

of VEGFR1 with the third Ig domain of VEGFR2. All of the VEGF-Trap variants were produced and purified from Chinese hamster ovary cells.

Pharmacokinetic Analysis of VEGF-Traps. BALB/c mice (25–30 g) were injected s.c. with 4 mg/kg of the various Traps and bled at 1, 2, 4, 6, 24, 48, 72, and 144 hr after injection. Levels of all VEGF-Traps were measured by an ELISA by using human VEGF₁₆₅ to capture and an antibody to the human Fc region as the reporter.

Extracellular Matrix (ECM)-Binding Assay. ECM-coated plates (Becton Dickinson no. 35–4607) were incubated with varying concentrations of VEGF-Traps for 1 hr at room temperature. They were washed and incubated with alkaline phosphatase-conjugated anti-human Fc antibody (Promega, 1:4,000 in PBS + 10% BCS) for 1 hr at room temperature. Plates were washed four times with PBS + 0.1% Triton-X 100 and reagent buffer added for color development. Plates were read at 405–570 nm.

VEGF-Trap-Binding Assay. Binding affinities of VEGF-Traps were measured by using a specific and sensitive ELISA (R&D Systems kit no. DVE00) for detecting free (unbound) human VEGF in mixtures of the VEGF-Traps (ranging in concentration from 0.1 to 160 pM) with human VEGF₁₆₅ (at 10 pM), incubated overnight at room temperature.

Human Umbilical Vein Endothelial Cell Phosphorylation Assay. Confluent monolayers of human umbilical vein endothelial cells [Vec Technologies (Rensselaer, NY) passage no. 5] were serum-starved for 2 hr and then challenged for 5 min with vehicle or 40 ng/ml of human VEGF₁₆₅, alone or preincubated with VEGF-Traps at 1.5-fold molar excess. Cells were then lysed, immunoprecipitated by using a VEGFR2-specific antibody, and immunoblotted with a phosphotyrosine-specific antibody (Upstate Biotechnology, 4G10 mAb).

VEGF-Induced Proliferation Assay. Cells that proliferate in response to VEGF were generated by stably transfecting NIH 3T3 cells with a VEGFR2/TrkB chimeric receptor (in which the cytodomain of VEGFR2 was replaced with that of TrkB, a receptor for brain-derived neurotrophic factor that effectively drives proliferation in these cells). Five thousand cells were plated per well of a 96-well plate, allowed to settle for 2 hr, incubated for 1 hr with VEGF-Trap variants (titrated from 40 nM to 20 pM), then challenged for 72 hr with human VEGF₁₆₅ at a concentration of 1.56 nM, followed by addition of [3-(4,5 dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, innersai and spectrophotometric analysis at 450/570 nm.

Acute Hypotension. Male Wistar-Kyoto rats (180–240 g) from Taconic Laboratories were maintained on a 12:12 light/dark cycle (lights on 0600) with food and water available *ad libitum*. Before challenge with VEGF, animals were pretreated with VEGF-Traps or PBS as indicated, anesthetized with 1.5–2% isoflurane in oxygen, and the left femoral artery catheterized for direct measurement of systolic blood pressure through a blood pressure transducer (IITC, Woodland Hills, CA) into a chart recorder (Linseis, Princeton Junction, NJ). Animals were then injected in the right jugular vein with a 200- μ l bolus containing 10 μ g of recombinant human VEGF₁₆₅. Systolic blood pressure was measured before VEGF injection and every minute thereafter for 20 min. Blood pressures were normalized to baseline preinjection and analyzed by using mixed factorial ANOVAs (see supporting information on the PNAS web site, www.pnas.org).

Tumor Growth Experiments. C6 glioma cells (1.0×10^6 cells/mouse) and A673 rhabdomyosarcoma cells (2.0×10^6 cells/mouse) were

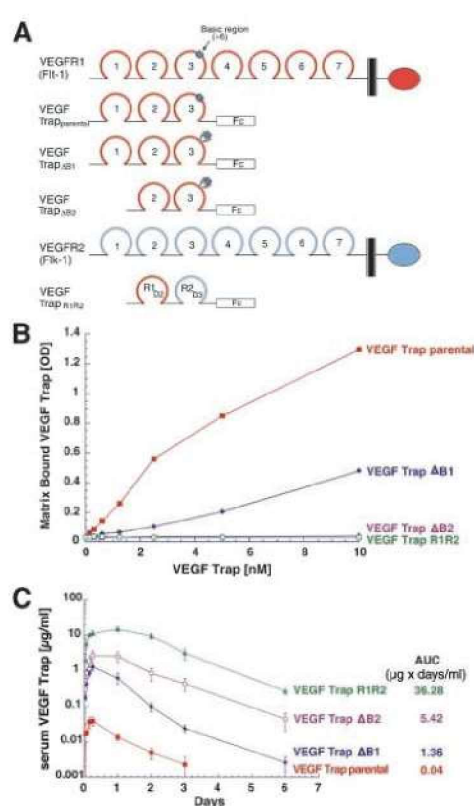


Fig. 1. Engineering of VEGF-Traps with improved pharmacokinetics. (A) Schematics of full-length VEGFR1 (red) and VEGFR2 (blue) are provided, indicating their seven Ig domains, transmembrane regions (black bars), and kinase domains (ovals). The parental VEGF-Trap contains the first three Ig domains of VEGFR1 (including the highly basic 10-aa stretch in Ig3, blue box) fused to the Fc portion of human IgG1. VEGF-Trap Δ B1 is identical to the parental VEGF-Trap, except that the basic stretch in Ig3 has been removed. VEGF-Trap Δ B2 is the same construct as Δ B1, except that the first Ig domain has been removed. VEGF-Trap Δ R1R2 possesses the second Ig domain of VEGFR1 and the third Ig domain of VEGFR2 fused to the Fc portion of human IgG1. (B) The four indicated VEGF-Traps were assayed *in vitro* for their capacity to bind to extracellular matrix, with only the parental VEGF-Trap and VEGF-Trap Δ B1 demonstrating binding. (C) Pharmacokinetic analysis of the VEGF-Traps reveals that the parental VEGF-Trap has the poorest profile, whereas VEGF-Trap Δ R1R2 showed the best profile.

obtained from American Type Culture Collection, and B16F10.9 melanoma cells (5.0×10^5 cells/mouse) were a generous gift from Charles Lin (Duke University, Durham, NC). Cells were suspended in serum-free medium and implanted s.c. on the shaved right flank of male C.B-17 SCID mice at the indicated concentrations. After tumor cell implantation and twice weekly thereafter for the duration of the experiment, mice received a s.c. injection (at the nape of the neck) of vehicle (PBS + 0.5% glycerol), VEGF-Trap, or DC101 (from American Type Culture Collection). After 2–3.0 weeks, animals were killed and tumors were measured *ex vivo* with calipers (tumor volume = length $\times$ width $\times$ height). For immunohistochemistry studies, mice were perfused with 4% paraformaldehyde, and tissue was processed as previously described (25).

Results

Reengineering Parental VEGF-Trap to Improve Its Pharmacokinetic Profile. On the basis of the previously reported high affinity of a soluble decoy receptor in which VEGFR1 is fused to the Fc portion of human IgG1 (16, 17), we produced this fusion protein to study its properties (see parental VEGF-Trap, Fig. 1A). Single s.c. injections of parental VEGF-Trap (4 mg/kg) into mice were