Supporting Text

Methods and Materials

Whole Blood Sunitinib Treatment

Blood from Balb/C mice was collected, pooled, and then aliquoted into Vacutainer (Becton Dickinson. Franklin Lakes, NJ) tubes containing K_2 EDTA (BD catalog no. 367861) to avoid clotting. After addition of sunitinib, samples were incubated at 4°C with gentle rotation for 24 h. Aliquots of each blood sample were then placed in red blood cell lysis buffer (0.155 M NH_4Cl/0.01 M $NaCO_3$/0.14 mM EDTA) supplemented with 7-amino-actinomycin D (7AAD) for 10 min, resuspended in FACS buffer (0.01 M EDTA/0.2 g of BSA wt/vol), and analyzed on a FACSCalibur (Becton Dickinson) to determine the apoptotic fraction. No significant differences were seen in cell viability between treatments (data not shown). Remaining blood samples were centrifuged at 4°C in Microtainer plasma separating tubes for plasma analysis.

Animals and Cell Lines

$PC3^{VEGF\text{-}HIGH}$ tumors were grown until they almost reached the institutionally allowed endpoint (1,700 mm^3) when a 7-day course treatment with sunitinib was initiated at varying doses. This duration of drug treatment was chosen to ensure a period sufficient for drug target inhibition (8) yet not sufficient to produce appreciable size-related antitumor effects. For confirmation of this latter point, daily measurements of tumors were taken, and no statistical difference in tumor size was seen between treatment groups (data not shown). Blood was drawn 24 h after final treatment (see description below), and mice were killed with tumors excised and weighed. For the measurement of plasma proteins in tumor-bearing mice, values were standardized to the tumor burden of the individual mouse by dividing levels of plasma VEGF (pg/ml) by total weight of the excised s.c. tumor (grams), yielding the units of “pg/ml/g” for comparison.

ELISA Analysis

Commercially available mouse sandwich ELISA assays were used with the following protocol modifications: for mouse VEGF, a 1:2 dilution was made instead of the recommended 1:5; for mouse sVEGFR-2, a 1:20-fold dilution was made instead of the recommended 1:15-fold. Mouse PlGF has only one isoform, corresponding to the human PlGF-2, and therefore mouse ELISA kit measurement is for PlGF-2 (9). For simplicity, mouse PlGF-2 is referred throughout the manuscript as "PlGF." Average plasma levels for vehicle-treated Balb/C mice used as controls for this study were the following: VEGF (59.63 ± 32.40 pg/ml), sVEGFR-2 (68.54 ± 13.48 ng/ml), PlGF (12.98 ± 3.6 pg/ml), SDF-1α (525.85 ± 124.15 pg/ml), OPN (68.01 ± 6.62 ng/ml) SCF (137.26 ± 68.33 pg/ml), IL-6 (8.64 ± 2.93 pg/ml), PDGF-AB (31.06 ± 55.47 pg/ml), PDGF-BB (12.8 ± 11.40 pg/ml), EPO (15.93 ± 10.55 pg/ml), sTIE-2 (1,370.89 ± 10.55 pg/ml) and G-CSF (67.61 ± 23.31 pg/ml).

Cell lysis and Protein Extraction

Tissues were homogenized with plastic pestle and then, following a 1-h incubation at 4°C, centrifuged at 15,000 rpm for 10 min and supernatant removed. For nuclear extraction, tissue fragments were fractionated into cytosol, membranes, nuclei, and cytoskeleton components using the ProteoExtract Subcellular Proteome Extraction Kit (CalBiochem) following the manufacturer's protocol. Protein lysates were quantified by using Bradford reagent (BioRad, Hercules, CA) and standardized by using known amounts of BSA (Gibco, New York).

Western Blotting

Proteins were resolved using 7.5% SDS/PAGE gels and blotted onto polyvinylidene difluoride membranes (Immobilon-P, Millipore Nepean, ON, Canada) by electrophoretic transfer (Hoefer Pharmacia Biotech, San Francisco, CA). Before immunoblotting, membranes were blocked in 10% nonfat milk in TBS-T buffer (10 mM Tris, pH 7.5/150

mM NaCl/0.1% Tween 20) and subjected to immunoblot analysis as described (10). Antibodies used for immunoblotting include: anti-HIF-1α mouse monoclonal IgG_{2b} (NB100-105; Novus Biologicals); anti-HIF-1β (NB 100-110, Novus Biologicals); anti-VEGFR-2 goat polyclonal antibody conjugated to horseradish peroxidase (HRP) was used as described (11) (R&D Systems, Inc., Minneapolis, MN); anti-β-actin (Clone AC-15; Sigma-Aldrich, St. Louis, MO); secondary antibodies used included anti-rabbit IgG HRP and anti-mouse IgG HRP (Promega, Madison, WI).

Mouse Angiogenesis Antibody Array

The Transignal Mouse Angiogenesis Array (Panomics, Redwood City, CA) was used according to the manufacturer's protocol for the detection of epidermal growth factor (EGF), fibroblast growth factor alpha and beta (FGFα, FGFβ), G-CSF, interleukins 1a, 1b, 4, 6, 12 (IL-1a, IL-1b, IL-4, IL-6, IL-12), leptin, tumor necrosis factor alpha (TNFα), transforming growth factor alpha and beta (TGF-α, TGF-β), IFN-γ (IFNγ), IFN-inducible protein 10 (IP-10), and tissue inhibitor of metalloproteinases 1 and 2 (TIMP-1, TIMP-2). VEGF levels were below the minimum threshold of detection of this assay and were removed from the analysis. All procedures followed the manufacturer's protocol. Briefly, a 1:10 dilution of plasma from Balb/C mice treated with either control (vehicle) or sunitinib (120 mg/kg/day) for 7 days was incubated on a membrane containing immobilized antibodies allowing for the capture of aforementioned proteins. After washing, membrane was incubated with biotin-conjugated antibodies to detect captured proteins and, after incubation with biotin-streptavidin, electrochemical luminescence (ECL) was added for detection on scientific imaging film (Kodak, Blue XB-1, Rochester, NY).

Densitometric Analysis

For densitometric analysis of the aforementioned protein array blots, exposures were imaged using a Molecular Imager GS-800 Calibrated Densitometer (BioRad, Hercules, CA) and quantified by using Quantity One 1-D Analysis Software (BioRad). Each dot

representing an individual protein was standardized to relative background and compared to negative controls of that particular blot. Aggregated values for treated groups were compared to aggregate values for controls. To ensure that quantification was performed in a linear range, at least three immunoblot exposures of varying intensity were analyzed with only non-saturated blots subjected to quantification.

Evaluation of Hypoxia

Mice received an intravenous injection of pimonidazole hydrochloride (60 mg/kg; Chemicon International, Inc., Temecula, CA) 90 min before death. This marker was used to assess tumor hypoxia as has been described (12-14). Pimonidazole hydrochloride is a bioreductive chemical probe activation of which occurs at pO_2 levels <10 mm Hg (15).

Tissue Processing and Immunohistochemistry

Organs were surgically removed and immediately placed in Tissue-Tek optimum cutting temperature compound (Miles Inc., Elkhart, IN), frozen in dry ice, and then kept at –70°C. Five-micrometer-thick spleen cryosections were stained with either Hypoxyprobe-1 mouse monoclonal antibody (1:200 dilution, Chemicon International) followed by an FITC-conjugated anti-mouse antibody (1:200 dilution, Jackson Laboratories), or an anti-HIF1α rabbit polyclonal antibody (1:50 dilution, Novus Biologicals) followed by a Cy3-conjugated anti-rabbit antibody (1:100, Jackson Laboratories). Sections were counterstained with DAPI (1:3500 dilution, Molecular Probes). Staining with mouse IgG2a (Dako Cytomation, Carpenteria, CA) was used as a negative control.

Image Acquisition and Quantification

Sections were visualized under a Carl Zeiss Axioplan 2 microscope, using the following fluorescence filters: Cy3 (540 nm excitation), FITC (470 nm) and DAPI (350 nm). Images were captured with a Zeiss Axiocam camera connected to the microscope using AxioVision 3.0 software. Images of four fields were taken of each organ section at a

magnification of 100x (10x objective/10x eyepiece). Positive pixels (FITC, for hypoxyprobe-1, CY3, for HIF-1α) were quantified using Adobe Photoshop 7.0.

1. Faivre S, Delbaldo C, Vera K, Robert C, Lozahic S, Lassau N, Bello C, Deprimo S, Brega N, Massimini G, *et al.* (2006) *J Clin Oncol* 24:25-35.

2. Motzer RJ, Michaelson MD, Redman BG, Hudes GR, Wilding G, Figlin RA, Ginsberg MS, Kim ST, Baum CM, Deprimo SE, *et al.* (2006) *J Clin Oncol* 24:16-24.

3. Norden-Zfoni A, Desai J, Manola J, Beaudry P, Force J, Maki R, Folkman J, Bello C, Baum C, Deprimo SE, *et al.* (2007) *Clin Cancer Res* 13:2643-2650.

4. Giles FJ, Bellamy WT, Estrov, Z., O'brien SM, Verstovsek S, Ravandi F, Beran M, Bycott P, Pithavala Y, Steinfeldt H, *et al.* (2005) *Leuk Res* 30:801-811.

5. Rosen LS, Kurzrock R, Mulay M, Van Vugt A, Purdom M, Ng C, Silverman J, Koutsoukos A, Sun YN, Bass, M. B. *et al.* (2007) *J Clin Oncol* 25:2369-2376.

6. Lee L, Sharma S, Morgan B, Allegrini P, Schnell C, Brueggen J, Cozens R, Horsfield M, Guenther C, Steward WP, *et al.* (2006) *Cancer Chemother Pharmacol* 57:761-771.

7. Drevs J, Zirrgiebel U, Schmidt-Gersbach CI, Mross K, Medinger M, Lee L, Pinheiro J, Wood J, Thomas AL, Unger C, *et al.* (2005) *Ann Oncol* 16:558-565.

8. Mendel DB, Laird AD, Xin X, Louie SG, Christensen JG, Li G, Schreck RE, Abrams TJ, Ngai TJ, Lee LB, *et al.* (2003) *Clin Cancer Res* 9:327-337.

9. Nagy JA, Dvorak AM, Dvorak HF (2003) *Trends Cardiovasc Med* 13:169-175.

10. Ebos JM, Tran J, Master Z, Dumont D, Melo JV, Buchdunger E, Kerbel RS (2002) *Mol Cancer Res* 1:89-95.

11. Ebos JML, Bocci G, Man.S., Thorpe PE, Hicklin DJ, Zhou D, Jia X, Kerbel RS (2004) *Mol Cancer Res* 2:315-326.

12. Franco M, Man S, Chen L, Emmenegger U, Shaked Y, Cheung AM, Brown AS, Hicklin DJ, Foster S, Kerbel RS (2006) *Cancer Res* 66:3639-3648.

13. Varia MA, Calkins-Adams DP, Rinker LH, Kennedy AS, Novotny DB, Fowler WC, Jr, Raleigh JA (1998) *Gynecol Oncol* 71:270-277.

14. Raleigh JA, Chou SC, Calkins-Adams DP, Ballenger CA, Novotny DB, Varia MA (2000) *Clin Cancer Res* 6:855-862.

15. Raleigh JA, Chou SC, Arteel GE, Horsman MR (1999) *Radiat Res* 151:580-589.