SAR131675, a potent and selective VEGFR-3-TK inhibitor with anti-lymphangiogenic, anti-tumoral and anti-metastatic activities.

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Running title: SAR131675 a potent and selective VEGFR-3 inhibitor

Precis: SAR131675, a highly active and specific VEGFR-3 inhibitor, reduces lymphangiogenesis and angiogenesis and also tumor associated macrophages infiltration and as consequences, it reduces tumor growth and metastasis

Keywords: Angiogenesis / lymphangiogenesis / tyrosine kinase / VEGF-R3 / macrophages/ metastasis

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Abstract

SAR131675 is a potent and selective VEGFR-3 inhibitor. It inhibited VEGFR-3 tyrosine kinase (TK) activity and VEGFR-3 autophosphorylation in HEK cells with IC\textsubscript{50}s of 20nM and 45nM, respectively. SAR131675 dose-dependently inhibited the proliferation of primary human lymphatic cells, induced by the VEGFR-3 ligands VEGFC and VEGFD, with an IC\textsubscript{50} of about 20nM. 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 anti-proliferative activity on a panel of 30 tumors and primary cells, further demonstrating its high specificity and indicating that SAR131675 is not a cytotoxic or cytostatic agent. SAR131675 was very well tolerated in mice and demonstrated a potent antitumoral effect in several orthotopic and syngenic models including mammary 4T1 carcinoma and RIP1.Tag2 tumors. Interestingly, it significantly reduced lymph node invasion and lung metastasis, demonstrating its anti-lymphangiogenic activity \textit{in vivo}. Moreover, treatment of mice before resection of 4T1 primary tumors was sufficient to prevent metastasis. Tumor Associated Macrophages (TAMs) 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 anti-tumoral and anti-metastatic activities \textit{in vivo} through inhibition of lymphangiogenesis and TAM invasion.
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 vascular endothelial growth factor 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 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 etc. (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 (TAMs) (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 trans-differentiating 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.

In order 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 present anti-VEGFR-3 activity, currently there is no specific VEGFR-3-TK inhibitor under evaluation. Here 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 hemoglobin were obtained from Sigma®. The proteome array Phospho-MAPK, 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 Upstate, and huVEGFR-3-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 Braunschweig, Germany or the American Tissue Culture Collection Frederick, MA, USA. No further authentication was performed. 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, Grand Island, NY) containing 10% heat-inactivated FCS (Gibco Laboratories), 2 mM L-glutamine, 1mM sodium pyruvate, 100 UI/ml penicillin, 100 µg/ml streptomycin (Gibco Laboratories).

Adult HLMVEC (Human Lymphatic Micro Vascular Endothelial Cells), were purchased from Cambrex, maintained in culture as described previously (35) and were
tested and authenticated in the lab 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 ATTC (CRL-2539) and were maintained in RPMI 1640 containing 10% heat-inactivated FCS and 2 mM 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 performed in the presence of kinase buffer (10X: 50 mM HEPES buffer, pH 7.4, 20 mM MgCl₂, 0.1 mM MnCl₂, and 0.2 mM Na₃VO₄) supplemented with ATP and DMSO for the positive control (C⁺) or SAR131675 (ranging from 3 nM to 1000 nM). ATP was used at 30 µM for VEGFR-1 and VEGFR-3 and at 15 µM for VEGFR-2. The phosphorylated ployGT was probed with a phosphotyrosine specific monoclonal antibody 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 M H₂SO₄ and absorbance was determined using an Envision spectrophotometer at 492 nm.

Percent inhibition (I%) was calculated using the following formula: 

\[ I\% = 100 - \left( \frac{[\text{SAR131675}] - \text{C}^-}{\text{C}^+ - \text{C}^-} \right) \times 100 \]

where [SAR131675] represents the value obtained in the presence of the indicated concentration of SAR131675.

**Autophosphorylation in HEK cells and survival and migration assays of HLMVC:** Experiments were performed as described previously (35). Twenty four hours after transfection, the cells were treated during 1 hour with orthovanadate (100 mM),
harvested, collected and, after counting, distributed in 5 ml tubes in the presence of the indicated concentration of SAR131675. After 30 min of incubation, the reaction was stopped by the addition of cold PBS supplemented by orthovanadate. Cells were then lysed with 150 µl of RIPA buffer over 15 min at 4°C, and then centrifuged for 10 min at 10,000xg. 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 2mM MgCl₂. The reaction was stopped with 50µl of 2N H₂SO₄ and the signal was read using an Envision spectrophotometer at 485 and 530 nm.

**Survival assay:** HLMVEC were seeded in 96-well plates coated with 0.3% gelatine (5x10³ cells per well). Cells were incubated in RPMI 0.1% FCS with VEGFA (10ng/ml) VEGFC (300ng/ml), VEGFD (300ng/ml) or FGF2 (10ng/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, Madison, WI) as previously described (35).

**Migration assay:** The migration assay was performed with the BD BioCoat Angiogenesis System-Endothelial Cell Migration kit (BD Biosciences, San Jose, CA). HLMVEC (1 x 10⁵ cells per well in RPMI) were added in triplicate in the upper chambers. The lower chambers were loaded with RPMI alone, RPMI with 50 ng/ml VEGF-A or 100 ng/ml VEGF-C. After 24 hours at 37°C cells were labeled with calcein AM (Molecular probes, Inc. OR) according to the manufacturer's instructions. Fluorescence of cells that had migrated was measured on a TECAN GENios microplate reader (TECAN, Maennedorf, Switzerland).
Proteome Array and Erk phosphorylation: HLMVEC were plated in 6-well tissue culture plates at a density of 2 x10^5 cells for 48h. Cells were then serum deprived for 2h and stimulated or not for 10mn with VEGFC (500ng/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 anesthetized with tricaine and the number of intersegmental vessels was analyzed by fluorescence microscopy (2.5x magnification).

Angiogenesis and lymphangiogenesis induced in a sponge implant mouse model: Sterile sponge discs (Cellspin®, Interchim) impregnated with 200µg of FGF2 or PBS were sub-cutaneously introduced on the back of anaesthetized mice. FGF2 was reinjected 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
euthanatized and the sponges were removed, harvested and lysed in RIPA buffer at 4°C. After a centrifugation at 6,000xg, the supernatants were collected for further analysis.

**RIP1.Tag2/transgenic mouse models:** For the prevention study, treatment of mice (9/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/group) started at 12 weeks of age and mice were monitored daily to detect moribund mice.

**4T1 mammary carcinoma model:** 4T1 cells (10^5) were implanted into mammary fat pads of BALB/c mice (15/group) (37). Daily oral treatment with SAR131675 (30 and 100mg/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 x a^2 x 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 two groups (12 mice/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.
**Immunochemistry:** Tissues were fixed with 10% accustain® 24 hours, dehydrated and embedded in paraffin. Immunostaining was done in sections incubated with anti–mouse CD31 Ab (1/50), LYVE1 (1/1000, 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 DAB peroxidase substrate and counterstained with hematoxylin. Images were captured with a camera (SONY) mounted on a Nikon microscope.

**Statistical Analysis:**

IC$_{50}$ 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 two-analysis of variance (ANOVA) following Dunnett’s test or confidence interval calculation as specified in the results part. Statistical analysis of data from the survival study was performed using a log-rank test. For tumor regression analysis, two-way ANOVA with repeated measures was performed.
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\(_{50}\) of 23 ± 7nM (Fig.1B, n=4). Under the same conditions SU11248 (sunitinib) inhibited VEGFR-3-TK activity with an IC\(_{50}\) of 10nM (a representative set of data with sunitinib is shown in supplementary Fig.1). SAR131675 is an ATP-competitive inhibitor; it inhibited VEGFR-3-TK activity with a Ki of about 12nM, similar to its IC\(_{50}\) value (supplementary Fig.1).

The effect of SAR131675 on VEGFR-3 auto-phosphorylation 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 IC\(_{50}\) ranging from 30-50nM (Fig.1C). In this assay, sunitinib inhibited VEGFR-3 autophosphorylation with an IC\(_{50}\) ranging from 10-30nM (not shown). SAR131675 was also evaluated on two 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 (supplemental Fig.1).

The selectivity of SAR131675 towards 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 IC\(_{50}\) > 3µM (I%= 31 at 3µM) and VEGFR-2-TK activity with an IC\(_{50}\) of 235nM (n=2) (Table 1).
In the same assay, sunitinib inhibited VEGFR-1 and VEGFR-2 with an IC\textsubscript{50} of 64nM and 14nM, respectively (not shown). In the autophosphorylation assay in HEK cells, SAR131675 inhibited VEGFR-1 autophosphorylation with an IC\textsubscript{50} of about 1\muM and VEGFR-2 with an IC\textsubscript{50} of about 280nM (Table 1). These results confirm that SAR131675 moderately inhibits VEGFR-2 and has very little effect on VEGFR-1, demonstrating a good selectivity for VEGFR-3, which is not the case for sunitinib.

In order to further confirm the activity of SAR131675 on VEGFR-2, we used Porcine Aortic Endothelial Cells (PAEC) stably expressing human VEGFR-2. SAR131675 inhibited VEGFA-induced VEGFR-2 phosphorylation in a dose dependent manner, with an IC\textsubscript{50} of 239nM, confirming the results obtained in HEK293T cells after VEGFR-2 overexpression (Fig.1D, E).

The selectivity of SAR131675 was further confirmed as it was inactive on a panel of 65 kinases, on a panel of 107 non-kinase enzymes and receptors and on 21 ion channels (supplementary tables 1, 2A and 2B, respectively). We ruled out the eventuality of any non-specific 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\muM (supplementary table 3), thus confirming the specificity of SAR131675.

Together, these results show that SAR131675 is a potent and selective inhibitor of VEGFR-3-TK with moderate activity towards 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 figure 2A, SAR131675 potently inhibited lymphatic cell survival induced by VEGFC and VEGFD (IC$_{50}$ of 14 and 17nM, respectively). It inhibited VEGFA-induced survival with an IC$_{50}$ of 664nM, confirming its moderate activity on VEGFR-2 and had no effect on FGF2-induced proliferation. In the same experiment, sunitinib inhibited VEGFA, - C and -D induced survival with an IC$_{50}$ of about 5nM and, consistent with reported data (38), it had no effect on FGF2-induced survival (supplementary Fig.1).

We then evaluated the effect of SAR131675 on HLMVEC migration induced by VEGFA or VEGFC in Boyden’s chambers. SAR131675 potently inhibited VEGFC-induced migration with an IC$_{50}<$30nM (Fig.2B) and VEGFA-induced migration with an IC$_{50}$ of about 100nM (Fig.2C).

Using the PhosphoMAP array in HLMVEC, Erk was identified as the major phosphorylated kinase upon VEGFC-stimulation (Fig.2D). SAR131675, significantly and dose dependently inhibited VEGFC-induced Erk phosphorylation, with an IC$_{50}$ of about 30nM (Fig.2E), confirming that SAR131675 is a potent inhibitor of VEGFR-3 signaling.

**Effect of SAR131675 on angiogenesis and lymphangiogenesis in non tumoral 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 post fertilization. Embryos treated with 3µM SAR131675 for 24h showed fewer
intersegmental vessels in the tail compared to control embryos and at the dose of 10 µM 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 disc 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.3I).

Treatment of mice with SAR131675 (30, 100 or 300 mg/kg/day) started on the day of sponge implantation. Seven days later, SAR1316765 at 100 mg/kg/day 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/day 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.2). Since 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
300mg/kg is able to inhibit both VEGFR-2 and VEGFR-3 signaling in vivo. In consequence 100mg/kg was the highest dosage used in subsequent in vivo studies.

**Effect of SAR131675 on carcinogenesis in the RIP1.Tag2 mice 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 to 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 two studies a significant decrease of CD31 positive vessels was observed in the SAR131675 treated mice compared to control mice. In the survival study, where 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 demonstrate 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 100mg/kg/day) from day 5 to day 21 after implantation.

SAR131675 was well tolerated since no loss of body weight was observed in the two treated groups. Treatment with SAR131675 significantly reduced the tumor volume (24% p<0.05 and 50% p<0.001 at 30 and 100mg/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.3A and C, respectively). Interestingly, as illustrated in supplementary Fig.3B and D, SAR131675 seems to reduce both parameters. Moreover, SAR131675 at 30 and 100mg/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 anti-lymphangiogenic properties.

Since 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 (R²=0.998, supplementary Fig.3E and F). Interestingly, the osteopontin content dramatically increased in sentinel lymph nodes, indicating an infiltration by metastatic 4T1 cells (Fig.5C). SAR131675, at 100mg/kg significantly reduced the osteopontin content in lymph nodes (56%, p<0.01), indicating that SAR131675 has a potent effect on lymph node invasion by 4T1 cells (Fig. 5C).

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.4A), it is interesting to
note that in this model, sunitinib at 50mg/kg/d had no effect on the number of lung metastases (supplementary Fig.4A).

Altogether these results demonstrate that SAR131675 is a potent antitumoral agent, acting through anti-angiogenic and anti-lymphangiogenic 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 anti-angiogenic 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 indicates that SAR131675 blocks early events of tumor escape and metastasis and suggests that treatment prior to 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 lung metastases, whatever the treatment schedule (supplementary Fig.4B).

**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-post implantation, 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 figure 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 was also observed in the angiogenic islets and the tumors from SAR131675-treated mice (supplementary Fig. 3G). These results indicate that reduction of tumor growth induced by SAR131675 in the RIP1.Tag2 model may also arise from a reduction in TAM infiltration.

Together, these results from two 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 but 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 therapeutical 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 on 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 100mg/kg/d did not result in any mortality.

Vascular complications have emerged as relevant toxicities associated with angiogenesis inhibitors. Bevacizumab, a monoclonal antibody targeting VEGFA, has been linked to hemorrhage, arterial and venous thrombosis, as well as hypertension (45). However, at 100mg/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 100mg/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 demonstrate 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 the 4T1 model, SAR131675, but not sunitinib, significantly reduced lymph node invasion and distant metastasis. In addition, short-term treatment prior to tumor resection with SAR131675 was sufficient to reduce lung metastasis, but this was not the case with sunitinib.

In addition to its anti-metastatic 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 which showed that blocking the VEGFR-3 pathway may block primary tumor growth (49). Three major mechanisms could explain the role of VEGFR-3 in primary tumor growth: an autocrine effect on tumor cells, pathological 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 and 4T1 tumors confirmed that VEGFR-3 is not induced on these tumor cells in vivo (not shown).
Although VEGFR-3 is not expressed on adult blood vessels (3), high expression of VEGFR-3 has been reported in angiogenic sprouts and blocking VEGFR-3 signaling with monoclonal antibodies results in decreased sprouting and vascular density in mouse angiogenesis models (16). Thus, the reduction of tumor growth by SAR131675 could be due to inhibition of angiogenesis. This would be consistent with the reduction of angiogenesis in the sponge model, which expresses high levels of VEGFR-3 and also with the decrease in vascular density in the 4T1 and the RIP1.Tag2 models. Thus SAR131675 may block angiogenesis through different non-exclusive mechanisms: by inhibiting VEGFR-3 signaling in endothelial cells or by blocking VEGFA- or VEGFC-induced VEGFR-2/VEGFR-3 heterodimers. It is worth noting that the moderate VEGFR-2 activity of SAR131675 may also contribute to its activity on tumor angiogenesis.

Finally, the anti-angiogenic effect may result from the effect of SAR131675 on TAM infiltration. TAMs promote angiogenesis by releasing pro-angiogenic factors such as VEGFA and VEGFC, and thereby induce tip-cell formation and fusion (16, 29, 32). It is well established that macrophages and also TAMs express VEGFR-3 (28-30). Moreover, VEGFR-3 inhibition decreased dendritic cell recruitment to the spleen and resulted in prolonged rat cardiac allograft survival (50). Here we have shown that 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 anti-tumoral immune response is under evaluation.
Acknowledgements:

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References


46. Ober X. VEGFc is required for vascular development and endoderm morphogenesis in zebrafish. EMBO reports 2004; 5:78-84.


Table 1: Effect of SAR131675 on human VEGFR-1 and VEGFR-2 using recombinant enzymes or after overexpression in HEK cells. The IC50 geometric mean are represented

<table>
<thead>
<tr>
<th></th>
<th>VEGFR-1</th>
<th>VEGFR-2</th>
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<tbody>
<tr>
<td>rhVEGFR-1</td>
<td>HEK-VEGFR-1</td>
<td>rhVEGFR-2</td>
</tr>
<tr>
<td>SAR131675</td>
<td>I%= 31 at 3µM</td>
<td>I%= 48 at 1µM</td>
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Figure Legends

**Fig1: Effect of SAR131675 on VEGFR Tyrosine kinases.** A) Structure of SAR131675. B) Effect of SAR131675 on kinase activity of recombinant human VEGFR-3 measured in the presence of 30µM of ATP. C) Effect of SAR131675 on VEGFR-3 auto-phosphorylation after overexpression in HEK cells. These data are representative of several assays. C) Effect of VEGFA on VEGFR-2 phosphorylation in PAEC stably expressing VEGFR-2. D) Effect of SAR131675 on VEGFA-induced VEGFR-2 phosphorylation.

**Fig2. Effect of SAR131675 on human primary lymphatic cells.** A) Effect on lymphatic cell survival. Cells were plated in medium containing 0.1% FCS. Cells were then left unstimulated or stimulated with VEGFA (10ng/ml) VEGFC (300ng/ml), VEGFD (300ng/ml) and bFGF (10ng/ml) in the presence of increasing SAR131675 concentrations. Data are expressed as percentage of inhibition, and plotted on SPEED software. Results are representative of 3 independent experiments. B and C) Migration assay of HMVECs. Cells were seeded in triplicate in the upper wells of a fluoroblok chamber. RPMI alone or containing VEGF-A 50 ng/mL or VEGF-C 100 ng/mL was added in the bottom wells in the presence of increasing doses of SAR131675 (30-300 nM for VEGFC and 100-1000nM for VEGFA). The amount of migrating cells was quantified using calcein labeling. Data are shown as mean ± standard error of the mean. D) Effect of VEGFC on MAP-kinases. HMVEC were left unstimulated or stimulated for 10mn with VEGFC (500ng/ml) and lysed. +: Represents positive controls, the spots corresponding to Erk and Akt are indicated. Cell lysates were than incubated with array membranes. E) Effect of SAR131675 on Erk phosphorylation in lymphatic cells. Cells were stimulated as
described in B in the presence of increasing concentration of SAR131675. Erk phosphorylation was quantified by ELISA. Results are expressed as mean ± standard error of the mean, significant differences are assessed by ANOVA followed by Dunnett test vs VEGF-C, * p<0.05; ** p<0.01; *** p<0.001.

**Fig3: In vivo effect of SAR131675 on embryonic and adult angiogenesis and lymphangiogenesis.** A) Lateral view of zebrafish embryos which specifically express the fluorescent protein copGFP in the vascular system. Controls show the usual pattern of intersegmental blood vessels (ISV) which failed to form after treatment with 3μM and 10μM of SAR131675 (arrowhead). B and C) Induction of angiogenesis and lymphangiogenesis by FGF2 (200 ng) in a sponge implanted in the back of mice. Angiogenesis and lymphangiogenesis were evaluated at day7 by immunochemistry staining with CD31 (D) and anti LYVE-1 antibodies (E), respectively. Visual effect of vehicle (F) and SAR131675 (100mg/kg/d, G) on FGF2-induced angiogenesis and lymphangiogenesis. Dose effect of SAR131675 on VEGFR-3 levels (H) and on hemoglobin content (I) in the sponge. Results are expressed as mean ± standard error of the mean, by ANOVA followed by Dunnett test vs FGF-2 stimulated group. ** p<0.01; *** p<0.001.

**Fig.4 Effect of SAR131675 on a spontaneous multistage tumor model (RIP1.Tag2)** (A) Studies designed to target discrete stages of tumoral growth in the RIP1.Tag2 transgenic mice. In the prevention study (B), mice were treated with vehicle or with SAR131675 (100mg/kg/d), from Week 5 to Week 10 and the number of angiogenic islets (the transition from pre-vascular hyperplasia to highly vascularized and progressively outgrowing tumors), were measured at 10 weeks of age. For the intervention study (C)
mice were treated with vehicle or with SAR131675 (100mg/kg/d), starting at Week 10 and the total tumor volume was calculated at Week 12 by measuring each tumor volume with calipers. Error bars represent the SEM of data from 9 mice/group, * p<0.05 and *** p<0.001, Student’s test vs control group. (D) Monitoring of RIP1.Tag2 mice survival following daily treatment with vehicle or with SAR131675 (100mg/kg) starting from Week 12 (n=20 mice/group, p=0.01, log-rank test).

Fig 5: Antitumoral effect of SAR131675 treatment on a murine mammary carcinoma model. A) Tumor volume monitoring in Balb/C mice in control or mice treated with SAR131675 at 30 and 100 mg/kg/d. B) Murine VEGFR3 levels were quantified by ELISA in the tumor lysates of each treated group at the end of the experiment (day 21).

C) Quantification of osteopontin levels by ELISA in the lymph nodes after vehicle or SAR131675 treatment. Results are expressed as mean ± standard error of the mean, *p<0.05; ** p<0.01; *** p<0.001, ANOVA followed by Dunnett test vs vehicle group. In A, two-way ANOVA analysis with repeated measures was performed. D) Number of metastases at the surface of the lungs for each treated group.

Fig 6: Anti metastatic effect of SAR131675 after 4T1 tumor resection. A) Experiment schedule. SAR131675 treatment at 100mg/kg/d starts 5 days after 4T1 cell implantation in mammary fat pads, at day 15 primary tumors were removed in all groups and the tumor weight was evaluated (B). In the group noted 10 days, the SAR131675 treatment stopped at day 15 and mice were studied untreated until day 26. In the group noted 20 days, the treatment was continued until the end of the experiment (day 26). C) Murine VEGFR3 levels were quantified by ELISA in tumors lysates at day 15. D) The number of
metastases was counted at the lung surface at day 26. E) Illustration of the number of lung metastases after Bouin fixation. Results are expressed as mean ± standard error of the mean, significant differences between groups are assessed by ANOVA followed by Dunnett test, * p<0.05; ** p<0.01; *** p<0.001.

**Fig 7: Effect of SAR131675 on macrophage infiltration at different stages of tumor development.** A) Immunohistochemical staining of F4-80 in 4T1 mammary tumors at day 12 and day 21 in control and SAR131675 treated mice (40x; 400x magnification). B) Quantification of F4-80 levels by ELISA dosage in the 4T1 mammary tumor lysates at day 21 in vehicle and SAR131675 treated mice (100mg/kg/d).
Fig. 1

A

B

C

D

E
Fig. 2

A

% inhibition

VEGF-D
VEGF-C
VEGF-A
FGF-2

[SAR131675] (nM)

B

Luminescence (AU)

VEGF-C + SAR131675 (nM)

C

Luminescence (AU)

VEGF-A + SAR131675 (nM)

D

E

VEGFC + SAR131675 (nM)

0
10
100
300
1000

0
100
300
1000

0
10
100
300
1000

0
0.2
0.4
0.6
0.8

NS VEGF-D

VEGF-C

VEGF-A

FGF-2

VEGF-C + SAR131675 (nM)

VEGF-A + SAR131675 (nM)

VEGFC + SAR131675 (nM)

500ng/ml 10mn

VEGF-C 1 3 10 30 100 300

VEGF-C + SAR131675 (nM)

0
30
100
300

VEGF-C

VEGF-C + SAR131675 (nM)

VEGF-A + SAR131675 (nM)

VEGFC + SAR131675 (nM)

500ng/ml 10mn

0
30
100
300

VEGF-C

VEGF-C + SAR131675 (nM)

VEGF-A + SAR131675 (nM)

VEGFC + SAR131675 (nM)

500ng/ml 10mn

VEGF-C 1 3 10 30 100 300

VEGF-C + SAR131675 (nM)

VEGF-C 1 3 10 30 100 300

VEGF-C + SAR131675 (nM)
Fig. 4

A

Week 5 → Week 10 → Week 12

Prevention → Intervention → survival

B

SAR131675 @ 10W

C

SAR131675 @ 12W

D

% mice survival

weeks

SAR131675 → Control

p=0.01
Fig. 6:

**A**
Day 0: 4T1 cells implantation
Day 5: Start of p.o. treatment
SAR131675 (20d treatment)
SAR131675 (10d treatment)
Day 15: Excision
SAR131675 (10d treatment)
Day 26
Lung Metastasis number

**B**

<table>
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<th>Tumor weight at day 15 (g)</th>
<th>Vehicle</th>
<th>SAR131675</th>
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<tr>
<td></td>
<td>0.5</td>
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***

**C**

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<th>VEGFR3 level at day 15 (pg/ml)</th>
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**D**

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<th>Vehicle</th>
<th>10days</th>
<th>20days</th>
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<tr>
<td></td>
<td>120</td>
<td>60</td>
<td>30</td>
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**E**

Vehicle
SAR131675 (10 days treatment group)
Fig. 7:

**A**

F4/80 IHC on 4T1 mammary carcinoma model

<table>
<thead>
<tr>
<th>Day 12</th>
<th>Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Control</td>
</tr>
</tbody>
</table>

*40x*  
*400x*

**B**

![Graph showing F4/80 IHC on 4T1 mammary carcinoma model.](image)

Vehicle  
SAR131675  
100mg/kg/d

Arbitrary Unit/tumor

![Bar graph showing comparison between control and SAR131675 treatments.](image)

***
Molecular Cancer Therapeutics

SAR131675, a potent and selective VEGFR-3-TK inhibitor with antilymphangiogenic, anti-tumoral and anti-metastatic activities.

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

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