SAR131675, a Potent and Selective VEGFR-3–TK Inhibitor with Antilymphangiogenic, Antitumoral, and Antimetastatic Activities

Antoine Alam1, Isabelle Blanc1, Geneviève Gueguen-Dorbes1, Olivier Duclos2, Jacques Bonnin1, Pauline Barron1, Marie-Claude Laplace1, Gaelle Morin1, Florence Gaujarengues1, Frédérique Dol1, Jean-Pascal Hérault1, Paul Schaeffer1, Pierre Savi1, and Françoise Bono1

Abstract

SAR131675 is a potent and selective VEGFR-3 inhibitor. It inhibited VEGFR-3 tyrosine kinase activity and VEGFR-3 autophosphorylation in HEK cells with IC50 values of 20 and 45 nmol/L, respectively. SAR131675 dose dependently inhibited the proliferation of primary human lymphatic cells, induced by the VEGFR-3 ligands VEGFC and VEGFD, with an IC50 of about 20 nmol/L. SAR131675 was found to be highly selective for VEGFR-3 versus 107 receptors, enzymes, ion channels, and 65 kinases. However, it was moderately active on VEGFR-2 with a VEGFR-3/VEGFR-2 ratio of about 10. SAR131675 had no antiproliferative activity on a panel of 30 tumors and primary cells, further showing its high specificity and indicating that SAR131675 is not a cytotoxic or cytostatic agent. SAR131675 was very well tolerated in mice and showed a potent antitumoral effect in several orthotopic and syngenic models, including mammary 4T1 carcinoma and RIP1.Tg2 tumors. Interestingly, it significantly reduced lymph node invasion and lung metastasis, showing its antilymphangiogenic activity in vivo. Moreover, treatment of mice before resection of 4T1 primary tumors was sufficient to prevent metastasis. Tumor-associated macrophages (TAM) play an important role in tumor growth and metastasis. The expression of VEGFR-3 on TAMs has been recently described. F4/80 immunostaining clearly showed that SAR131675 significantly reduced TAM infiltration and aggregation in 4T1 tumors. Taken together, SAR131675 is the first highly specific VEGFR-3-TK inhibitor described to date, displaying significant antitumoral and antimetastatic activities in vivo through inhibition of lymphangiogenesis and TAM invasion.

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Introduction

In most solid tumors, metastases in regional lymph nodes occur frequently in the initial process of cancer dissemination and are a strong indicator of poor patient prognosis (1). A number of clinical and experimental data suggest that migration of tumor cells into the lymph nodes is greatly facilitated by tumor lymphangiogenesis (2).

One key molecule mediating tumor lymphangiogenesis is the VEGF receptor-3 (VEGFR-3), a tyrosine kinase receptor recognized and activated by VEGFC and VEGFD, commonly expressed in malignant and tumor-infiltrating stromal cells (3). In experimental tumor models, VEGFC and VEGFD expression induces lymphangiogenesis and correlates with lymphatic invasion and nodal metastasis (4, 5). In addition, agents neutralizing VEGFC and VEGFD or blocking VEGFR-3 signaling suppress development of new lymphatic vessels and tumor metastasis in experimental cancer models (6, 7).

Although VEGFC and VEGFD are the sole ligands described to date as binding directly to VEGFR-3 (8, 9), blunting of both ligands at the same time does not result in the embryonic blood vasculature defects seen in VEGFR-3−/− mice (10–12). This suggests that additional ligands exist or that ligand-independent signaling occurs. Indeed, whereas VEGFA binds only to VEGFR-2, we and others have shown that VEGFA may induce formation of VEGFR-2-/3 heterodimers (13, 14). This suggests that when cells express both receptors, VEGFR-3 inhibitors may also block some VEGFA signaling.

In adults, VEGFR-3 is found only on endothelial cells of lymphatic vessels. However, in malignant tumors, VEGFR-3 expression is found on vascular endothelial cells and particularly on angiogenic sprouts. Genetic targeting of VEGFR-3 or blocking of VEGFR-3 signaling with monoclonal antibodies results in decreased sprouting and

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Corresponding Author: Françoise Bono, Sanofi Recherche et Développement, Early to Candidate DPU, Toulouse; and Sanofi Recherche et Développement, Fibrosis TSU, Chilly Mazarin, France

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vascular density, suggesting that VEGFR-3 plays a crucial role in tumor angiogenesis (15, 16).

There is considerable evidence of the expression of VEGFR-3 in several primary tumors, including colorectal, ovarian, gastric, and others (17–19), and its upregulation in breast cancer has been shown to precede tumor cell invasion (20). Interestingly, coexpression of VEGFR-3 with VEGFC in human cancer cells was associated with increased metastasis and poorer survival, suggesting a possible autocrine loop between VEGFC and VEGFR-3 in cancer cells (21, 22).

Expression of VEGFR-3 has also been reported on macrophages after brain ischemia, kidney transplantation, chronic airway inflammation, or corneal injury (23–27) and also on tumor-associated macrophages (TAM) (28, 29). TAMs are critical regulators of angiogenesis and lymphangiogenesis; they express VEGFA and VEGFC and induce tip-cell formation and fusion (16, 30–33). In addition, they are thought to directly contribute to lymphatic vessel formation by transdifferentiating into lymphatic endothelial cells (25, 28, 33, 34).

Collectively, these data implicate VEGFR-3 in different processes of tumor growth and metastasis, suggesting that therapeutic targeting of VEGFR-3 might selectively reduce tumor growth and metastasis.

To obtain maximal beneficial effects without causing severe side effects, VEGFR-3 selective compounds are required. Although several marketed multikinase inhibitors, including sunitinib and sorafenib, have been reported to reduce lymphangiogenesis, angiogenesis, and TAM selectivity for VEGFR-3 and show that SAR131675 is able to inhibit VEGFR-3–TK inhibitor under evaluation. Here we present anti–VEGFR-3 activity, currently there is no specific VEGFR-3–TK inhibitor under evaluation. We describe, for the first time, a small molecule with high selectivity for VEGFR-3 and show that SAR131675 is able to reduce lymphangiogenesis, angiogenesis, and TAM infiltration and consequently reduce tumor growth and metastasis.

Materials and Methods

Reagents

Accustain, Drabkin, and the human recombinant hemo-
globin were obtained from Sigma. The proteome array Phospho-MAPK (mitogen-activated protein kinase), the DuoSet ELISA Phospho-ERK1/2, and the recombinant proteins bFGF, VEGFA, and VEGFD were purchased from R&D and VEGFC from ReliaTech, huVEGFR-1-TK from Cell Signalling, and huVEGFR-2-TK from Cell Signalling: huVEGFR-2-TK was produced internally.

Animals

All animal treatment procedures described in this study were approved by the Animal Care and Use Committee of sanofi. Female BALB/cByJ mice and RIP1.Tag2 mice on the C57Bl/6J background were obtained from Charles River France.

Treatment

SAR131675 was dissolved on the day of use in a 0.6% methylcellulose/0.5% Tween 80 solution.

Cell lines

Human tumor cell lines were obtained from DSMZ or the American Type Culture Collection (ATCC). No further authentication was carried out. Cells were cultured in the culture medium in a humidified atmosphere of 5% CO2 and 95% air at 37°C. Cell viability was determined by trypan blue exclusion and always exceeded 95%.

Complete cell culture medium for human cell proliferation was RPMI-1640 (Gibco Laboratories) containing 10% heat-inactivated FCS (fetal calf serum; Gibco Laboratories), 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 100 UI/mL penicillin, and 100 µg/mL streptomycin (Gibco Laboratories).

Adult human lymphatic microvascular endothelial cells (HLMVEC) were purchased from Cambrex, maintained in culture as described previously (35), and were tested and authenticated in the laboratory for the expression of VEGFR-3 and Prox1 as lymphatic cell markers. Cells were used up to passage 6. 4T1 mouse mammary adenocarcinoma cells were obtained from the ATCC (CRL-2539) and were maintained in RPMI-1640 containing 10% heat-inactivated FCS and 2 mmol/L L-glutamine.

Authentication of the cell line was done by expression of osteopontin as described below and used up to passage 25.

Tyrosine kinase assay

Multiwell plates were precoated with a synthetic polymer substrate poly-Glu-Tyr (polyGT 4:1). The reaction was carried out in the presence of kinase buffer (10×; 30 mmol/L HEPES buffer, pH 7.4, 20 mmol/L MgCl2, 0.1 mmol/L MnCl2, and 0.2 mmol/L Na3VO4) supplemented with ATP and dimethyl sulfoxide (DMSO) for the positive control (C+) or SAR131675 (ranging from 3–1,000 nmol/L). ATP was used at 30 µmol/L for VEGFR-1 and VEGFR-3 and at 15 µmol/L for VEGFR-2. The phosphorylated poly-Glu-Tyr was probed with a phosphotyrosine specific monoclonal antibody (mAb) conjugated to horseradish peroxidase (HRP; 1/30,000; clone PT66; Sigma) and developed in the dark with the HRP chromogenic substrate (OPD). The reaction was then stopped by the addition of 100 µL 1.25 mol/L H2SO4, and absorbance was determined using an Envision spectrophotometer at 492 nm.

Percent inhibition (%) was calculated using the following formula: % = 100 – ([SAR131675] – C–)/([C+ – C–] · 100, in which [SAR131675] represents the value obtained in the presence of the indicated concentration of SAR131675.

Autophosphorylation in HEK cells and survival and migration assays of HLMVCs

Experiments were conducted as described previously (35). Twenty-four hours after transfection, the cells were treated during 1 hour with orthovanadate (100 mmol/L), harvested, collected, and, after counting, distributed in 5 mL tubes in the presence of the indicated concentration of SAR131675. After 30 minutes of incubation, the reaction was stopped by the addition of cold PBS supplemented by ATP.
orthovanadate. Cells were then lysed with 150 μL of radioimmunoprecipitation assay (RIPA) buffer over 15 minutes at 4°C and then centrifuged for 10 minutes at 10,000 × g. Supernatants were distributed in duplicate on 96-well plates precoated with the anti-Flag and left for 1 hour at room temperature. After 3 washes, the anti–phosphotyrosine conjugated to the HRP was added and incubated for 1 hour at room temperature. Wells were then washed 3 times with TBS buffer containing 0.5% Tween 20 and 2 mmol/L MgCl2. The reaction was stopped with 50 μL of 2N H2SO4, and the signal was read using an Envision spectrophotometer at 485 and 530 nm.

**Survival assay**

HLMVECs were seeded in 96-well plates coated with 0.3% gelatin (5 × 10⁵ cells per well). Cells were incubated in RPMI 0.1% FCS with VEGFA (10 ng/mL) VEGFC (300 ng/mL), or FGF2 (10 ng/mL) in the absence or presence of SAR131675. Five days later, viable cells were quantified with the cell Titer-glo luminescent cell viability assay (Promega) as previously described (35).

**Migration assay**

The migration assay was carried out with the BD Bio-Coat Angiogenesis System-Endothelial Cell Migration Kit (BD Biosciences). HLMVECs (1 × 10⁵ cells per well in RPMI) were added in triplicate in the upper chambers. The lower chambers were loaded with RPMI alone, RPMI 0.1% FCS with VEGFA (10 ng/mL) VEGFC (300 ng/mL) or FGF2 (10 ng/mL) in the presence or not of the indicated concentration of SAR131675, lysed and subjected to phospho-MAPK array or to phospho Erk ELISA according to the manufacturer’s instructions. Fluorescence of cells that had migrated was measured on a TECAN GENios Microplate reader (TECAN).

**Proteome array and Erk phosphorylation**

HLMVECs were plated in 6-well tissue culture plates at a density of 2 × 10⁵ cells for 48 hours. Cells were then serum deprived for 2 hours and stimulated or not for 10 minutes with VEGFC (500 ng/mL) in the presence or not of the indicated concentration of SAR131675, lysed and subjected to phospho-MAPK array or to phospho Erk ELISA according to manufacturer’s instructions.

**Quantification of VEGFR-2 phosphorylation by ELISA**

Quantification of VEGFR-2 phosphorylation by ELISA was carried out as described previously using porcine aortic endothelial cells (PAEC) expressing VEGFR-2 (35).

**Blood vessel formation in zebrafish**

Embryos from transgenic zebrafish (*Danio rerio*) that specifically express the fluorescent protein copGFP in the vascular system were used. The chorion of the 24 hpf (hours post fertilization) embryos were removed, and embryos were dispensed in a 96-well assay plate in embryo water mixed with SAR131675 solubilized in DMSO. After 24 hours at 28.5°C, the embryos were anes-theorized with tricaine, and the number of intersegmental vessels was analyzed by fluorescence microscopy (×2.5 magnification).

**Angiogenesis and lymphangiogenesis induced in a sponge implant mouse model**

Sterile sponge disks (Cellspon; Interchim) impregnated with 200 μg of FGF2 or PBS were subcutaneously introduced on the back of anaesthetized mice. FGF2 was reimplanted into the sponges the first 2 days. Daily oral treatment with SAR131675 (30, 100, and 300 mg/kg/d) started the day of sponge implantation. Seven days later, the animals were euthanized and the sponges were removed, harvested, and lysed in RIPA buffer at 4°C. After a centrifugation at 6,000 × g, the supernatants were collected for further analysis.

**RIPL.Tag2 transgenic mouse models**

For the prevention study, treatment of mice (9 per group) started at 5 weeks of age for 5 weeks and then the number of Langherans islets (red islets) was determined after retrograde perfusion with collagenase solution through the common bile duct (36). For the intervention study, mice were treated daily from 10 weeks of age for 16 days and tumor volume was measured and calculated as for 4T1 tumors below. The tumor burden was calculated as the sum of individual tumor volumes for each mouse. For the survival study, daily treatment (20 mice per group) started at 12 weeks of age and mice were monitored daily to detect moribund mice.

**4T1 mammary carcinoma model**

4T1 cells (10⁵) were implanted into mammary fat pads of BALB/c mice (15 per group; ref. 37). Daily oral treatment with SAR131675 (30 and 100 mg/kg/d) started at day 5. Tumors were measured 2 to 3 times weekly with calipers. The tumor volume (V) was calculated using the formula $V = 0.52 \times a^{2} \times b$ (a: smallest tumor diameter and b: largest tumor diameter). At day 21, the tumors, the lungs, and the axillary lymph nodes were removed. The number of metastases at the surface of each lung was counted. The tumors and the lymph nodes were lysed in RIPA buffer at 4°C. The osteopontin level in lymph nodes was quantified using an ELISA kit (Assay Design).

**4T1 tumor excision model**

Implantation of cells and treatment was started as described above, but primary tumors were removed at day 15 in all groups and the tumor weight was evaluated. The mice treated with SAR131675 were then divided into 2 groups (12 mice per group); in the first group, treatment with SAR131675 was stopped whereas in the second, mice were treated up to day 26. At day 26, lung metastatic foci were counted.

**Immunohistochemistry**

Tissues were fixed with 10% acetic acid for 24 hours, dehydrated, and embedded in paraffin. Immunostaining
was done in sections incubated with anti–mouse CD31 Ab (1/50), LYVE1 (1/1,000; Santa Cruz) or F4-80 (1/50; eBiosciences, BD PharMingen) following incubation with the Vectastain ABC Kit (Vector laboratories) appropriate to the species of primary antibody, and antigens were developed with 3,3′-diaminobenzidine peroxidase substrate and counterstained with hematoxylin. Images were captured with a camera (SONY) mounted on a Nikon microscope.

**Statistical analysis**

IC\textsubscript{50} values were obtained using internal software Biost@t-SPEED v2.0 with the 4-parameter logistic model. For the *in vivo* studies, results are given as mean ± SEM. Differences between groups were examined for statistical significance using 2-way ANOVA following Dunnett test or confidence interval calculation as specified in the results part. Statistical analysis of data from the survival study was conducted using a log-rank test. For tumor regression analysis, 2-way ANOVA with repeated measures was carried out.

**Results**

**In vitro effect of SAR131675 on VEGFR-3 tyrosine kinase activity**

SAR131675 is the result of high-throughput screening and chemical optimization (Fig. 1A). Its ability to inhibit the kinase activity of VEGFR-3 was first determined by ELISA using recombinant human (rh)-VEGFR-3, with ATP and polyGT as substrates. SAR131675 dose dependently inhibited rh-VEGFR-3–TK activity with an IC\textsubscript{50} of 23 ± 7 nmol/L (Fig. 1B, n = 4). Under the same conditions, SU11248 (sunitinib) inhibited VEGFR-3–TK activity with an IC\textsubscript{50} of 10 nmol/L (a representative set of data with sunitinib is shown in Supplementary Fig. S1). SAR131675 dose dependently inhibited rh-VEGFR-3–TK activity with an IC\textsubscript{50} of 23 ± 7 nmol/L (Fig. 1B, n = 4). Under the same conditions, SU11248 (sunitinib) inhibited VEGFR-3–TK activity with an IC\textsubscript{50} of 10 nmol/L (a representative set of data with sunitinib is shown in Supplementary Fig. S1). SAR131675 is an ATP-competitive inhibitor; it inhibited VEGFR-3–TK activity with a K\textsubscript{i} of about 12 nmol/L, similar to its IC\textsubscript{50} value (Supplementary Fig. S1).
The effect of SAR131675 on VEGFR-3 autophosphorylation was evaluated after overexpression in HEK cells. SAR131675 was seen to be cell permeable and inhibited VEGFR-3 autophosphorylation in a dose-dependent manner with an IC₅₀ ranging from 30 to 50 nmol/L (Fig. 1C). In this assay, sunitinib inhibited VEGFR-3 autophosphorylation with an IC₅₀ ranging from 10 to 30 nmol/L (not shown). SAR131675 was also evaluated on 2 different variants of VEGFR-3 and also on murine flt4. The results indicated that SAR131675 has a similar inhibitory effect on both human variants and murine VEGFR-3 (Supplementary Fig. S1).

The selectivity of SAR131675 toward VEGFR-1 and VEGFR-2 was evaluated using rh-VEGFR-1 and rh-VEGFR-2 ELISA assays as for VEGFR-3. SAR131675 inhibited VEGFR-1–TK activity with an IC₅₀ > 3 µmol/L (I% = 31 at 3 µmol/L) and VEGFR-2–TK activity with an IC₅₀ of 235 nmol/L (I% = 48 at 1 µmol/L, Table 1).

In the same assay, sunitinib inhibited VEGFR-1 and VEGFR-2 with an IC₅₀ of 64 and 14 nmol/L, respectively (not shown). In the autophosphorylation assay in HEK cells, SAR131675 inhibited VEGFR-1 autophosphorylation with an IC₅₀ of about 1 µmol/L and VEGFR-2 with an IC₅₀ of about 280 nmol/L (Table 1). These results confirmed that SAR131675 moderately inhibits VEGFR-2 and has very little effect on VEGFR-1, showing a good selectivity for VEGFR-3, which is not the case for sunitinib.

To further confirm the activity of SAR131675 on VEGFR-2, we used PAECs stably expressing human VEGFR-2. SAR131675 inhibited VEGF-induced phosphorylation in a dose-dependent manner, with an IC₅₀ of 239 nmol/L, confirming the results obtained in HEK293T cells after VEGFR-2 overexpression (Fig. 1D and E).

The selectivity of SAR131675 was further confirmed as it was inactive on a panel of 65 kinases, on a panel of 107 nonkinase enzymes and receptors, and on 21 ion channels (Supplementary Tables S1, S2A and B, respectively). We ruled out the eventuality of any nonspecific cytotoxic or cytostatic effect of SAR131675 by investigating its effect on the proliferation of tumor cell lines. SAR131675 did not significantly inhibit the proliferation of any of the cell lines studied at concentrations up to 10 µmol/L (Supplementary Table S3), thus confirming the specificity of SAR131675.

Together, these results showed that SAR131675 is a potent and selective inhibitor of VEGFR-3–TK with moderate activity toward VEGFR-2 and with no cytostatic or cytotoxic effects on tumor or primary cells.

**In vitro effect of SAR131675 on lymphatic cell survival and migration**

We evaluated the effect of SAR131675 on the in vitro survival of primary human lymphatic cells induced by specific VEGFR-3 ligands, VEGFC and VEGFD, and by unrelated growth factors, VEGFA and FGF-2. As shown in Fig. 2A, SAR131675 potently inhibited lymphatic cell survival induced by VEGFC and VEGFD (IC₅₀ of 14 and 17 nmol/L, respectively). It inhibited VEGFA-induced survival with an IC₅₀ of about 100 nmol/L, confirming its moderate activity on VEGFR-2 and had no effect on FGF2-induced proliferation. In the same experiment, sunitinib inhibited VEGFA-α, VEGFA-C, and VEGFA-D-induced survival with an IC₅₀ of about 100 nmol/L and, consistent with reported data (38), it had no effect on FGF2-induced survival (Supplementary Fig. S1).

We then evaluated the effect of SAR131675 on HLMVEC migration induced by VEGFA or VEGFC in Boyden chambers. SAR131675 potently inhibited VEGFC-induced migration with an IC₅₀ of about 30 nmol/L (Fig. 2B) and VEGFA-induced migration with an IC₅₀ of about 100 nmol/L (Fig. 2C).

Using the Phospho-MAP array in HLMVECs, Erk was identified as the major phosphorylated kinase upon VEGFC stimulation (Fig. 2D). SAR131675, significantly and dose dependently inhibited Erk phosphorylation, with an IC₅₀ of about 30 nmol/L (Fig. 2E), confirming that SAR131675 is a potent inhibitor of VEGFR-3 signaling.

**Effect of SAR131675 on angiogenesis and lymphangiogenesis in nontumoral models in vivo**

The in vivo activity of SAR131675 was first investigated in embryonic angiogenesis using the zebrafish model (Danio rerio) expressing GFP under the control of a vascular specific promoter. Treatment with SAR131675 started when blood flow began, 24 hours postfertilization. Embryos treated with 3 µmol/L SAR131675 for 24 hours showed fewer intersegmental vessels in the tail compared with control embryos and at the dose of 10 µmol/L intersegmental vessels largely failed to form (Fig. 3A). Thus, in this model, SAR131675 efficiently impaired embryonic vasculogenesis.
Using a corneal model, a high concentration of FGF2 has been reported to induce VEGFR-3-dependent lymphangiogenesis (39). We therefore developed a similar approach by subcutaneous implantation of a sterile sponge disk impregnated with FGF2 in mice (Fig. 3B). In this model, FGF2 induces angiogenesis that is measured by CD31 immunostaining and hemoglobin content (Fig. 3D), as well as lymphangiogenesis that is measured by LYVE-1 immunostaining and by VEGFR-3 quantification using ELISA (Fig. 3E). FGF2 induced a 3- to 5-fold increase of hemoglobin and VEGFR-3, thus confirming that in these conditions, FGF2 induces angiogenesis and lymphangiogenesis (Fig. 3D).

Treatment of mice with SAR131675 (30, 100, or 300 mg/kg/d) started on the day of sponge implantation.

Seven days later, SAR1316765 at 100 mg/kg/d had significantly reduced the levels of VEGFR-3 (Fig. 3H) and hemoglobin content (Fig. 3I) by about 50%, (P < 0.001). Treatment with 300 mg/kg/d gave levels of VEGFR-3 and hemoglobin that were similar to those of the control group. Thus, SAR131675 efficiently abrogates lymphangiogenesis and angiogenesis induced in vivo by FGF2.

Furthermore, at 100 mg/kg SAR131675 did not show any effect on arterial pressure whereas at 300 mg/kg, a minor and transient increase of blood pressure was observed (Supplementary Fig. S2). Because the inhibition of VEGFR-2 signaling is known to increase blood pressure and given our in vitro results, we concluded that SAR131675 at a dose of 300 mg/kg is able to inhibit both VEGF-2 and VEGF-3 signaling in vivo. In consequence...
100 mg/kg was the highest dosage used in subsequent in vivo studies.

**Effect of SAR131675 on carcinogenesis in the RIP1.Tag2 model**

The RIP1.Tag2 transgenic mouse is a well-characterized multistep carcinogenesis model, which arises from targeted oncogene expression in the insulin-producing beta cells (40). We investigated the efficacy of SAR131675 on the angiogenic switch (prevention study), on asymptomatic small tumors (intervention study), and finally on advanced near-end-stage cancers (survival study) as illustrated in Fig. 4A.

In the prevention study, 5 weeks treatment with SAR131675 was well tolerated and the number of angiogenic islets in the pancreas of SAR131675-treated mice was significantly decreased (42%, \( P < 0.001 \)) compared with the vehicle-treated group (Fig. 4B). In the intervention study, daily oral administration of SAR131675 from week 10 to week 12.5 caused a significant decrease in tumor burden (62%, \( P < 0.05 \), Fig. 4C). In these 2 studies a significant decrease of CD31-positive vessels was observed in the SAR131675-treated mice compared with control mice. In the survival study, in which treatment started at week 12 and continued until the death of the mice, mice under vehicle treatment started to die at 12 weeks with a median survival age of 13 weeks. Daily treatment with SAR131675 significantly extended the median survival age of the mice to 15 weeks (Fig. 4D, \( P < 0.01 \)). Taken together, these results showed that SAR131675 is able to prevent the angiogenic switch and slow tumor growth.

**Effect of SAR131675 on growth and metastases of 4T1 mammary carcinoma tumors in mice**

4T1 cells were implanted orthotopically into mammary fat pads in mice who were then orally treated with vehicle or SAR131675 (30 and 100 mg/kg/d) from days 5 to 21 after implantation.

SAR131675 was well tolerated as no loss of body weight was observed in the 2 treated groups. Treatment with SAR131675 significantly reduced the tumor volume (24% \( P < 0.05 \) and 50% \( P < 0.001 \) at 30 and 100 mg/kg/d, respectively; Fig. 5A). Development of 4T1 tumors was associated with important vascularization and peritumoral lymphangiogenesis.
as measured by CD31 and anti-LYVE1 immunostaining (Supplementary Fig. S3A and C, respectively). Interestingly, as illustrated in Supplementary Fig. S3B and D, SAR131675 seems to reduce both parameters. Moreover, SAR131675 at 30 and 100 mg/kg/d significantly reduced VEGFR-3 levels in the tumors by 39% ($P = 0.05$) and 51% ($P < 0.01$), respectively (Fig. 5B), thus confirming its potent antilymphangiogenic properties.

Because human breast cancer leads to high levels of osteopontin, we quantified osteopontin levels in 4T1 cells in vitro and showed a good correlation with cell number ($R^2 = 0.998$, supplementary Fig. S3E and F). Interestingly, the osteopontin content dramatically increased in sentinel lymph nodes, indicating an infiltration by metastatic 4T1 cells (Fig. 5B). SAR131675, at 100 mg/kg, significantly reduced the osteopontin content in lymph nodes (56%, $P < 0.001$), thus confirming its potent antilymphangiogenic properties.

At day 21, the number of macroscopic lung metastases was reduced in the treated groups by 17% (ns) and 28% ($P < 0.05$) at 30 and 100 mg/kg/d, respectively (Fig. 5D). Although sunitinib strongly reduced tumor growth (82%, Supplementary Fig. S4A), it is interesting to note that in this model, sunitinib at 50 mg/kg/d had no effect on the number of lung metastases (Supplementary Fig. S4A).

Altogether, these results showed that SAR131675 is a potent antitumoral agent, acting through antiangiogenic and antilymphangiogenic effects. Moreover, it reduces the migration of cancer cells into lymph nodes and lungs.

**Effect of preoperative treatment with SAR131675 on distant metastasis**

Recent reports suggest that antiangiogenic compounds may promote tumor invasion after resection of the primary tumor (41). We evaluated the effect of SAR131675 on distant metastasis in the 4T1 model after resection of the primary tumor (Fig. 6A). Treatment was started on day 5 post cell injection, and the primary tumors were excised by surgery at day 15. By this time, the antitumoral effect of SAR131675 was already significant (Fig. 6B and C).
SAR131675 induced an important decrease in the number of lung metastases (Fig. 6D), whether the treatment was pursued after primary tumor resection or not. This indicated that SAR131675 blocks early events of tumor escape and metastasis and suggests that treatment before tumor resection could be sufficient to prevent tumor metastasis. As before, sunitinib was more efficient in reducing tumor growth, but showed no significant effect on the number of metastases, whatever the treatment schedule (Supplementary Fig. S4B).

**Effect of SAR131675 on macrophage infiltration**

TAMs play an important role in tumor promotion and metastasis (42). We analyzed macrophage infiltration (with F4/80 immunostaining), using sections from 4T1 mammary tumors and from the RIP1.Tag2 pancreas.

In 4T1 tumors, macrophages were mainly located at the periphery of the tumor at day 12 postimplantation, whereas they infiltrated the tumor and formed clusters in more advanced tumors (day 21, Fig. 7A). Treatment with SAR131675 decreased macrophage infiltration and clustering as illustrated in Fig. 7A and consistently reduced F4/80 levels in tumors as measured by an ELISA assay (Fig. 7B).

A strong decrease of F4/80 staining in the RIP1.Tag2 model may also arise from a reduction in TAM infiltration.

Together, these results from 2 models corroborate the hypothesis that tumor reduction by SAR131675 is associated with a reduction in TAM infiltration.

**Discussion**

It is well established that VEGFR-3 is a key player in the control of lymphangiogenesis and also angiogenesis. In addition, an increasing number of studies have shown its upregulation in tumor cells and also on TAMs, suggesting that selectively targeting VEGFR-3 might be an interesting therapeutic approach to reduce tumor growth and metastasis. Identification of such selective inhibitors could also have the advantage of providing maximal therapeutic effect without causing severe side effects. Here we describe, for the first time, a small molecule with highly restricted selectivity for VEGFR-3. SAR131675 is a potent and selective VEGFR-3 inhibitor, with a moderate activity against VEGFR-2. It is able to block angiogenesis, lymphangiogenesis, and TAM infiltration and in consequence, it reduces tumor growth and metastasis.

It has been reported that targeting VEGFR-3 with specific inhibitors may block new lymphatic growth exclusively, without any effect on the survival or function of existing lymphatic vessels in adult mice (43, 44). In agreement with these reports, SAR131675 is able to block the formation of new lymphatic vessels in a sponge model as well during tumor-induced lymphangiogenesis, without
any sign of lymphedema. Moreover, treatment of mice with SAR131675 for about 3 months at 100 mg/kg/d did not result in any mortality.

Vascular complications have emerged as relevant toxicities associated with angiogenesis inhibitors. Bevacizumab, a mAb targeting VEGFA, has been linked to hemorrhage, arterial, and venous thrombosis, as well as hypertension (45). However, at 100 mg/kg, SAR131675 did not induce any significant hypertension in rats, suggesting that inhibition of the VEGFR-3 pathway is not responsible for this adverse event. This also shows that at 100 mg/kg, any effect of SAR131675 on VEGFR-2 activity is not sufficient to induce hypertension in vivo. This minimal activity on VEGFR-2 in vivo was also confirmed in zebrafish embryo studies. Indeed, in agreement with results obtained with a morpholino antisense nucleotide of VEGFC or through overexpression of a soluble form of VEGFR-3 (46), treatment with SAR131675 affected only intersegmental vessel development but did not give rise to side effects such as the defects in artery development seen in the VEGFR-2 KO zebrafish (11).

Although angiogenesis inhibitors show antitumor effects in several mouse models, they have been reported to concomitantly elicit increase of lymphatic and distant metastasis. Indeed, an anti–VEGFR-2 neutralizing antibody reduces tumor vasculature and volume and also increases incidence of lymph node and liver metastasis in RIP1.Tag2 mice (47, 48). In this same model, SAR131675 reduced tumor volume in both prevention and intervention studies without any prominent invasive fronts in the treated animals. Increased metastasis was also observed in mice receiving short-term therapy with sunitinib (41).

In addition to its antimetastatic activity, SAR131675 significantly reduced the tumor volume in several experimental tumor models, including colon, mammary and prostate, and in RIP1.Tag2 mice. These data are consistent with those reported previously that showed that blocking the VEGFR-3 pathway may block primary tumor growth.
Three major mechanisms could explain the role of VEGFR-3 in primary tumor growth: an autocrine effect on tumor cells, pathologic angiogenesis, and TAMs.

An increasing number of studies have shown the expression of VEGFR-3 on tumor cells in human cancer patients (5, 17–19, 21–22). However, this phenomenon has never been reported in mouse tumor models. In agreement with this, VEGFR-3 immunostaining of RIP1-Tag2 mice (100 mg/kg/d).

SAR131675 strongly reduced TAM infiltration in the 4T1 and the RIP1.Tag2 models. This reduction was associated with reduced tumor growth, reduced metastasis, and extended median survival of RIP1.Tag2 mice. The effect of SAR131675 on macrophage differentiation and recruitment and on antitumoral immune response is under evaluation.

Disclosure of Potential Conflicts of Interest
P. Schaeffer has ownership interest (including patents) in Sanofi stock.

Authors’ Contributions
Conception and design: A. Alam, I. Blanc, G. Gueguen-Dorbes, O. Duclos, P. Schaeffer, F. Bono
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Alam, I. Blanc, G. Gueguen-Dorbes, J. Bonnin, P. Barron, M.-C. Laplace, G. Morin
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Alam, I. Blanc, G. Gueguen-Dorbes, O. Duclos, P. Barron, G. Morin, P. Schaeffer, F. Bono
Writing, review, and/or revision of the manuscript: A. Alam, I. Blanc, G. Gueguen-Dorbes, F. Del, J.-P. Héraut, P. Schaeffer, F. Bono
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Alam, J. Bonnin, P. Barron, F. Gueguen-Dorbes
Study supervision: A. Alam, I. Blanc, G. Gueguen-Dorbes, O. Duclos, F. Del, P. Schaeffer, P. Savi, F. Bono

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Antoine Alam, Isabelle Blanc, Geneviève Gueguen-Dorbes, et al.

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