Vascular Biology, Atherosclerosis and Endothelium Biology

Inhibition of Chronic and Acute Skin Inflammation by Treatment with a Vascular Endothelial Growth Factor Receptor Tyrosine Kinase Inhibitor

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Although vascular remodeling is a hallmark of many chronic inflammatory disorders, antivascular strategies to treat these conditions have received little attention to date. We investigated the effects of a newly identified vascular endothelial growth factor (VEGF) receptor tyrosine-kinase inhibitor, NVP-BAW2881, on endothelial cell function *in vitro* and its anti-inflammatory activity in different animal models. NVP-BAW2881 inhibited proliferation, migration, and tube formation by human umbilical vein endothelial cells and lymphatic endothelial cells *in vitro*. In a transgenic mouse model of psoriasis, NVP-BAW2881 reduced the number of blood and lymphatic vessels and infiltrating leukocytes in the skin, and normalized the epidermal architecture. NVP-BAW2881 also displayed strong anti-inflammatory effects in models of acute inflammation; pretreatment with topical NVP-BAW2881 significantly inhibited VEGF-A-induced vascular permeability in the skin of pigs and mice. Furthermore, topical application of NVP-BAW2881 reduced the inflammatory response elicited in pig skin by UV-B irradiation or by contact hypersensitivity reactions. These results demonstrate for the first time that VEGF receptor tyrosine-kinase inhibitors might be used to treat patients with inflammatory skin disorders such as psoriasis. (*Am J Pathol* 2008, 173:265–277; DOI: 10.2353/ajpath.2008.071074)

Angiogenesis and lymphangiogenesis have important roles in tumor growth and metastasis.^{1,2} Vascular remodeling also accompanies many inflammatory and autoimmune disorders, such as renal transplant rejection, rheu-

matoid arthritis, inflammatory bowel disease, and the chronic inflammatory skin disease, psoriasis.^{3–6} Vascular endothelial growth factor (VEGF)-A levels are elevated in inflamed tissue associated with these conditions.^{7–9} Epidermal VEGF-A expression is strongly up-regulated⁸ and blood and lymphatic vessels are increased¹⁰ in human psoriatic skin lesions, indicating the involvement of VEGF-A in psoriasis pathogenesis. Furthermore, specific genetic polymorphisms of the gene encoding VEGF-A are correlated with psoriasis severity.¹¹

A variety of strategies have been pursued to inhibit neovascularization by blocking VEGF-A binding to VEGF receptors (VEGFRs)—particularly to VEGFR-2. VEGFR-2 is believed to be the major mediator of VEGF-A's (lymph)angiogenic activity in blood vascular and lymphatic endothelial cells.¹² Thus far however, these approaches have primarily focused on the development of cancer therapeutics. The most successful anti-angiogenic strategy to date has been the development of a monoclonal antibody directed against VEGF-A (bevacizumab, Avastin).^{1,13} Several other agents designed to block VEGF-A signaling are in late stages of preclinical and clinical development; these include antibodies that target VEGFR-2,¹⁴ soluble VEGFR-2 decoy receptors,¹⁵ and small-molecule inhibitors of VEGFR tyrosine kinase (TK) activity.^{16,17}

Surprisingly, anti-(lymph)angiogenic substances have received little attention as potential therapeutics for chronic inflammatory conditions. In particular, the efficacy of small-molecule inhibitors of VEGFR TK activity

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has only been evaluated in a few *in vivo* studies of chronic inflammation.¹⁸ The potentially low cost of production of these organic molecules, as compared with those of anti-VEGF-A biopharmaceuticals, as well as their potential for oral or topical administration, make these compounds attractive for the treatment of patients with debilitating chronic inflammatory conditions. Furthermore, simultaneously interfering with all VEGFR-mediated signaling pathways with small-molecule TK inhibitors may be advantageous to antibody-mediated blockade of only one particular VEGF isoform or of one particular VEGFR.

We investigated the *in vitro* and *in vivo* activity of the recently identified VEGFR TK inhibitor NVP-BAW2881, which inhibits human and mouse VEGFR TK activity at nanomolar concentrations. This compound blocked VEGF-A-induced proliferation, migration, and tube formation of human umbilical vein endothelial cells (HUVECs) and lymphatic endothelial cells (LECs) *in vitro*. In a mouse model of psoriasis, both oral and topical administration of NVP-BAW2881 strongly reduced psoriasis-like inflammation in ear skin. Topical NVP-BAW2881 also effectively reduced VEGF-A-induced vascular permeability in the skin of mice and domestic pigs, whose skin more closely resembles human skin. Furthermore, the compound given topically significantly inhibited acute skin inflammation in pigs, namely contact hypersensitivity (CHS) reactions and UV-B (UVB)-induced erythema. These findings indicate that orally or topically applied VEGFR TK inhibitors might be used to treat patients with psoriasis and other inflammatory skin disorders.

Materials and Methods

Compound

NVP-BAW2881 is a low-molecular weight compound developed for the VEGFR TK inhibitor program at Novartis. For *in vitro* functional assays, NVP-BAW2881 was dissolved in a 1 mmol/L stock solution of dimethyl sulfoxide. For topical treatment of mice and pigs, the compound was applied at 0.1% to 0.5% in a mixture of ethanol (30%) and propylene glycol (70%). NVP-BAW2881 was dissolved in polyethylene glycol-200 and orally administered to mice in a dose of 25 mg/kg/day in 10 ml/kg. The IC₅₀ values of NVP-BAW2881 for various kinases were determined in a scintillation proximity assay, using recombinant glutathione-S-transferase-labeled kinases as substrates, as previously described.^{19,20} Cellular autophosphorylation assays were performed as described previously.¹⁹

Cells

Human dermal LECs were isolated from neonatal human foreskins as described²¹ and cultured in LEC medium containing endothelial basal medium (Cambrex, Verviers, Belgium), supplemented with 20% fetal bovine serum (Gibco, Paisley, UK), antibiotic antimycotic solution, L-glutamine (2 mmol/L), hydrocortisone (10 µg/ml), and N⁶,2'-O-dibutyryl adenosine 3',5'-cyclic monophosphate

sodium salt (2.5×10^{-2} mg/ml; all from Fluka, Buchs, Switzerland). Cells were passaged up to 10 times. HUVECs (Cambrex) were cultured in LEC medium supplemented with endothelial cell growth supplement (Cambrex) for up to 8 passages. HUVECs were transferred to LEC medium 3 days before functional assays were performed.

Proliferation and Migration Assays

HUVECs or LECs (1.2×10^3) were seeded into fibronectin-coated 96-well plates. After 24 hours, the cells were transferred into LEC medium containing 2% fetal bovine serum and incubated for an additional 24 hours. Cells (eight wells/condition) were incubated with medium alone (control), 20 ng/ml VEGF-A (Biological Resources Branch, National Cancer Institute, Washington DC), or a combination of 20 ng/ml VEGF-A and 1 nmol/L to 1 µmol/L NVP-BAW2881. Proliferation was also assayed in LECs incubated with 500 ng/ml VEGF-C (Cys156Ser; R&D Systems, Abingdon, UK). The dimethyl sulfoxide concentration was adjusted to 0.1% in all wells. After 72 hours, cells were incubated with 5-methylumbelliferylheptanoate for subsequent fluorescent quantification of viable cells, using a SpectraMax Gemini electron microscope (Bücher Biotec AG, Basel, Switzerland) as described.²²

Chemotactic cell migration toward 20 ng/ml VEGF-A was assayed in FluoroBlok transwell chambers (BD Falcon) as described.²³ Cells were incubated in endothelial basal medium containing 0.2% bovine serum albumin for 10 minutes at 37°C in the presence or absence of NVP-BAW2881 (10 nmol/L or 1 µmol/L). Lower chambers (3 to 5 per condition) were filled with 500 µl medium, VEGF-A (20 ng/ml) and NVP-BAW2881 (0 nmol/L, 10 nmol/L or 1 µmol/L). Only medium was added to control wells. Cells (10^5 in 100 µl) that were exposed to the various concentrations of NVP-BAW2881 were seeded into the upper chambers and incubated for 3 hours at 37°C. Cells that had migrated to the bottom side of the filter plate were stained with Calcein AM (Molecular Probes). Fluorescence intensity (proportional to the number of transmigrated cells) was measured using a SpectraMax Gemini electron microscope.

Tube Formation Assay

Tube formation assays were performed as described.²⁴ LECs or HUVECs were grown to confluence in fibronectin-coated 24-well plates. Each well (3 to 4 per condition) was overlaid with 0.5 ml of neutralized isotonic bovine dermal collagen type I (Invitrogen, Palo Alto, CA; 1 mg/ml for LECs, 1.2 mg/ml for HUVECs) containing VEGF-A (20 ng/ml) and NVP-BAW2881 (0 nmol/L, 10 nmol/L, or 1 µmol/L). Controls wells were overlaid with collagen only. The wells were incubated for 20 hours at 37°C and representative images were captured (four per well). The total length of tube-like structures per image was measured using IPlab software (BD Biosciences).

Animals

Female hemizygous K14/VEGF-A TG mice (FVB background) that overexpress mouse VEGF-A164 in the epidermis under control of the human keratin 14 promoter²⁵ were bred and housed in the animal facility of ETH. Female hairless SKH1 mice were purchased from Charles River Laboratories, Inc. (Austria). Young castrated male domestic pigs (German Edelschwein × Landrace; Josef Semrad, Mü-nichstal, Austria) weighed 12 to 18 kg at the time of experimentation. Experiments were performed in accordance with the animal protocols approved by the “Kantonales Veterinäramt Zürich” (animal protocol Nr. 123/2005) and the “Landesregierung Wien” (study protocols MA 58-05120/05, MA 58-03893/2005/5, MA 58-03896/2005/5, and MA 58-03894/2005/7).

Mouse Model of Psoriasis

A contact hypersensitivity response was induced in the ear skin of 8-week-old female K14/VEGF-A TG mice as described.¹⁰ Mice (10/group) were anesthetized by i.p. injection of medetomidine (0.2 mg/kg) and ketamine (80 mg/kg) and sensitized by topical application of 2% oxazolone (4-ethoxymethylene-2 phenyl-2-oxazoline-5-one; Sigma, St. Louis, MO) in acetone/olive oil (4:1 v/v) to the shaved abdomen (50 μ l) and to each paw (5 μ l). Five days after sensitization (day 0), the right ear was challenged by topical application of 10 μ l oxazolone (1%) on each side. Starting on day 7, once-daily oral doses of 25 mg/kg NVP-BAW2881 or twice-daily topical doses of 0.5% NVP-BAW2881 were administered for 14 days. Control groups were given vehicles alone. The ear thickness was measured every other day using calipers. On day 21, mice were sacrificed and the weight of each ear and of its draining retro-auricular lymph node (LN) was determined.

Miles Assay

Female hairless SKH1 mice (three per group, two experiments performed) were treated topically on both flanks with 100 μ l of 0.5% NVP-BAW2881 or vehicle alone. To avoid oral uptake, mice were housed individually and fitted with ruffs. After 2 hours, mice were given i.v. injections of 200 μ l Evans Blue solution (0.5% in saline). Ten minutes later, 100 ng VEGF-A (in 50 μ l PBS) were injected intradermally on both sites; and 25 minutes later, mice were sacrificed and the test sites were excised using a biopsy punch (8 mm diameter; Stiefel, Austria).

Alternatively, 1.0 ml of 0.5% NVP-BAW2881 was applied to test areas of approximately 75 cm² on the right ventral abdomen of domestic pigs ($n = 6$). Contralateral sites were treated with vehicle only. The solutions were applied 30, 7, and 3 hours before intradermal injection of VEGF-A. At time 0 hours, animals were sedated with ketamine and xylazine (16 and 4 mg/kg, i.m.) and 2% Evans Blue solution was injected i.v. (2 ml/kg body weight). Ten minutes later, 10 ng VEGF-A in 50 μ l PBS was injected at four sites of the test areas. After 30

minutes, animals were sacrificed and 8-mm punch biopsies were taken from the injection sites. To analyze VEGF-A-induced extravasation of Evans Blue, samples were incubated in 0.5 ml formamide at room temperature for 48 hours. Subsequently, the concentration of Evans Blue in the supernatant was measured photometrically at 650 nm.

In additional experiments, female hairless SKH1 mice (5 per group) were topically treated with 100 μ l 1% NVP-BAW or vehicle alone on both flanks. Two hours later, 250 μ l of a 0.5% Evans Blue solution was injected i.v. Extravasation was induced 10 minutes later at two sites of the pretreated areas either with 50 ng platelet-activating factor (PAF) (Novabiochem, Merck, Darmstadt, Germany) or with 25 μ g histamine (Sigma), injected intradermally in 50 μ l volumes. 30 minutes later, the mice were sacrificed and from the three injection sites in each mouse 8 mm punch biopsies were collected for photometrical measurement of extracted Evans Blue, as described above.

UVB-Induced Erythema in Domestic Pigs

In 10 animals, a total of 26 focal erythemas (approximately 4 cm²) were elicited on the shaved dorsolateral back with UVB (72 mJ/cm²), generated with TL20W lamps (Philips). Contralateral test sites were treated with 50 μ l of 0.5% NVP-BAW2881 or vehicle immediately after irradiation, and again 3 and 6 hours later. After 6 and 24 hours, test sites were examined by reflectometry (Chromameter CR 400, Minolta) using a^* values ($L^*a^*b^*$ system) for skin redness and with laser Doppler flowmetry (PF 5000, Perimed) for measurement of microperfusion.^{26,27} In addition, erythema was scored at each site: 0 (absent), 1 (scarcely visible, small spotted), 2 (mild, large spotted), 3 (pronounced, confluent), and 4 (severe or livid discoloring, homogenous redness).

CHS Response in the Skin of Domestic Pigs

Animals ($n = 8$) were sensitized by applying 2, 4-dinitro fluorobenzene (DNFB, 10%, dissolved in dimethyl sulfoxide: acetone: olive oil [1:5:3, v/v/v]) onto the basis of both ears and onto both groins (100 μ l/site). Eight days later, the animals were challenged with 15 μ l of a 1% DNFB solution on eight test sites on both sides on the dorsolateral back. After 0.5 and 6 hours, contralateral test sites were treated with NVP-BAW2881 (0.1 or 0.5%) or vehicle. Test sites (in total 16 treated with 0.1%, 16 with 0.5%, and 32 with vehicle) were clinically examined 24 hours after challenge, when inflammation peaked. The changes were scored on a scale from 0 to 4, allowing a combined maximal score of 12 per designated site (Table 1).²⁸

Immunohistology

On day 21 after challenge, mice were sacrificed and their ears and auricular LNs were collected and weighed. Tissues were embedded in optimal cutting temperature compound (Sakura Finetek, Torrance, CA) and frozen on dry ice, and 6- μ m cryostat sections were cut. H&E staining was

Table 1. Scoring System Used to Evaluate CHS-Induced Skin Inflammation in Domestic Pigs

Score	Erythema/Intensity	Erythema/Extent	Induration
0	Absent	Absent	Absent
1	Scarcely visible	Small spotted	Scarcely palpable
2	Mild	Large spotted	Mild hardening
3	Pronounced	Confluent	Pronounced hardening
4	Severe (or livid discoloring)	Homogenous redness	Pronounced and elevated hardening

Erythema intensity, erythema extent, and induration of the skin were examined by optical inspection. Each parameter was scored on a scale from 0 to 4, allowing a combined maximal score of 12 per site examined.

performed as described.¹⁰ Immunofluorescence was performed as described²⁹ using the following antibodies: anti-mouse LYVE-1 (Angiobio, Del Mar, CA), MECA-32, anti-mouse CD31, anti-mouse CD45, anti-mouse CD11b (all from BD Biosciences), anti-mouse keratin 6, keratin 10, and loricrin (all from Covance Research Products, Berkeley, CA). Alexa488- or Alexa594-coupled secondary antibodies and Hoechst 33342 were purchased from Molecular Probes (Invitrogen, Basel, Switzerland).

Computer-Assisted Analyses of Ear Sections

CD45, LYVE-1, or MECA-32-labeled ear sections from three mice per treatment group were examined on an Axioskop 2 mot plus microscope (Carl Zeiss, Feldbach, Switzerland), equipped with an AxioCam MRc camera (Zeiss) and a Plan-APOCHROMAT 10_/0.45 objective (Zeiss). Images of three individual fields of view were acquired per section. Computer-assisted analysis of digital images was performed using the IP-LAB software (Scanalytics, Fairfax, VA), as previously described.³⁰ To quantify leukocyte infiltration, the percentage of ear tissue (covering the entire field of view, between the cartilage backbone and the epidermis) that stained positive for CD45 was determined. Furthermore, the average size of lymphatic vessels present in the entire field of view was determined. To identify changes in the blood vascular compartment, the percentage of tissue that stained positive for MECA-32 in the upper dermis (ie, the region located up to 120 μ m below the epidermis) was determined. The latter parameter was chosen because chronically inflamed ears of control-treated VEGF-A TG mice presented numerous sponge-like, tortuous blood vessels in the upper dermis, making it difficult to identify single vessels and to quantify microvessel density.

Statistical Analysis

Data are shown as mean $\pm$ SE (SEM) and were analyzed with a Student's *t*-test (paired or unpaired, depending on

the assay). Differences were considered statistically significant when $P < 0.05$.

Results

Nanomolar Concentrations of NVP-BAW2881 Inhibit Human and Mouse VEGFR TKs

NVP-BAW2881 is a low molecular weight compound developed for the VEGFR TK inhibitor program at Novartis. In biochemical assays, NVP-BAW2881 was shown to primarily target the VEGFR TK family with IC₅₀ values in the low nanomolar range. The IC₅₀ value for this compound against human VEGFR-2 (hVEGFR2, also termed KDR) was 37 nmol/L (Table 2). NVP-BAW2881 was highly selective for VEGFR, although it also demonstrated activity against Tie2 (IC₅₀ = 650 nmol/L) and RET (IC₅₀ = 410 nmol/L). The IC₅₀ values of NVP-BAW2881 toward a wide panel of other kinases were $>10 \mu$ mol/L (data not shown).

In cellular assays, NVP-BAW2881 inhibited VEGF-A-induced phosphorylation of VEGFR-2 in HUVECs and in VEGFR-2-transfected Chinese hamster ovary cells, with IC₅₀ values of 2.9 and 4.2 nmol/L, respectively (Table 3). NVP-BAW2881 also inhibited RET (NIH3T3 cells stably expressing a constitutively active RET mutant), platelet-derived growth factor receptor (PDGFR) β (in PDGFR β expressing A31 mouse embryonic fibroblasts), and c-Kit (GIST882 cells expressing an activating KIT mutation) TK activities, with IC₅₀ values of 45, 47, and 74 nmol/L, respectively. The compound showed moderate potency against cytoplasmic Bcr-Abl kinase (IC₅₀ of approximate 0.6 μ mol/L) while it was not effective (IC₅₀ values $>10 \mu$ mol/L) against a range of other kinases (data not shown). NVP-BAW2881 was also profiled in a panel of BaF3 cell lines, rendered interleukin-3-independent by transduction with constitutively active tyrosine kinases. The compound inhibited proliferation of the BaF3 cell line

Table 2. NVP-BAW2881 Inhibits Human and Mouse VEGFR TKs at Nanomolar Concentrations

Receptor	hVEGFR1	hVEGFR1	hVEGFR3	mVEGFR2
IC ₅₀ (μ M)	0.82 $\pm$ 0.019	0.037 $\pm$ 0.08	0.42 $\pm$ 0.015	0.165 $\pm$ 0.064

The IC₅₀ values of NVP-BAW2881 toward different human and mouse VEGFRs were determined in a scintillation proximity assay, using recombinant fusions of glutathione S-transferase (GST) with VEGFR tyrosine kinases. Incorporation of ³²P into the peptidic substrates was quantified. IC₅₀ values of a wide panel of other kinase tested were $>10 \mu$ mol/L.

Table 3. Inhibitory Activity of NVP-BAW2881 Toward Various Kinases, Determined in Cellular Assays

Receptor	Cell type	Readout	IC ₅₀ (nM)
VEGFR2	HUVEC	Phosphorylation	2.9
VEGFR2	CHO	Phosphorylation	4.2
RET	NIH3T3	Phosphorylation	45
PDGFR β	A31	Phosphorylation	47
c-kit	GIST882	Phosphorylation	74
Bcr-Abl	32D	Phosphorylation	578
VEGFR2	BAF3	Proliferation	1
PDGFR β	BAF3	Proliferation	13
PTC3-RET	BAF3	Proliferation	152
Bcr-Abl	BAF3	Proliferation	4750

The IC₅₀ values of NVP-BAW2881 toward different human kinases were determined in cellular assays. To this end, cell lines were transfected with the respective kinase and the activity of NVP-BAW2881 on either tyrosine kinase phosphorylation or cellular proliferation was determined (only in the case HUVECs, the activity toward endogenous VEGFR2 was analyzed).

Tel-VEGFR-2 with an IC₅₀ of 1 nmol/L, Tel-PDGFR- β (13 nmol/L) and PTC3-RET (152 nmol/L), and with weaker activity against Bcr-ABL (4750 nmol/L) while being inactive (IC₅₀ >10 μ mol/L) against a panel of transduced or parental BaF3 cells (data not shown).

Therefore, NVP-BAW2881 is highly selective; it is ≥ 10 -fold more selective for VEGFR-2 than for other VEGFR TK family members or for related TKs such as PDGFR, RET, or Tie-2.

NVP-BAW2881 Potently Inhibits VEGF-A-Induced Proliferation and Migration of Endothelial Cells in Vitro

VEGF-A induces proliferation of blood vascular cells and LEC *in vitro* and *in vivo*.^{1,12,31,32} To determine whether NVP-BAW2881 had a blocking effect on VEGF-A-induced proliferation of endothelial cells, HUVECs were incubated in VEGF-A-containing medium in the presence or absence of NVP-BAW2881. Although VEGF-A promoted HUVEC cell proliferation (1.3-fold more cells, compared with control; $P < 0.0001$), the lowest concentration of NVP-BAW2881 tested (1 nmol/L) potently inhibited this response (Figure 1A). Similarly, when testing the effects of this compound on LEC proliferation, we found that 1 nmol/L NVP-BAW2881 significantly reduced VEGF-A-induced proliferation, compared with controls ($P < 0.001$; Figure 1B). Since VEGF-C is thought to be main mediator of lymphangiogenesis *in vivo*, we tested whether NVP-BAW2881 could also block VEGF-C-induced proliferation of LECs. In this experiment, we used a mutant form of VEGF-C that selectively binds to and activates VEGFR-3.³³ Although 1 μ mol/L NVP-BAW2881 significantly reduced VEGF-C-induced LEC proliferation ($P = 0.042$, Figure 1C), lower concentrations of the compound did not have a significant effect. Furthermore, 10 nmol/L and 1 μ mol/L concentrations of NVP-BAW2881 completely inhibited the migration of

Figure 1. NVP-BAW2881 inhibits VEGF-induced proliferation and migration of HUVECs and LECs *in vitro*. **A–C:** The effect of increasing concentrations of NVP-BAW2881 on VEGF-A- or VEGF-C-induced proliferation of cultured HUVECs and LECs was assayed. **A:** Effect of NVP-BAW2881 on HUVEC proliferation induced by VEGF-A. **B:** Effect of NVP-BAW2881 on LEC proliferation induced by VEGF-A. **C:** Effect of NVP-BAW2881 on LEC proliferation induced by VEGF-C. **D and E:** The effect of either 10 nmol/L or 1 μ mol/L NVP-BAW2881 on migration of HUVECs (**D**) or LECs (**E**) toward VEGF-A, as assayed in transwell plates. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. In each panel, the results from one representative of 2 to 3 experiments are shown. NVP-BAW2881 is abbreviated as BAW2881.

HUVECs and of LECs toward VEGF-A in *in vitro* transmigration assays (Figure 1, D and E).

NVP-BAW2881 Inhibits VEGF-A-Induced Tube Formation in Endothelial Cells

To further assess the anti-angiogenic effects of NVP-BAW2881, *in vitro* tube formation assays were performed. Confluent monolayers of either HUVECs or LECs were overlaid with collagen type I (control) or with collagen that contained only VEGF-A, or VEGF-A in combination with 10 nmol/L or 1 μ mol/L NVP-BAW2881. VEGF-A alone induced a significant increase in the formation of tube-like structures in both HUVECs (Figure 2, A and C) and LECs (Figure 2, B and D). By contrast, the presence of NVP-BAW2881 (10 nmol/L or 1 μ mol/L) significantly inhibited VEGF-A-induced tube formation in both cell types (Figure 2, A–D). Taken together, these data demonstrate that NVP-BAW2881 potently inhibits VEGF-A-induced cellular processes, such as proliferation, migration and tube formation, in both lymphatic and blood vascular endothelial cells *in vitro*.

Oral and Topical Treatment with NVP-BAW2881 Reduces Symptoms of Ear Inflammation in K14/VEGF-A Transgenic Mice

We tested the effects of NVP-BAW2881 in a transgenic mouse model of psoriasis; the K14/VEGF-A mice consti-

tutively express VEGF-A in the epidermis under the control of the keratin K14 promotor.³⁴ Unlike wild-type mice, homozygous and hemizygous K14/VEGF-A mice are unable to down-regulate skin inflammation induced by a CHS response and develop chronic, psoriasis-like inflammatory skin lesions that are characterized by edema formation, epidermal hyperproliferation, and leukocyte infiltration.^{10,25} Immunofluorescence analysis has also revealed an increase in blood vascular and lymphatic vessels in the inflamed skin of these mice, similar to those observed in the inflamed skin of patients with psoriasis.¹⁰

Psoriasis-like lesions were induced in the right ears of K14/VEGF-A mice by inducing a CHS response, and NVP-BAW2881 was administered, topically or orally, beginning 7 days later. A significant reduction in ear swelling was observed within 5 days in groups given oral doses (-21% , $P = 0.003$) and within 7 days in groups given topical applications (-25% , $P = 0.006$) of NVP-BAW2881 (Figure 3, A and B, respectively). At the defined study endpoint (14 days after treatment began; study day 21), ear swelling had decreased by 56% ($P < 0.0001$) in the oral treatment group, as compared with the control group. Similarly, topical treatment with NVP-BAW2881 reduced ear swelling by 43% ($P = 0.0001$) as compared with controls.

Ear redness was qualitatively evaluated on study day 21 (14 days after treatment began) using a scoring system ranging from 0 (normal ear color) to 3 (dark red). The clinical score of mice given oral doses of NVP-BAW2881 was markedly reduced, as compared with controls

Figure 2. NVP-BAW2881 inhibits VEGF-A-induced tube formation of HUVECs and LECs *in vitro*. Tube formation assays were performed by covering confluent monolayers of HUVECs or LECs with collagen type I containing no additives (ctr), containing VEGF-A alone (VEGF-A), or containing VEGF-A together with either 10 nmol/L or 1 μ mol/L NVP-BAW2881. **A** and **B**: Representative pictures showing the effect of NVP-BAW2881 on tube formation in HUVECs (**A**) and LECs (**B**). **C** and **D**: Quantification of the effect of NVP-BAW2881 on tube formation in HUVECs (**C**) and LECs (**D**). For quantification, the total tube length per picture was measured and normalized to the tube length measured in the control picture. ** $P < 0.01$; *** $P < 0.001$. Each panel shows the results from one representative out of 2 to 3 experiments. NVP-BAW2881 is abbreviated as BAW2881.

Figure 3. Oral or topical treatment with NVP-BAW2881 reduces symptoms of chronic ear inflammation in K14/VEGF-A mice. Heterozygous female K14/VEGF-A TG mice were sensitized with oxazolone on the belly and paws on study day -5 . On study day 0, mice were challenged by application of oxazolone onto the ears. Treatment with NVP-BAW2881 was started on day 7 and repeated once (oral NVP-BAW2881) or twice (topical NVP-BAW2881) each day until the end of the study (day 21). Control (ctr) mice were given vehicle alone. **A and B:** Effect of oral (**A**) or topical (**B**) administration of NVP-BAW2881 on inflammation-induced ear swelling. **C and D:** The effect of oral (**C**) or topical (**D**) administration of NVP-BAW2881 on ear redness was qualitatively evaluated on day 21 with a scoring system ranging from 0 (normal ear color) to 3 (dark red). **E and F:** Mice were sacrificed on day 21 and the effect of oral (**E**) or topical (**F**) administration of NVP-BAW2881 on ear weight was determined. **G and H:** Effects of oral (**G**) or topical (**H**) administration of NVP-BAW2881 on the weight of the ear-draining auricular LN was determined (day 21). ** $P < 0.01$; *** $P < 0.001$. NVP-BAW2881 is abbreviated as BAW2881.

(-56% , $P < 0.0001$, Figure 3C). Similarly, ear redness was reduced compared with controls (-33% , $P = 0.002$), after topical administration of NVP-BAW2881, albeit to a lesser extent than mice given oral doses (Figure 3D).

The observed anti-inflammatory effects of NVP-BAW2881 were also apparent when ear weights were measured at the endpoint of the study (21 days after challenge) (Figure 3, E and F). In the group given oral doses of NVP-BAW2881, the mean ear weight was 26% less than that of the controls ($P < 0.0001$). Similarly, topical administration of NVP-BAW2881 reduced the mean ear weight by 24% ($P < 0.0001$), compared with controls. No differences in ear thickness or weight were detected in the uninflamed left ears of groups given NVP-BAW2881, as compared with control mice (data not shown).

At the same time, the weights of the ear-draining auricular LN, which typically correlate with nodal cellularity (C.H. & H.F., data not shown), were also determined. NVP-BAW2881 significantly reduced LN weight in mice following oral (54%, $P < 0.0001$) and topical administra-

tion (44%, $P < 0.0001$) compared with controls (Figure 3, G and H).

NVP-BAW2881 Reduces Leukocyte Infiltration and the Number and Size of Blood and Lymphatic Vessels in the Inflamed Tissue

To better characterize the efficacy of NVP-BAW2881 in reducing psoriasis-like skin inflammation, immunohistochemical analysis was performed on ear sections from mice given NVP-BAW2881 and from controls. H&E staining revealed the presence of massive leukocytic infiltrates in the inflamed ears of the control mice, as well as epidermal hyperproliferation and hyperkeratosis (Figure 4A). These symptoms were reduced in ear sections from mice given oral or topical doses of NVP-BAW2881 (Figure 4A). Immunofluorescence revealed the presence of numerous CD45⁺ leukocytes in sections from control mice, but fewer numbers in ears of both NVP-BAW2881 treatment groups (Figure 4B). Similarly, fewer CD11b⁺ macro-

Figure 4. Oral or topical administration of NVP-BAW2881 reduces leukocyte infiltration and vascular abnormalities in the inflamed skin. A CHS response to oxazolone was induced in the ears of K14/VEGF-A TG mice and treated by either oral or topical application of NVP-BAW2881 7 to 21 days after CHS induction. Histological analysis was performed on study day 21. **A:** H&E staining. **B:** Immunofluorescence for CD45 (leukocytes, red) and LYVE-1 (lymphatic vessels, green). **C:** Immunofluorescence for CD11b⁺ (red), in combination with the lymphatic vessel marker LYVE-1 (green). **D:** Immunofluorescence for MECA-32 (blood vessels, red) and LYVE-1 (lymphatic vessels, green). Scale bars = 50 μm . **E–G:** Changes in leukocyte infiltration and in vascular parameters were quantified by computer-assisted image analysis: **E**, Leukocyte infiltration, measured as the percentage of ear tissue that stained positively for the leukocyte marker CD45; **F**, average size of LYVE-1-positive lymphatic vessels; and **G**, percentage of tissue area in the upper dermis (area up to 120 μm below the epidermis) that stained positively for the blood vascular marker MECA-32. NVP-BAW2881 is abbreviated as BAW2881.

phages were detected in sections from NVP-BAW2881-treated animals, compared with control mice (Figure 4C). Importantly, immunofluorescence staining of MECA-32-positive blood vessels and LYVE-1-positive lymphatic vessels revealed that oral and topical administration of NVP-BAW2881 led to a decrease in tissue vascularization (Figure 4D). The number of blood vessels was reduced and lymphatic vessels were generally small and collapsed in NVP-BAW2881-treated mice, whereas the ears of control mice had many enlarged lymphatic vessels (Figure 4D). The changes in leukocyte infiltration and vascular

morphology induced by NVP-BAW2881 treatment could also be quantified by computer-assisted image analyses, performed on stained ear sections. Both oral and topical treatment with NVP-BAW2881 led to a significant reduction of the tissue area that stained positively for CD45 (Figure 4E). Similarly, the average size of LYVE-1-positive lymphatic vessels was markedly reduced after treatment with NVP-BAW2881 (Figure 4F). Furthermore, the presence of MECA-32-positive blood vascular structures was significantly reduced in the upper dermis of mice treated either orally or topically with NVP-BAW2881 (Figure 4G).

Figure 5. Oral or topical administration of NVP-BAW2881 reduces epidermal hyperproliferation in the inflamed skin. A CHS response to oxazolone was induced in the ears of K14/VEGF-A TG mice and treated by either oral or topical application of NVP-BAW2881 7 to 21 days after CHS induction. Histological analysis was performed on study day 21. **A:** Immunofluorescence for the epidermal marker loricrin (red), in combination with the nuclear stain Hoechst 33342 (blue). **B and C:** Immunofluorescence for the epidermal marker keratin 6 (**B**) and keratin 10 (**C**) (both in red), in combination with CD31 (green). Scale bars = 50 μ m. NVP-BAW2881 is abbreviated as BAW2881.

NVP-BAW2881 Normalizes the Epidermal Architecture in the Inflamed Tissue

In ear sections from mice given NVP-BAW2881, loricrin, a marker of epidermal cornification, was restricted to the upper granular layer, as typically observed in normal epidermis (Figure 4E and data not shown). By contrast, its staining was markedly increased and present in several keratinocyte layers of epidermis in ears of control mice (Figure 5A). Expression patterns of keratin 6 and keratin 10, markers of epidermal hyperproliferation and differentiation, respectively, were also analyzed. Keratin 6 is normally absent from the interfollicular epidermis whereas keratin 10 is expressed in the suprabasal layers of the normal epidermis. During psoriatic hyperproliferation of keratinocytes, both display a much broader staining pattern. Oral or topical treatment with NVP-BAW2881 reverted the expression of both keratin 6 and keratin 10 to a staining pattern much more similar to the one expected in uninfamed epidermis (data not shown), as compared with the inflamed epidermis of control mice (Figure 5, B and C).

Taken together, the immunohistological studies revealed that the major characteristic components of psoriatic skin lesions (epidermal hyperplasia and altered differentiation, inflammatory cell infiltration, vascular abnormalities) were markedly reduced following oral or topical administration of NVP-BAW2881.

Topical NVP-BAW2881 Significantly Reduces VEGF-A-Induced Vascular Permeability

VEGF-A induces vascular leakage of blood vessels *in vivo*.¹² To test whether topical NVP-BAW2881 administration could prevent this immediate effect of VEGF-A, a modified Miles assay³⁵ was performed in mice. The back skin of hairless SKH1 mice was exposed to 0.5% NVP-BAW2881 or vehicle (control); VEGF-A was intradermally injected and then extravasation of Evans Blue dye was quantified spectrophotometrically. Pretreatment with 0.5% NVP-BAW2881 reduced VEGF-A-induced extravasation by 41% ($P < 0.001$) compared with control mice (Figure 6A). Notably, NVP-BAW2881 was only effective in blocking VEGF-A-mediated vascular permeability; pretreatment with the compound showed no effect in reducing vascular permeability induced by other factors, such as PAF or histamine (Figure 6B). In the skin of domestic pigs, which is more similar to human skin than is mouse skin,³⁶ topical exposure to 0.5% NVP-BAW2881 (cumulative pretreatment at 30, 7, and 3 hours before VEGF-A injection) significantly decreased VEGF-A-induced vascular leakage (by 53%, $P < 0.001$) compared with control mice (Figure 6C).

Topical NVP-BAW2881 Reduces Signs of Acute Inflammation Induced by UVB Irradiation and by a CHS Response

The effects of topical NVP-BAW2881 were also tested in two different models of acute inflammation in domestic

Figure 6. Topical pretreatment with NVP-BAW2881 blocks VEGF-A-induced vascular permeability in mouse and pig skin. VEGF-A-induced extravasation of intravenously injected dye into the skin of mice or domestic pigs was measured spectrophotometrically. **A:** In mice, one pretreatment of the skin with NVP-BAW2881 for 2 hours before intradermal injection of VEGF-A, significantly reduced the extravasation of Evans Blue. **B:** Pretreatment with NVP-BAW2881 did not affect vascular permeability induced by intradermal injection of PAF or histamine into the skin of mice. **C:** In domestic pigs, three epicutaneous pretreatments with NVP-BAW2881 (at -30, -7, and -2 hours) led to a significant reduction in Evans Blue extravasation. *** $P < 0.001$. NVP-BAW2881 is abbreviated as BAW2881.

pigs. In a model of UVB-induced erythema, signs of inflammation were significantly reduced when NVP-BAW2881 was applied after irradiation (0, 3, and 6 hours later). After 6 and 24 hours, clinical scoring of the overall appearance of the irradiated skin regions revealed a significant reduction of inflammatory symptoms in NVP-BAW2881-treated skin compared with controls -11%, $P = 0.045$, and -24%, $P = 0.003$, respectively; Figure 7A). At 24 hours after irradiation, skin redness (measured by reflectometry) and microperfusion (measured by laser Doppler flowmetry), were significantly reduced in NVP-BAW2881-treated skin regions compared with controls

(20% reduction, $P = 0.015$, and 31% reduction, $P = 0.0006$, respectively; Figure 7B and 7C).

Furthermore, the effectiveness of topical NVP-BAW2881 in reducing inflammatory symptoms elicited by a CHS response was assessed. To this end, young domestic pigs were sensitized to DNFB and challenged 8 days later by re-exposure of the skin to DNFB. After 0.5 and 6 hours, the test sites were treated with either 0.1% or 0.5% NVP-BAW2881. Analysis of the skin 24 hours after challenge revealed a dose-dependent inhibitory effect of NVP-BAW2881 on the clinical score (Figure 7D) and the redness (Figure 7E) of the inflamed skin area.

Figure 7. Signs of acute skin inflammation are significantly reduced after topical treatment with NVP-BAW2881. The anti-inflammatory effects of NVP-BAW2881 were tested in UVB-induced erythema and in acute contact dermatitis (CHS response) in the skin of domestic pigs. **A–C:** A phototoxic inflammatory response was induced by irradiation with UVB. Treatment with NVP-BAW2881 at 0, 3, and 6 hours after irradiation resulted in a significant reduction in clinical inflammatory symptoms (**A**), measured skin redness (reflectometry) (**B**), and microperfusion of the exposed skin (**C**), as compared with controls (ctr). **D** and **E:** A CHS response toward DNFB was elicited on the back skin domestic pigs. At 0.5 and 6 hours after challenge, the test sites were treated with 0.1% or 0.5% of NVP-BAW2881. At 24 hours after challenge, the inflammatory response was evaluated by clinical score (**D**) and reflectometry (**E**). Topical NVP-BAW2881 caused a dose-dependent reduction of inflammatory symptoms (erythema and induration). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. NVP-BAW2881 is abbreviated as BAW2881.

Taken together, these data reveal that topical treatment with a VEGFR TK inhibitor reduces symptoms of the acute inflammatory response in pigs, whose skin physiology is closely related to human skin.

Discussion

In this study, we have analyzed the *in vitro* and *in vivo* activity of NVP-BAW2881, a new VEGFR TK inhibitor that inhibits mouse and human VEGFR TKs at nanomolar concentrations. Whereas most studies performed to date with VEGFR TK inhibitors have focused on the use of these compounds in oncology, we investigated whether inhibition of VEGFR signaling with a small molecule might be useful for treatment of inflammatory skin disorders. Using a mouse model of the chronic inflammatory skin disease psoriasis, we found that oral or topical administration of NVP-BAW2881 effectively reduced psoriasis-like inflammatory symptoms in the diseased skin of mice. Importantly, the three major components of the disease pathogenesis—infiltration of leukocytes, hyperproliferation and abnormal differentiation of epidermal keratinocytes, and occurrence of vascular abnormalities—were markedly improved following treatment with NVP-BAW2881. These findings indicate that therapeutic intervention at the level of the vasculature could be sufficient to reduce also the immune-mediated and epidermal components of the disease.

The involvement of VEGF-A in the pathogenesis of psoriasis has been well documented: epidermal VEGF-A expression is up-regulated⁸ and the number and size of lymphatic and blood vessels are increased¹⁰ in human psoriatic skin lesions. Elevated levels of VEGF-A can be detected in the serum of human psoriasis patients and correlated with disease activity.³⁷ Furthermore, a recent study revealed that genetic polymorphisms of the VEGF-A gene correlate with psoriasis severity.¹¹ Moreover, in one of the animal models used in this study, chronic overexpression of VEGF-A in the skin of mice led to the development of psoriasis-like skin inflammation.^{10,25} Although not studied as extensively as in psoriasis, VEGF-A is also up-regulated in other chronic inflammatory skin disorders, such as atopic dermatitis³⁸ and bullous diseases.³⁹

In addition to its impact on chronic inflammation, blockade of VEGFR signaling reduced symptoms of the acute inflammatory response. Miles assays performed in the skin of mice and of domestic pigs demonstrated that topical application of NVP-BAW2881 prevented vascular leakage induced by injection of exogenous VEGF-A into the skin. Furthermore, inflammatory symptoms (eg, blood flow and redness) elicited by UVB-irradiation of the skin of pigs were significantly reduced after topical treatment with NVP-BAW2881. Since UVB irradiation induces VEGF-A expression in the skin,⁴⁰ NVP-BAW2881 probably acts by preventing VEGFR signaling triggered by endogenous VEGF-A. In fact, we have previously shown in mice that systemic inhibition of VEGF-A with a blocking antibody can prevent UVB-induced skin damage.⁴¹ Similar to its effect on UVB-induced skin damage, NVP-

BAW2881 also reduced inflammation elicited by a CHS response in the skin of domestic pigs. The inhibitory effects of NVP-BAW2881 on acute inflammation observed in the latter model are in agreement with a recent mouse study in which combined blockage of VEGFR-1 and VEGFR-2 by systemically administered antibodies significantly inhibited CHS-induced ear-skin inflammation.¹⁰ Interestingly, blockage of either VEGFR-1 or VEGFR-2 alone had no major effect on skin inflammation.¹⁰ This indicates that in addition to VEGFR-2, which is generally considered to be the major mediator of the angiogenic effects of VEGF-A,¹² VEGFR-1 signaling is also involved in VEGF-A-dependent acute inflammation. It can therefore be assumed that the anti-inflammatory activity of NVP-BAW2881 is mediated by inhibition of both VEGFR-1 and VEGFR-2.

In addition to inhibiting VEGFR-1 and VEGFR-2 signaling, this study indicates that NVP-BAW2881 blocks the activity of VEGFR-3. VEGFR-3 is expressed on lymphatic, but not on blood vascular endothelium and is the receptor for the LEC-specific growth factor VEGF-C.⁴² In a proliferation assay with LECs, NVP-BAW2881 effectively blocked VEGF-C-induced proliferation. VEGF-C exists in differently processed forms that activate both VEGFR-2 and VEGFR-3. However, the VEGF-C protein used in the LEC proliferation assays of this study contained a mutation that makes it a high-affinity agonist of VEGFR-3.³³ Overall, these data, together with our biochemical studies on isolated VEGFR TKs, demonstrate that NVP-BAW2881 blocks TK activity of all three types of VEGFRs.

With regard to the *in vivo* mechanism behind the anti-inflammatory effects of NVP-BAW2881, it is likely that the compound blocks VEGFR signaling both in endothelial cells and also in other skin-resident cell types. Besides their role in (lymph)angiogenesis, VEGFs are well documented regulators of various aspects of the inflammatory response. VEGF-A is a major inducer of fluid leakage out of blood vessels, leading to tissue swelling during inflammation.¹² Furthermore, chronic overexpression of VEGF-A in the skin of K14/VEGF-A TG mice promotes leukocyte rolling and adhesion in microvascular skin capillaries, because of the higher levels of adhesion molecules (eg, E- and P-selectin) expressed on the blood vascular endothelium.³⁴ Besides endothelial cells, monocytes and macrophages also express VEGFR-1 and VEGFR-3 and have been shown to migrate chemotactically toward VEGF-A and VEGF-C.^{43,44} Taken together, these findings could explain why NVP-BAW2881 reduces leukocyte infiltration into the skin, as observed in our immunofluorescence study on ear sections of K14/VEGF-A TG mice. Interestingly, it has been recently reported that cultured epidermal keratinocytes respond to VEGF-A,^{45,46} indicating the possibility that this cell type might also be affected by treatment with NVP-BAW2881.

Recent evidence indicates that besides angiogenesis, lymphangiogenesis is also involved in certain inflammatory and autoimmune conditions. For example, lymphatic hyperplasia is frequently found in rejected renal transplants^{3,47} and in psoriatic skin lesions.¹⁰ Furthermore, recent studies in mice have shown that bacterial infection

of the airways or inflammation of the skin induce a strong lymphangiogenic response in the affected tissue and in draining LNs.^{48–50} The exact role of inflammation-induced lymphangiogenesis is unclear at this point, but it is likely that lymphatic vessel formation and remodeling participates in the regulation of the immune response, by affecting the transport of antigen and leukocytes to draining LNs.^{49,51}

In recent years, several studies have documented the anti-angiogenic properties of different VEGFR TK inhibitors, and clinical trials with various compounds are generating promising data regarding the efficacy of these molecules in cancer therapy.¹⁷ In contrast to the currently developed biopharmaceuticals that target VEGF-A or its receptors, most VEGFR TK inhibitors under clinical evaluation can be administered orally. However, for certain VEGF-A-dependent pathological conditions, particularly for the treatment of chronic inflammatory skin disorders, the availability of a topically administered compound might be advantageous in that it would allow delivery of the active compound directly to the inflamed skin. Furthermore, topical administration could lower the risks of adverse effects associated with systemic administration of anti-angiogenic therapies.^{52,53} The latter may be particularly relevant when treating inflammatory disorders, which, in contrast to cancer, are only rarely life threatening. Taken together, our study provides the first proof of concept that it might be possible to treat inflammatory skin diseases by topical or oral administration of an anti-angiogenic compound.

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References

- Folkman J: Angiogenesis. *Annu Rev Med* 2006, 57:1–18
- Cao Y: Opinion: emerging mechanisms of tumour lymphangiogenesis and lymphatic metastasis. *Nat Rev Cancer* 2005, 5:735–743
- Kerjaschki D, Regele HM, Moosberger I, Nagy-Bojarski K, Watschinger B, Soleiman A, Birner P, Krieger S, Hovorka A, Silberhumer G, Laakkonen P, Petrova T, Langer B, Raab I: Lymphatic neoangiogenesis in human kidney transplants is associated with immunologically active lymphocytic infiltrates. *J Am Soc Nephrol* 2004, 15:603–612
- Bainbridge J, Sivakumar B, Paleolog E: Angiogenesis as a therapeutic target in arthritis: lessons from oncology. *Curr Pharm Des* 2006, 12:2631–2644
- Danese S, Sans M, de la Motte C, Graziani C, West G, Phillips MH, Pola R, Rutella S, Willis J, Gasbarrini A, Fiocchi C: Angiogenesis as a novel component of inflammatory bowel disease pathogenesis. *Gastroenterology* 2006, 130:2060–2073
- Leong TT, Fearon U, Veale DJ: Angiogenesis in psoriasis and psoriatic arthritis: clues to disease pathogenesis. *Curr Rheumatol Rep* 2005, 7:325–329
- Koch AE, Harlow LA, Haines GK, Amento EP, Unemori EN, Wong WL, Pope RM, Ferrara N: Vascular endothelial growth factor. A cytokine modulating endothelial function in rheumatoid arthritis. *J Immunol* 1994, 152:4149–4156
- Detmar M, Brown LF, Claffey KP, Yeo KT, Kocher O, Jackman RW, Berse B, Dvorak HF: Overexpression of vascular permeability factor/vascular endothelial growth factor and its receptors in psoriasis. *J Exp Med* 1994, 180:1141–1146
- Kanazawa S, Tsunoda T, Onuma E, Majima T, Kagiya M, Kikuchi K: VEGF, basic-FGF, and TGF-beta in Crohn's disease and ulcerative colitis: a novel mechanism of chronic intestinal inflammation. *Am J Gastroenterol* 2001, 96:822–828
- Kunstfeld R, Hirakawa S, Hong YK, Schacht V, Lange-Asschenfeldt B, Velasco P, Lin C, Fiebigler E, Wei X, Wu Y, Hicklin D, Bohlen P, Detmar M: Induction of cutaneous delayed-type hypersensitivity reactions in VEGF-A transgenic mice results in chronic skin inflammation associated with persistent lymphatic hyperplasia. *Blood* 2004, 104:1048–1057
- Young HS, Summers AM, Bhushan M, Brenchley PE, Griffiths CE: Single-nucleotide polymorphisms of vascular endothelial growth factor in psoriasis of early onset. *J Invest Dermatol* 2004, 122:209–215
- Ferrara N, Gerber HP, LeCouter J: The biology of VEGF and its receptors. *Nat Med* 2003, 9:669–676
- Kerr DJ: Targeting angiogenesis in cancer: clinical development of bevacizumab. *Nat Clin Pract Oncol* 2004, 1:39–43
- Lu D, Shen J, Vil MD, Zhang H, Jimenez X, Bohlen P, Witte L, Zhu Z: Tailoring in vitro selection for a picomolar affinity human antibody directed against vascular endothelial growth factor receptor 2 for enhanced neutralizing activity. *J Biol Chem* 2003, 278:43496–43507
- Rudge JS, Thurston G, Davis S, Papadopoulos N, Gale N, Wiegand SJ, Yancopoulos GD: VEGF trap as a novel antiangiogenic treatment currently in clinical trials for cancer and eye diseases, and VEGF-based discovery of the next generation of angiogenesis targets. *Cold Spring Harb Symp Quant Biol* 2005, 70:411–418
- Underiner TL, Ruggeri B, Gingrich DE: Development of vascular endothelial growth factor receptor (VEGFR) kinase inhibitors as anti-angiogenic agents in cancer therapy. *Curr Med Chem* 2004, 11:731–745
- Morabito A, De Maio E, Di Maio M, Normanno N, Perrone F: Tyrosine kinase inhibitors of vascular endothelial growth factor receptors in clinical trials: current status and future directions. *Oncologist* 2006, 11:753–764
- Grosios K, Wood J, Esser R, Raychaudhuri A, Dawson J: Angiogenesis inhibition by the novel VEGF receptor tyrosine kinase inhibitor. PTK787/ZK222584, causes significant anti-arthritis effects in models of rheumatoid arthritis. *Inflamm Res* 2004, 53:133–142
- Garcia-Echeverria C, Pearson MA, Marti A, Meyer T, Mestan J, Zimmermann J, Gao J, Brueggen J, Capraro HG, Cozens R, Evans DB, Fabbro D, Furet P, Porta DG, Liebetanz J, Martiny-Baron G, Ruetz S, Hofmann F: In vivo antitumor activity of NVP-AEW541-A novel, potent, and selective inhibitor of the IGF-IR kinase. *Cancer Cell* 2004, 5:231–239
- Traxler P, Allegrini PR, Brandt R, Brueggen J, Cozens R, Fabbro D, Grosios K, Lane HA, McSheehy P, Mestan J, Meyer T, Tang C, Wartmann M, Wood J, Caravatti G: AEE788: a dual family epidermal growth factor receptor/ErbB2 and vascular endothelial growth factor receptor tyrosine kinase inhibitor with antitumor and antiangiogenic activity. *Cancer Res* 2004, 64:4931–4941
- Kajiya K, Hirakawa S, Ma B, Drinnenberg I, Detmar M: Hepatocyte growth factor promotes lymphatic vessel formation and function. *EMBO J* 2005, 24:2885–2895
- Detmar M, Tenorio S, Hettmannsperger U, Ruszczak Z, Orfanos CE: Cytokine regulation of proliferation and ICAM-1 expression of human dermal microvascular endothelial cells in vitro. *J Invest Dermatol* 1992, 98:147–153
- Hong YK, Lange-Asschenfeldt B, Velasco P, Hirakawa S, Kunstfeld R, Brown LF, Bohlen P, Senger DR, Detmar M: VEGF-A promotes tissue repair-associated lymphatic vessel formation via VEGFR-2 and the alpha1beta1 and alpha2beta1 integrins. *FASEB J* 2004, 18:1111–1113
- Schacht V, Ramirez MI, Hong YK, Hirakawa S, Feng D, Harvey N, Williams M, Dvorak AM, Dvorak HF, Oliver G, Detmar M: T1alpha/podoplanin deficiency disrupts normal lymphatic vasculature formation and causes lymphedema. *EMBO J* 2003, 22:3546–3556
- Xia YP, Li B, Hylton D, Detmar M, Yancopoulos GD, Rudge JS: Transgenic delivery of VEGF to mouse skin leads to an inflammatory condition resembling human psoriasis. *Blood* 2003, 102:161–168
- El-Gamal S, Hoffmann K, Steinert P, Gassmüller J, Altmeyer P: Objective assessment of human skin reaction to sun and UV-B. *Nonin-*

- vasive Methods for the Quantification of Skin Functions. Edited by Frosch PJ, Kligman AM. Berlin, Springer, 1993, pp 133–144
27. Duteil I, Queille-Roussel C, Czernielewski J: Assessing treatment of psoriasis and eczema by noninvasive methods. *Noninvasive Methods for the Quantification of Skin Functions*. Edited by Frosch PJ, Kligman AM. Berlin, Springer, 1993, pp 223–240
28. Meingassner JG, Grassberger M, Fahrngruber H, Moore HD, Schuurman H, Stutz A: A novel anti-inflammatory drug. SDZ ASM 981, for the topical and oral treatment of skin diseases: in vivo pharmacology *Br J Dermatol* 1997, 137:568–576
29. Skobe M, Hawighorst T, Jackson DG, Prevo R, Janes L, Velasco P, Riccardi L, Alitalo K, Claffey K, Detmar M: Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. *Nat Med* 2001, 7:192–198
30. Oura H, Bertoncini J, Velasco P, Brown LF, Carmeliet P, Detmar M: A critical role of placental growth factor in the induction of inflammation and edema formation. *Blood* 2003, 101:560–567
31. Hirakawa S, Kodama S, Kunstfeld R, Kajiya K, Brown LF, Detmar M: VEGF-A induces tumor and sentinel lymph node lymphangiogenesis and promotes lymphatic metastasis. *J Exp Med* 2005 201:1089–1099
32. Tobler NE, Detmar M: Tumor and lymph node lymphangiogenesis—impact on cancer metastasis. *J Leukoc Biol* 2006, 80:691–696
33. Joukov V, Sorsa T, Kumar V, Jeltsch M, Claesson-Welsh L, Cao Y, Saksela O, Kalkinen N, Alitalo K: Proteolytic processing regulates receptor specificity and activity of VEGF-C. *EMBO J* 1997, 16:3898–3911
34. Detmar M, Brown LF, Schon MP, Elicker BM, Velasco P, Richard L, Fukumura D, Monsky W, Claffey KP, Jain RK: Increased microvascular density and enhanced leukocyte rolling and adhesion in the skin of VEGF transgenic mice. *J Invest Dermatol* 1998, 111:1–6
35. Miles AA, Miles EM: Vascular reactions to histamine, histamine-liberator and leukotaxine in the skin of guinea-pigs. *J Physiol* 1952, 118:228–257
36. Schmook FP, Meingassner JG, Billich A: Comparison of human skin or epidermis models with human and animal skin in in-vitro percutaneous absorption. *Int J Pharm* 2001, 215:51–56
37. Bhushan M, McLaughlin B, Weiss JB, Griffiths CE: Levels of endothelial cell stimulating angiogenesis factor and vascular endothelial growth factor are elevated in psoriasis. *Br J Dermatol* 1999, 141:1054–1060
38. Zhang Y, Matsuo H, Morita E: Increased production of vascular endothelial growth factor in the lesions of atopic dermatitis. *Arch Dermatol Res* 2006, 297:425–429
39. Brown LF, Harrist TJ, Yeo KT, Stahle-Backdahl M, Jackman RW, Berse B, Tognazzi K, Dvorak HF, Detmar M: Increased expression of vascular permeability factor (vascular endothelial growth factor) in bullous pemphigoid, dermatitis herpetiformis, and erythema multiforme. *J Invest Dermatol* 1995, 104:744–749
40. Kajiya K, Detmar M: An important role of lymphatic vessels in the control of UVB-induced edema formation and inflammation. *J Invest Dermatol* 2006, 126:919–921
41. Hirakawa S, Fujii S, Kajiya K, Yano K, Detmar M: Vascular endothelial growth factor promotes sensitivity to ultraviolet B-induced cutaneous photodamage. *Blood* 2005, 105:2392–2399
42. Cueni LN, Detmar M: New insights into the molecular control of the lymphatic vascular system and its role in disease. *J Invest Dermatol* 2006, 126:2167–2177
43. Sawano A, Iwai S, Sakurai Y, Ito M, Shitara K, Nakahata T, Shibuya M: Flt-1, vascular endothelial growth factor receptor 1, is a novel cell surface marker for the lineage of monocyte-macrophages in humans. *Blood* 2001, 97:785–791
44. Skobe M, Hamberg LM, Hawighorst T, Schirner M, Wolf GL, Alitalo K, Detmar M: Concurrent induction of lymphangiogenesis, angiogenesis, and macrophage recruitment by vascular endothelial growth factor-C in melanoma. *Am J Pathol* 2001, 159:893–903
45. Wilgus TA, Matthies AM, Radek KA, Dovi JV, Burns AL, Shankar R, DiPietro LA: Novel function for vascular endothelial growth factor receptor-1 on epidermal keratinocytes. *Am J Pathol* 2005, 167:1257–1266
46. Man XY, Yang XH, Cai SQ, Yao YG, Zheng M: Immunolocalization and expression of vascular endothelial growth factor receptors (VEGFRs) and neuropilins (NRPs) on keratinocytes in human epidermis. *Mol Med* 2006, 12:127–136
47. Kerjaschki D, Huttary N, Raab I, Regele H, Bojarski-Nagy K, Bartel G, Krober SM, Greinix H, Rosenmaier A, Karhofer F, Wick N, Mazal PR: Lymphatic endothelial progenitor cells contribute to de novo lymphangiogenesis in human renal transplants. *Nat Med* 2006, 12:230–234
48. Baluk P, Tammela T, Ator E, Lyubynska N, Achen MG, Hicklin DJ, Jeltsch M, Petrova TV, Pytowski B, Stacker SA, Yla-Herttuala S, Jackson DG, Alitalo K, McDonald DM: Pathogenesis of persistent lymphatic vessel hyperplasia in chronic airway inflammation. *J Clin Invest* 2005, 115:247–257
49. Angeli V, Ginhoux F, Llodra J, Quemeneur L, Frenette PS, Skobe M, Jessberger R, Merad M, Randolph GJ: B cell-driven lymphangiogenesis in inflamed lymph nodes enhances dendritic cell mobilization. *Immunity* 2006, 24:203–215
50. Halin C, Tobler NE, Vigl B, Brown LF, Detmar M: VEGF-A produced by chronically inflamed tissue induces lymphangiogenesis in draining lymph nodes. *Blood* 2007, 110:3158–3167
51. Halin C, Detmar M: An unexpected connection: lymph node lymphangiogenesis and dendritic cell migration. *Immunity* 2006, 24:129–131
52. Hurwitz H, Saini S: Bevacizumab in the treatment of metastatic colorectal cancer: safety profile and management of adverse events. *Semin Oncol* 2006, 33:S26–S34
53. Los M, Roodhart JM, Voest EE: Target practice: lessons from phase III trials with bevacizumab and vatalanib in the treatment of advanced colorectal cancer. *Oncologist* 2007, 12:443–450