSPK, but not the negative control peptide GRADSPK (Fig. 5*A*), exerted a similar FGF2-antagonist activity.

When tested at a fixed dose equal to 30 μM, SCH 221153 retained its full antagonist activity in the presence of increasing concentrations of FGF2 as high as 300 ng/ml, thus indicating that its mechanism of action is not competitive (data not shown). Accordingly, SCH 221153 did not affect the binding of ^{125}I-FGF2 to low-affinity heparin sulfate proteoglycans and high-affinity tyrosine kinase receptors in GM7373 cells.[4]

To assess its specificity of action, SCH 221153 was tested for the capacity to affect GM7373 cell proliferation triggered by different stimuli. Under the same experimental conditions, SCH 221153 inhibits the mitogenic activity exerted by FGF2, but not that exerted by FCS, epidermal growth factor, 12-*O*-tetradecanoyl phorbol 13-acetate, or 1,2-dioctanoyl-sn-glycerol (Fig. 5*B*). Again, SCH 216687 was ineffective on all of the mitogenic stimuli investigated.

In agreement with the data obtained in the short-term proliferation assay, SCH 221153 treatment resulted in dose-dependent inhibition of HUVEC proliferation in a long-term assay. Again, SCH 221153 inhibited the mitogenic activity of FGF2 with an IC_{50} equal to 3–10 μM, whereas SCH 216687 was ineffective (Fig. 6*A*). SCH 221153 retained its antagonist activity when HUVECs were stimulated with FGF2 in the presence of $VEGF_{165}$ (Fig. 6*B*).

Inhibition of FGF2-induced Angiogenesis in Chick CAM Assay by SCH 221153. To assess whether SCH 221153 was able to exert an anti-angiogenic activity *in vivo*, we tested SCH 221153 in the chick embryo CAM assay using FGF2 as an angiogenic stimulus. In this assay, chick embryos 10 days of age were used to induce angiogenesis in the CAM using filter discs soaked with FGF2. At least 10 embryos were used for each group in each experiment. After 24 h, either PBS or different concentrations of SCH 221153 were applied topically to the surface of the filter discs. After 48 h, the CAMs were dissected out and the representative areas were photographed. The blood vessel branch points present within the area defined by the filter disc were counted in a blinded fashion using a high power stereo microscope. As shown in Fig. 7*a* and *b*, SCH 221153 was effective in blocking FGF2-induced angiogenesis in a dose-dependent manner with an IC_{50} equal to 100 ng/implant. Interestingly, SCH 221153 had very little effect on preexisting blood vessels of the CAM, indicating that the compound exerts a specific action on proliferating microvessels (Fig. 7*a*).

Inhibition of Tumor Growth *in Vivo* by SCH 221153. Human melanoma LOX cells express very low levels of $\alpha_v\beta_5$. Accordingly, SCH 221153 does not affect their proliferative capacity *in vitro* when

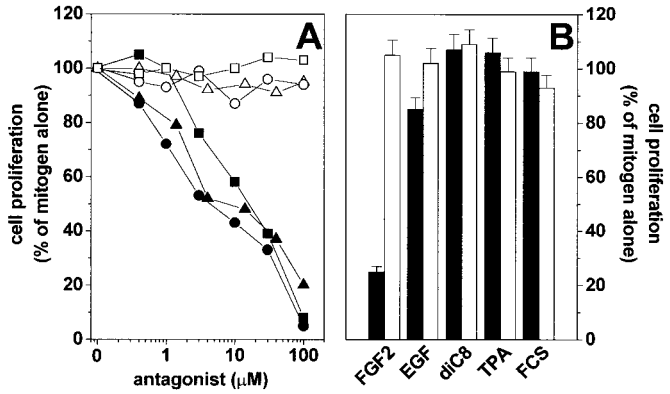


Fig. 5. Effect of SCH 221153 on endothelial GM 7373 cell proliferation. *A*, GM 7373 cells were incubated with 10 ng/ml FGF2 plus 0.4% FCS in the absence or in the presence of increasing concentrations of SCH 221153 (●), SCH 216687 (○), GRGDSPK (▲), or GRADSPK (△). Parallel cultures were incubated with 30 ng/ml VEGF plus 0.4% FCS in the presence of increasing concentrations of SCH 221153 (■) or SCH 216687 (□). *B*, GM 7373 cells were incubated with 0.4% FCS supplemented with 30 ng/ml FGF2, 30 ng/ml epidermal growth factor, 5 μg/ml 1,2-dioctanoyl-sn-glycerol, 10 ng/ml 12-*O*-tetradecanoyl phorbol 13-acetate, or 10% FCS in the absence or in the presence of 30 μM GRGDSPK (*black bar*) or GRADSPK (*open bar*). All cultures were trypsinized and counted 24 h after the beginning of treatment. The data are expressed as a percentage of the proliferation observed in cultures treated with the mitogen alone.

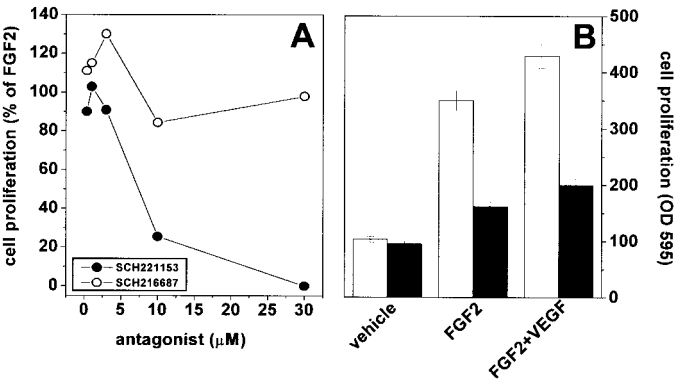


Fig. 6. Effect of SCH 221153 on HUVEC proliferation. *A*, HUVEC cells grown in 96-well plates were incubated with 10 ng/ml FGF2 in the absence or in the presence of increasing concentrations of SCH 221153(●) or SCH 216687(○). *B*, HUVEC cells were incubated with vehicle, 10 ng/ml FGF2, or 10 ng/ml FGF2 plus 30 ng/ml VEGF in the absence (*black bar*) or in the presence of 30 μM SCH 221153 (*open bar*). After 6 days, cells were stained with crystal violet and plates were read with a microplate reader at 595 nm. In *A*, data are expressed as the percentage of proliferation observed in cell cultures treated with FGF2 alone.

[4] M. Presta, unpublished observations.

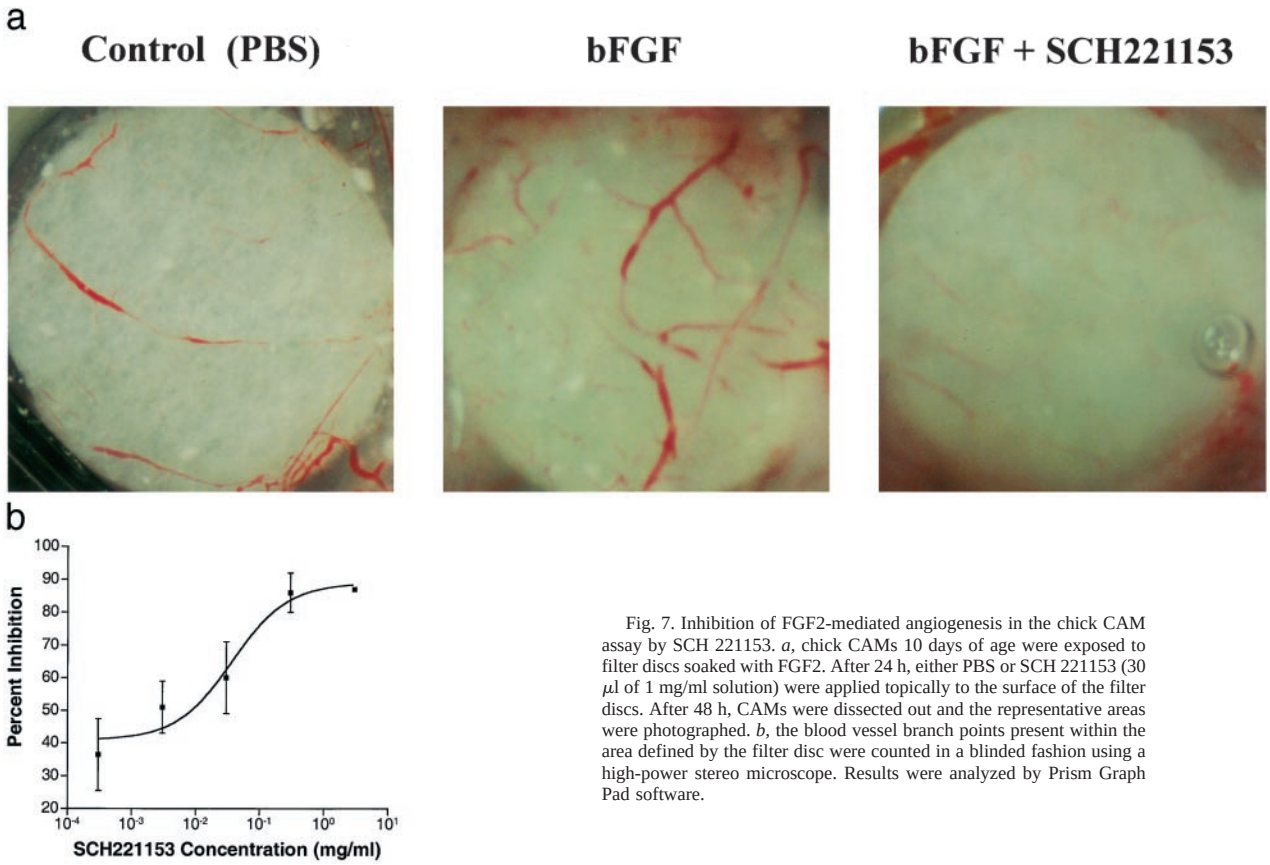


Fig. 7. Inhibition of FGF2-mediated angiogenesis in the chick CAM assay by SCH 221153. *a*, chick CAMs 10 days of age were exposed to filter discs soaked with FGF2. After 24 h, either PBS or SCH 221153 (30 μl of 1 mg/ml solution) were applied topically to the surface of the filter discs. After 48 h, CAMs were dissected out and the representative areas were photographed. *b*, the blood vessel branch points present within the area defined by the filter disc were counted in a blinded fashion using a high-power stereo microscope. Results were analyzed by Prism Graph Pad software.

tested at doses up to 30 μM.[5] On this basis, this cell line was chosen to assess the efficacy of SCH 221153 in inhibiting tumor growth in mice by acting as an anti-angiogenic agent. In one study, LOX cells were inoculated into SCID mice intradermally on day 0. Starting on day 1, SCH 221153 was administered through i.p. injection twice daily at various doses for 15 days. SCH 221153 was dissolved in 20% (w/v) HPβCD, and mice in the Vehicle Control group received 20% HPβCD. Each tumor was measured in three dimensions on days 8, 11, and 15. As shown in Fig. 8*a*, LOX tumor cells injected intradermally reached a maximum average tumor volume of 130 mm^3 on day 15, and treatment of mice with SCH 221153 resulted in dose-dependent inhibition of tumor growth with 71% inhibition being observed at 20 mpk. Increasing the dosage to 50 mpk body weight did not result in any additional increase in the inhibition of tumor growth. The skin sections surrounding the tumors were photographed to visualize the vasculature around the tumors. As shown in Fig. 8*c*, new blood vessel growth surrounding the tumors was significantly inhibited in animals dosed with SCH 221153 (50 mpk).

In the second study, LOX tumor cells injected s.c. into SCID mice grew to an average size of 1500 mm^3, about 10 times the size of tumors obtained in intradermal tumor models. Nevertheless, treatment of animals with SCH 221153 resulted in dose-dependent inhibition of tumor growth also under these experimental conditions. Again, the compound caused 68% and 71% inhibition of tumor growth when given at 20 and 50 mpk, respectively. No weight loss or any noticeable adverse effects were observed in SCH 221153-treated animals compared with control animals.

[5] Unpublished observations.

DISCUSSION

Inhibition of tumor-induced angiogenesis has been shown to suppress tumor growth in animal models, and a number of anti-angiogenic factors are currently being tested in clinical trials (2, 3). For many of these agents, the precise molecular target(s) has not been defined. Antagonists of integrin receptors $\alpha_v\beta_3$ and $\alpha_v\beta_5$ would represent a class of molecules with a precise molecular target(s) and a clear understanding of the mechanism of action (14). Antagonists of $\alpha_v\beta_3$, such as cRGDfV, humanized monoclonal antibody Vitaxin, and a number of peptidomimetic small-molecular-weight compounds are under investigation as anti-angiogenesis agents (29). These antagonists have been shown to induce the apoptosis of proliferating endothelial cells expressing $\alpha_v\beta_3$ without an apparent effect on normal, nonproliferating endothelium. Additionally, it has been demonstrated recently that in lymphocytes and MCF-7 cells, induction of apoptosis by RGD peptides is by direct activation of caspase-3 (30). Furthermore, it has been shown that exposure of endothelial cells to TNF-α and IFN-γ caused selective inhibition of integrin $\alpha_v\beta_3$-dependent cell adhesion and survival *in vitro* (31). Administration of TNF-α and IFN-γ to melanoma patients induced detachment and apoptosis of $\alpha_v\beta_3$-positive endothelial cells of tumor vasculature *in vivo* (31). These results implicate integrin $\alpha_v\beta_3$ in the antivascular activity of TNF-α and IFN-γ and demonstrate a new mechanism by which cytokines control cell adhesion via integrin receptors.

In this study, we have characterized an RGD peptidomimetic, SCH 221153, that is a potent inhibitor of integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ and exhibits selectivity against related $\alpha_{IIb}\beta_3$ and $\alpha_5\beta_1$ receptors. We have shown that SCH 221153 inhibits adhesion of 293-$\alpha_v\beta_3$ and 293-$\alpha_v\beta_5$ cells and endothelial cells to ECM proteins and to FGF2. In addition, we have shown that SCH 221153, but not an inactive derivative SCH 216687, is capable of inhibiting FGF2 and VEGF-induced proliferation of bovine

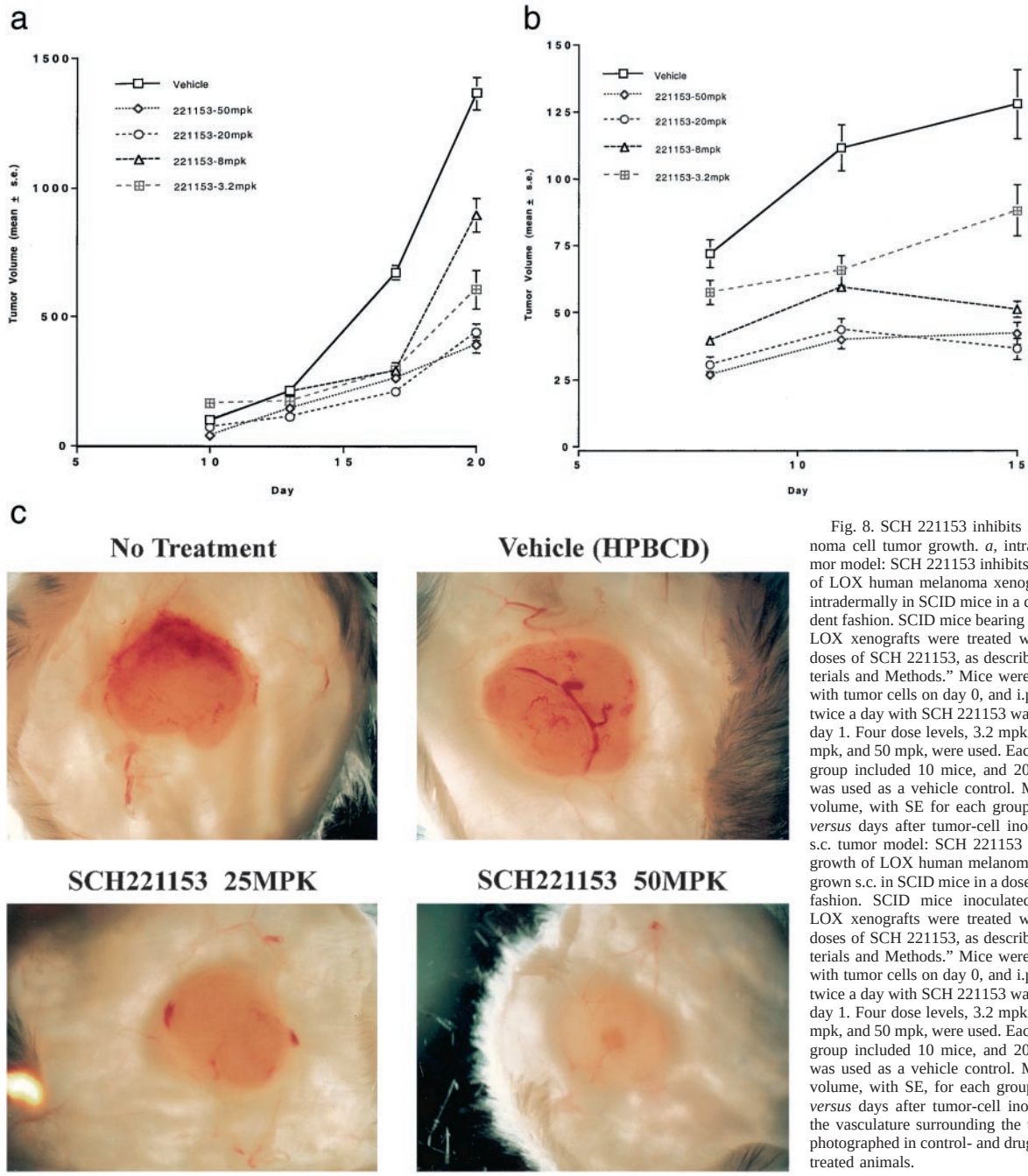


Fig. 8. SCH 221153 inhibits LOX melanoma cell tumor growth. *a*, intradermal tumor model: SCH 221153 inhibits the growth of LOX human melanoma xenograft grown intradermally in SCID mice in a dose-dependent fashion. SCID mice bearing intradermal LOX xenografts were treated with various doses of SCH 221153, as described in "Materials and Methods." Mice were inoculated with tumor cells on day 0, and i.p. treatment twice a day with SCH 221153 was started on day 1. Four dose levels, 3.2 mpk, 8 mpk, 25 mpk, and 50 mpk, were used. Each treatment group included 10 mice, and 20% HPβCD was used as a vehicle control. Mean tumor volume, with SE for each group, is plotted *versus* days after tumor-cell inoculation. *b*, s.c. tumor model: SCH 221153 inhibits the growth of LOX human melanoma xenograft grown s.c. in SCID mice in a dose-dependent fashion. SCID mice inoculated s.c. with LOX xenografts were treated with various doses of SCH 221153, as described in "Materials and Methods." Mice were inoculated with tumor cells on day 0, and i.p. treatment twice a day with SCH 221153 was started on day 1. Four dose levels, 3.2 mpk, 8 mpk, 25 mpk, and 50 mpk, were used. Each treatment group included 10 mice, and 20% HPβCD was used as a vehicle control. Mean tumor volume, with SE, for each group is plotted *versus* days after tumor-cell inoculation. *c*, the vasculature surrounding the tumors was photographed in control- and drug- (50 mpk) treated animals.

and human aortic endothelial cells. SCH 221153 also inhibited FGF2-induced angiogenesis in the CAM assay system and blocked the growth of LOX melanoma tumors in mouse xenograft models. These studies are in agreement with previous observations on the capacity of antibody and RGD-based peptide antagonists of integrin $\alpha_v\beta_3$ to inhibit angiogenesis on the chick CAM, leading to regression of human tumors (12–14). Furthermore, an antibody against $\alpha_v\beta_3$ blocked human breast cancer growth and angiogenesis in a nude mouse/human skin chimera model (13). In an extension of these studies, it was discovered that FGF2 and VEGF activate two angiogenic pathways mediated by integrin $\alpha_v\beta_3$ and $\alpha_v\beta_5$, respectively (22). In both the rabbit corneal eye pocket and the chick CAM assays, anti-$\alpha_v\beta_3$ monoclonal antibody blocked FGF2-induced angiogenesis, whereas anti$\alpha_v\beta_5$ antagonists blocked VEGF-induced angiogenesis (17). The biological significance of these distinct angiogenic pathways is unknown. VEGF is a potent mitogen and angiogenic agent secreted by several tumors and plays an important role in eliciting tumor-induced angiogenesis (32). Recent studies have shown that integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ are expressed by microvascular endothelium of high risk neuroblastomas, and their inhibition is associated with increased endogenous ceramide production, which may contribute to endothelial cell death (33). These studies clearly indicate that it may be necessary to inhibit both of these integrins to block angiogenesis *in vivo*. Here, we describe a dual antagonist of these integrins and demonstrate its *in vivo* efficacy in inhibiting angiogenesis and tumor growth.

Integrin selectivity was considered important because integrins, in general, bind to the RGD motif present in a number of ECM proteins. Of particular concern is the platelet fibrinogen receptor, $\alpha_{IIb}\beta_3$. Integrin $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ are related, in that they share a common β_3 subunit. More importantly, $\alpha_{IIb}\beta_3$ is a key player of platelet aggregation, and antagonists of $\alpha_{IIb}\beta_3$ may cause unwanted bleeding prob-

lems. Medicinal chemistry efforts produced SCH221153, which is 700-fold more selective toward $\alpha_v\beta_3$ compared with $\alpha_{IIb}\beta_3$.

Evidence that inhibition of tumor growth by SCH 2211153 is indirect, presumably via inhibition of angiogenesis comes from two observations. First, the melanoma-derived LOX cells express very low levels of the $\alpha_v\beta_3$-receptor, ruling out the possibility of direct effect of SCH 221153 on tumor cells. Indeed, SCH 221153 has no effect on LOX cell proliferation *in vitro*. Second, previous studies have shown that integrin $\alpha_v\beta_3$-antagonists inhibit angiogenesis in different *in vivo* assays (12–14). Accordingly, SCH 221153 inhibits FGF2-induced endothelial cell proliferation *in vitro* and angiogenesis in the *in vivo* CAM assay. Visualization of vasculature surrounding the LOX tumors in the *in vivo* efficacy study indicates that SCH 221153 causes a significant decrease in the number of blood vessels surrounding the xenograft tumors (Fig. 8*c*). Analysis of the pharmacokinetic profile of SCH 221153 in mice and rats indicated that this compound has a short half-life of about 12 min and is cleared rapidly from blood.[6] At 20-mpk doses administered twice daily in mice, the effective concentration required to inhibit cell attachment to matrix is maintained for only 4 h subsequent to i.p. dosing in mice. This analysis suggests that continuous exposure of newly synthesized endothelial cells to the antagonist is not required to inhibit endothelial cell adhesion and the formation of new blood vessels.

A number of integrin $\alpha_v\beta_3$ antagonists are being developed for use as angiogenesis inhibitors. These include a humanized form of anti-$\alpha_v\beta_3$ monoclonal antibody LM609 (Vitaxin), cRGD peptides, and synthetic RGD mimetics (34), (35). Small molecular weight compounds with oral bioavailability will have a number of advantages over antibody-based approaches. Whereas most of these compounds are targeted specifically toward the $\alpha_v\beta_3$ receptor, it is clear that the $\alpha_v\beta_5$ receptor also plays a critical role, and dual antagonists of both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ would have a definite therapeutic advantage. SCH 221153 is a small-molecular-weight compound that can inhibit both $\alpha_v\beta_3$- and $\alpha_v\beta_5$-receptors, and thus represents a class of molecules that would have a distinct advantage over $\alpha_v\beta_3$-specific antagonists. Studies by others have shown that $\alpha_v\beta_3$ plays a critical role in osteoclast-mediated bone resorption, and both anti-$\alpha_v\beta_3$ monoclonal antibodies and RGD peptides inhibit bone resorption both *in vitro* and *in vivo* models (36). Peptidomimetic antagonists of $\alpha_v\beta_3$ are also being developed for use in osteoporosis indication (34, 35). The combination of anti-angiogenic, antitumor, and anti-bone resorptive activities in a single pharmacological agent offers significant therapeutic opportunities.

ACKNOWLEDGMENTS

The authors thank Chiara Urbinati and Mirella Belleri for their skillful technical assistance.

REFERENCES

1. Folkman, J. The role of angiogenesis in tumor growth. Semin. Cancer Biol., *3:* 65–71, 1992.
2. Brower, V. Tumor angiogenesis—new drugs on the block. Nat. Biotechnol. (Lond.), *17:* 963–968, 1999.
3. Kumar, C. C., and Armstrong, L. Tumor-induced angiogenesis: a novel target for drug therapy? *In:* Emerging Drugs: The Prospects for New Medicines, pp. 175–190, 1997.
4. Varner, J. A., and Cheresh, D. A. Tumor angiogenesis and the role of vascular cell integrin $\alpha v\beta 3$. *In:* V. T. DeVita, S. Hellman, and S. A. Rosenberg (eds.), Important Advances in Oncology. Philadelphia: Lippincott-Raven Publishers, 1996.
5. Ruoslahti, E. RGD and other recognition sequences for integrins. Annu. Rev. Cell Dev. Biol., *12:* 697–715, 1996.
6. Varner, J. A., Brooks, P. C., and Cheresh, D. A. Review: the integrin $\alpha v\beta 3$: angiogenesis and apoptosis. Cell Adhes. Commun., *3:* 367–374, 1995.
7. Rusnati, M. T. E., Dell'Era, P., Gualandris, A., and Presta, M. $\alpha v\beta 3$ integrin mediates the cell-adhesive capacity and biological activity of basic fibroblast growth factor (FGF2) in cultured endothelial cells. Mol. Biol. Cell, *8:* 2449–2461, 1997.
8. Brooks, P. C., Stromblad, S., Sanders, L. C., von Schalscha, T. L., Aimes, R. T., Stetlet-Stevenson, W. G., Quigley, J. P., and Cheresh, D. A. Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin $\alpha v\beta 3$. Cell, *85:* 683–693, 1996.
9. Giancotti, F, G., and Ruoslahti, E. Integrin signaling. Science (Washington DC), *285:* 1028–1032, 1999.
10. Kumar, C. C. Signaling by integrin receptors. Oncogene, *17:* 1365–1373, 1998.
11. Stromblad, S., Becker, J. C., Yebra, M., Brooks, P. C, and Cheresh, D. A. Suppression of p53 activity and p21WAF1/CIP1 expression by vascular cell integrin $\alpha v\beta 3$ during angiogenesis. J. Clin. Investig., *98:* 426–433, 1996.
12. Brooks, P. C., Clark, R. A. F., and Cheresh, D. A. Requirement of vascular integrin $\alpha v\beta 3$ for angiogenesis. Science (Washington DC), *264:* 569–571, 1994.
13. Brooks, P. C., Stromblad, S., Klemke, R., Visscher, D., Sarkar, F., and Cheresh, D. Antiintegrin $\alpha v\beta 3$ blocks human breast cancer growth and angiogenesis in human skin. J. Clin. Investig., *96:* 1815–1822, 1995.
14. Eliceiri, B. P., and Cheresh, D. A. The role of integrins during angiogenesis: insights into potential mechanisms of action and clinical development. J. Clin. Investig., *103:* 1227–1230, 1999.
15. Shattil, S. J. Function and regulation of the $\beta 3$ integrins in hemostasis and vascular biology. Thromb. Haemost., *74:* 149–155, 1995.
16. Cheresh, D. A. Structure, function and biological properties of integrin $\alpha v\beta 3$ on human melanoma cells. Cancer Metastasis Rev., *10:* 3–10, 1991.
17. Friedlander, M., Theesfeld, C. T., Sugita, M., Fruttiger, M., Thomas, M. A ., Chang, S., and Cheresh, D. A. Involvement of integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ in ocular neovascular diseases. Proc. Natl. Acad. Sci. USA, *93:* 9764–9769, 1995.
18. Sipkins, D. A., Cheresh, D. A., Kazemi, M. R., Nevin, L. M., Bednarski, M. D., and Li, K. C. Detection of tumor angiogenesis *in vivo* by $\alpha v\beta 3$-targeted magnetic resonance imaging. Nat. Med., *4(5):* 623–626, 1998.
19. Albelda, S., M., Mette, S. A., Elder, D. E., Steward, R., Danjanovich, L., Herlyn, M., and Buck, C. A. Integrin distribution in malignant melanoma: association of the $\beta 3$ subunit with tumor progression. Cancer Res., *50:* 6757–6764, 1990.
20. Petitclerc, E., Stromblad, S., von Schalsck, T. L., Mitjans, F., Piulats, J., Montgomery, A. M. P., Cheresh, D. A., and Brooks, P. A. Integrin $\alpha v\beta 3$promotes M21 melanoma growth in human skin by regulating tumor cell survival. Cancer Res., *59:* 2724–2730, 1999.
21. Gladson, C., and Cheresh, D. A. Glioblastoma expression of vitronectin and $\alpha v\beta 3$ integrin. J. Clin. Investig., *88:* 1924–1932, 1991.
22. Friedlander, M., Brooks, P. C., Shaffer, R. W., Kincaid, C. M., Varner, J. A., and Cheresh, D. A. Definition of two angiogenic pathways by distinct αv integrins. Science (Washington DC), *270:* 1500–1502, 1995.
23. Elicieri, B. P., Paul, R., Schwartzberg, P. L., Hood, J. D., Leng, J., and Cheresh, D. A. Selective requirement for Src kinases during VEGF-induced angiogenesis and vascular permeability. Mol. Cell. Biol., *4:* 915–924, 1999.
24. Isacchi, A., Statuto, M., Chiesa, R., Bergonzoni, L., Rusnati, M., Sarmientos, P., Ragnotti, G., and Presta, M. A six amino acid deletion in basic fibroblast growth factor dissociates its mitogenic activity from its plasminogen activator-inducing capacity. Proc. Natl. Acad. Sci. USA, *88:* 2628–2632, 1991.
25. Kumar, C. C., Nie, H., Rogers, C. P., Malkowski, M., Maxwell, E., Catino, J. J., and Armstrong, L. Biochemical characterization of the binding of echistatin to integrin $\alpha v\beta 3$ receptor. J. Pharmacol. Exp. Ther., *283:* 843–853, 1997.
26. Grinspan, J. B., Mueller, S. N., and Levine, E. M. Bovine endothelial cells transformed *in vitro* with benzo(*a*)pyrene. J. Cell. Physiol., *114:* 328–338, 1983.
27. Presta, M., Maier, J. A. M., and Ragnotti, G. The mitogenic signaling pathway but not the plasminogen activator-inducing pathway of basic fibroblast growth factor is mediated through protein kinase C in fetal bovine aortic endothelial cells. J. Cell Biol., *109:* 1877–1884, 1989.
28. Liu, M., Bryant, M. S., Chen, J., Lee, S., Yaremko, B., Lipari, P., Malkowski, M., Ferrari, E., Nielsen, L., Prioli, N., Dell, J., Sinha, D., Syed, J., Korfmacher, W. A., Nomeir, A. A., Lin, C. C., Wang, L., Taveras, A. G., Doll, R. J., Njoroge, F. G., Mallams, A. K., Remiszewski, S., Catino, J. J., Girijavallabhan, V. M., and Bishop, W. R. Antitumor activity of SCH 66336, an orally bioavailable tricyclic inhibitor of farnesyl protein transferase, in human tumor xenograft models and wap-*ras* transgenic mice. Cancer Res., *58:* 4947–4956, 1998.
29. Kerr, J. S., Slee, M. A., and Mousa, S. A. Small molecule αv integrin antagonists: novel anticancer agents. Expert Opin. Investig. Drugs, *9:* 1271–1279, 2000.
30. Buckley, C. D., Pilling, D., Henriquez, G., Threlfall, K., Scheel-Toellner., Simmons, D. L., Akbar, A. N., Lord, J. M., and Salmon, M. RGD peptides induce apoptosis by direct caspase-3 activation. Nature (Lond.), *397:* 534–539, 1999.
31. Ruegg, C., Yilmaz, A., Bieler, G., Bamat, J., Chaubert, P., and Lejeune, J. F. Evidence for the involvement of endothelial cell integrin $\alpha v\beta 3$ in the disruption of the tumor vasculature induced by TNF and IFN-γ. Nat. Med., *4:* 408–414, 1998.
32. Thomas, K. A. Vascular endothelial growth factor, a potent and selective angiogenic agent. J. Biol. Chem., *271:* 603–606, 1996.
33. Erdreich-Epstein, A., Shimada, H., Groshen, S., Liu, M., Metelitsa, L. S., Kim, K., Stins, M. F., Seeger, R. C., and Durden, D. L. Integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ are expressed by endothelium of high-risk neuroblastoma and their inhibition is associated with increased endogenous ceramide. Cancer Res., *60:* 712–721, 2000.
34. Miller, W. H., Bondinell, W. E., Cousins, R. D., Erhard, K. F., Jakas, D. R., Keenan, R. M., Ku, T. W., Newlander, K. A., Ross, S. T., Haltiwanger, R. C., *et al.* Orally bioavailable nonpeptide vitronectin receptor antagonists with efficacy in an osteoporosis model. Bioorg. Med. Chem. Lett., *9:* 1807–1812, 1999.
35. Miller, W. H., Alberts, D. P., Bhatnagar, P. K., Bondinell, W. E., Callahan, J. F., Calvo, R. R., Cousins, R. D., Erhard, K. F., Heerding, D. A., Keenan, R. M., *et al.* Discovery of orally active nonpeptide vitronectin receptor antagonists based on a 2-benzazepine Gly-Asp mimetic. J. Med. Chem., *43:* 22–26, 2000.
36. Hartman, G. D., and Duggan, M. E. $\alpha v\beta 3$ integrin antagonists as inhibitors of bone resorption. Expert Opin. Investig. Drugs, *9:* 1281–1291, 2000.

[6] Unpublished observations.

Inhibition of Angiogenesis and Tumor Growth by SCH221153, a Dual $\alpha_v\beta_3$ and $\alpha_v\beta_5$ Integrin Receptor Antagonist

C. Chandra Kumar, Michael Malkowski, Zizhang Yin, et al.

Cancer Res 2001;61:2232-2238.