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Original Research

Targeting FGFR2 with alofanib (RPT835) shows potent activity in tumour models

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Abstract Alofanib (RPT835) is a novel selective allosteric inhibitor of fibroblast growth factor receptor 2 (FGFR2). We showed previously that alofanib could bind to the extracellular domain of FGFR2 and has an inhibitory effect on FGF2-induced phoshphorylation of FRS2α. In the present study, we further showed that alofanib inhibited phosphorylation of FRS2α with the half maximal inhibitory concentration (IC50) values of 7 and 9 nmol/l in cancer cells expressing different FGFR2 isoforms. In a panel of four cell lines representing several tumour types (triple-negative breast cancer, melanoma, and ovarian cancer), alofanib inhibited FGF-mediated proliferation with 50% growth inhibition (GI50) values of 16–370 nmol/l. Alofanib dose dependently inhibited the proliferation and migration of human and mouse endothelial cells (GI50 11–58 nmol/l) compared with brivanib and bevacizumab. Treatment with alofanib ablated experimental FGF-induced angiogenesis *in vivo*. In a FGFR-driven human tumour xenograft model, oral administration of alofanib was well tolerated and resulted in potent antitumour activity. Importantly, alofanib was effective in FGFR2-expressing models. These results show that alofanib is a potent FGFR2 inhibitor and provide strong rationale for its evaluation in patients with FGFR2-driven cancers.

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1. Introduction

Fibroblast growth factors (FGFs) and fibroblast growth factor receptor 2 (FGFR2) regulate cellular proliferation, survival, migration and differentiation. Deregulation of FGF/FGFR2 signalling in cancer is now well understood [2,12,22,28]. The mechanisms of dysregulation of FGFR2 include activating mutations in the extracellular and kinase domains of the receptor [7,25], gene amplification [14,35], chromosomal translocations [32], altered splicing [31,34] and germline single nucleotide polymorphisms [19]. Finally, some studies showed that FGF(R) mediates resistance to vascular endothelial growth factor receptor (VEGFR) targeting by reactivating tumour angiogenesis [9,23,26].

On the basis of the evidence for FGFR dysregulation in tumours, several companies have discovered small-molecule tyrosine kinase inhibitors targeting the ATP-binding site of the intracellular tyrosine kinase domain of FGFRs or monoclonal antibodies blocking the active site of the extracellular domain of FGFRs [1,8,10,27,29,36].

Alofanib (RPT835) is a novel small-molecule selective allosteric inhibitor binding to the extracellular domain of FGFR2 (Fig. 1A). Alofanib had a dramatic inhibitory effect with IC50 <10 nM on FGF2-induced phosphorylation of FRS2α in KATO III cells [21]. However, alofanib had no direct effect on FGF2-dependent FGFR1 and FGFR3 phosphorylation levels in either cell lines. In a non-radioactive high-throughput binding assay and in a binding assay with anti-FGF2 antibody coated wells, there were no effects of alofanib on FGF2-FGFR2 binding.

In this report, we describe the preclinical profile of alofanib.

2. Materials and methods

2.1. Alofanib

3-(*N*-(4-methyl-2-nitro-5-(pyridin-3-yl)phenyl)sulfamoyl)benzoic acid (RPT835, CAS Registry Number 1612888-66-0; Ruspharmtech LLC, Fig. 1B) was synthesized by EcoSynth (Oostende, Belgium) according to the processes described in the International Patent Application Publication Number PCT/EA2014/000013. The free base of alofanib (molecular weight = 413.4 g/mol) was used in all the preclinical studies.

2.2. Cell lines

The following cell lines were purchased: SKOV3, HS578T, NCI-H226, SVEC-4-10, HUVEC, and hFOB from the American Type Culture Collection; SUM 52PE from Asterand; and Mel Kor from the N.N. Blokhin Russian Cancer Research Center (Russian Patent Number 2287578).

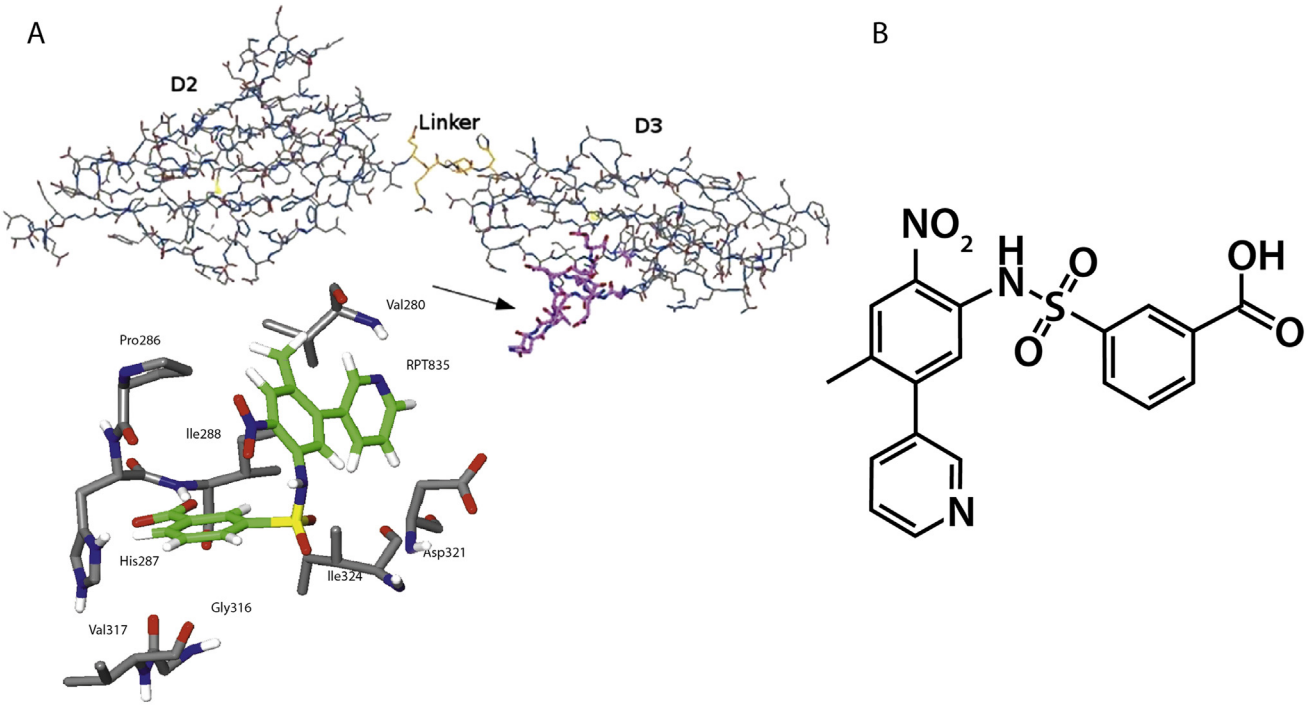

Fig. 1. A, Location of the proposed binding site of alofanib (RPT835) indicated by the arrow. The carbon atoms of the linker between D2 and D3 of fibroblast growth factor receptor 2 are colored orange. The carbon atoms of amino acids 313–325 are colored magenta. B, Chemical structure of alofanib.(For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.3. Inhibition of FGF-mediated cellular proliferation

Cell lines were plated into 96-well plates and routinely grown in RPMI-1640 (SKOV3, HS578T, and SUM 52PE) supplemented with 10% foetal calf serum (FCS; HyClone) and 2 mmol/l L-glutamine (Paneco Ltd) for 72 h. The hFOB cells were cultured according to the American Type Culture Collection (ATCC) protocol and references described as before [11].

To assess the efficacy of alofanib on FGF-mediated cell proliferation, cells were treated with serially diluted compound. Six hours after dosing, FGF2 (BD Bioscience) was added at a concentration of 25 ng/ml. Negative control cells were left untreated. Cell growth inhibition was determined using Cell Titer-Glo® (Promega) assay. To differentiate between a cytostatic and cytotoxic drug effect, the concentration that causes 50% growth inhibition (GI50) was determined by correcting for the cell count at time zero (time of treatment) and plotting data as percentage of growth relative to vehicle-treated cells.

2.4. Immunoblot analysis

hFOB cells with low expression of FGFR2 IIIc and SUM 52PE cells with expression of FGFR2 IIIb were incubated with/without FGF1 (30 ng/ml; Sino Biological) and treated with alofanib or vehicle (0.1% dimethyl sulfoxide [DMSO]) for 1 h. Whole cell lysates were analysed by electrophoresis and membranes immunoblotted with antibodies (Santa Cruz Biotechnology) against phospho-FRS2α. IC50 values were calculated. For immunoblot analysis, the SKOV3, HS578T, and Mel Kor cells were treated with the alofanib and then collected in lysis buffer (Cell Signalling). Total protein was quantified using Coomassie protein assay reagent (Bio-Rad). An equal amount of protein (60 μg) was separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and electrotransferred onto nitrocellulose membrane. The following primary antibodies were used: cleaved poly(ADP-ribose) polymerase (PARP), cleaved caspase 3, and human Bcl-2 (Cell Signalling). β-actin (Sigma) was measured as control for equal loading. Immunoblot analysis of FGFR1–3 protein expression in SUM 52PE, HS578T, and NCI-H226 cell lines with primary antibodies (Santa Cruz Biotechnology) was performed.

2.5. Human in vitro angiogenesis assay

SVEC-4-10 and HUVEC cells were plated into 24-well plates and were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Paneco Ltd) containing 10% FCS, 2 mmol/l L-glutamine, and 1% penicillin/streptomycin for 24 h. Alofanib or vehicle (0.01% DMSO in DMEM) was added to the cultures. Brivanib (Selleck Chemicals) or bevacizumab (Genentech) in different concentrations was used as a positive control. FGF2 (BD Bioscience) was added at a concentration of 25 ng/ml. Endothelial cell proliferation was determined. To assess the cell migration, wound healing assay was used [18]. SVEC-4-10 cells were cultured in DMEM and were treated the next day with compound (10–100 nM). To quantify tubule growth, SVEC-4-10 cells were plated into 24-well plates with Matrigel (100 μl per well, density 8.4 mg/ml; BD Bioscience) and were cultured. Tubule formation was examined at day 10 following fixing and staining of tubules for CD31 (platelet/endothelial cell adhesion molecule 1). Comparative treatment with bevacizumab (10 μg/ml) was also examined.

2.6. Inhibition of FGF-induced angiogenesis in vivo

In vivo Matrigel plug assay was performed as described by [16] using either VEGF or FGF2 as an angiogenic factor. Briefly, Matrigel (1 ml; BD Biosciences) containing heparin (60 units/ml; BD Biosciences) was diluted in VEGF (200 ng/ml; BD Biosciences) or FGF2 (100 ng/ml; BD Biosciences) and injected subcutaneously into C57Bl/6 × DBA/2 F1 mice of 22–30 g (five per group). Alofanib (15 mg/kg/d), or bevacizumab (10 mg/kg/d) or vehicle (water) was given intraperitoneally (0, 3 and 6 d). Matrigel plugs were recovered on day 7 and were fixed in neutral formalin and embedded in paraffin for haematoxylin and eosin staining, histological and immunohistochemical (rabbit anti-CD31/PECAM-1 antibodies; Abcam, EnVision™+ kit, Dako) analysis.

2.7. Xenograft studies

Three cell lines with different expression of FGFR2 were selected for xenograft studies. Immunocompromised nude mice (4- to 6-week old males) were purchased from the Harlan laboratories and were used for xenotransplantation of FGFR2 high-expressing SUM 52PE cells [20], FGFR2 low-expressing HS578T cells [24], and FGFR2-negative NCI-H226 cells [30]. Tumour xenografts were established by subcutaneous injection into the right flank with 2×10^6 tumour cells mixed 1:1 with Matrigel (BD Biosciences). Injection was performed at respective day 0.

In each study, 20 animals with measurable tumours were selected on day 10 and randomized into treatment group (alofanib, 30 mg/kg; gavage, daily, N = 10) or vehicle (water, gavage, daily, N = 10). Measurements of tumour volume (cubic millimetre) were performed by digital calipers every 3 d during 40 d after tumour inoculation. Animals were weighed daily for the first 5 d of the study and twice weekly thereafter. Acceptable toxicity was defined as group mean body weight loss of less than 20% during the study and not more than one treatment-related death among ten treated animals in each study.

All animal studies were carried out under a protocol approved by the Institutional Animal Care and Use Committee.

3. Results

3.1. Alofanib has potent in vitro effects on FGFR2-mediated cancer cell proliferation and apoptosis

Five tumour cell lines were selected on the basis of known different abnormalities in FGFR2 expression and signalling. Expression of FGFR2 has been shown in Figs. 2, 8D. SUM 52PE is a triple-negative breast cancer cell line that expresses wild-type FGFR2 IIIb, and hFOB is an osteoblast cell line that expresses wild-type FGFR2 IIIc. SKOV3 and HS578T are ovarian and triple-negative breast cancer cell lines with the expression of FGFR2 [6,24]; Mel Kor is a melanoma cell that do not express FGFR2 (http://www.ntpo.com/patents_medicine/medicine_12/medicine_239.shtml).

As shown in Fig. 3A, alofanib potently inhibited growth of cells expressing FGFR2 IIIb and IIIc, with GI50 values of 12 and 16 nmol/l, respectively. To confirm target inhibition, we assessed the effects of alofanib on phosphorylation of FRS2α as an important substrate of FGFR2 in each cell line and found that compound inhibited phosphorylation of FRS2α with IC50 values of 7 and 9 nmol/l, respectively (Fig. 3B).

Alofanib inhibited growth of SKOV3 and HS578T cells with GI50 values of 0.37 and 0.21 μmol/l (Fig. 3A). In contrast, compound did not potently inhibit growth of Mel Kor cells that do not contain FGFR2 (GI50 > 10 μmol/l).

We evaluated the status of apoptotic markers in Fig. 4B and observed marked mitochondrial-mediated apoptosis with cleavage of caspase 3 and PARP in SKOV3 and HS578T cells compared with resistant Mel Kor cell line. The expression of Bcl-2 was analysed and major changes in expression of this protein were observed following treatment in SKOV3 cell line.

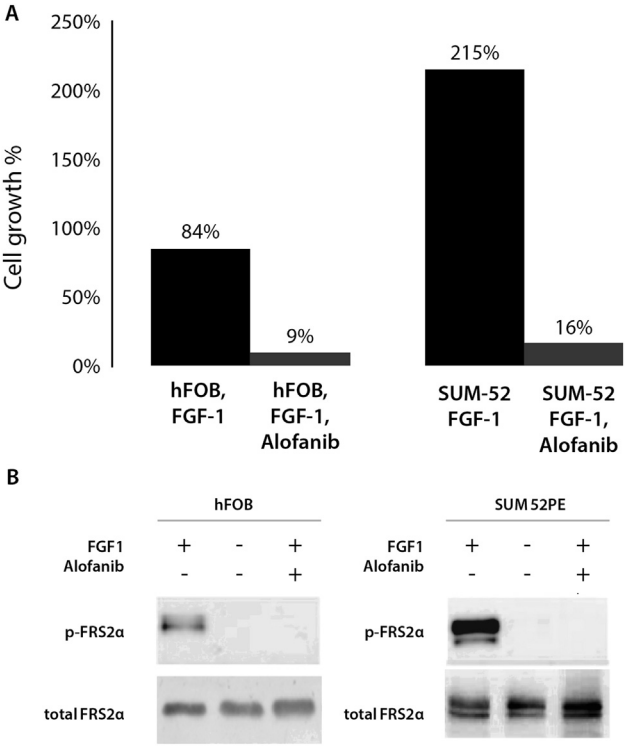

Fig. 3. Inhibition of fibroblast growth factor receptor (FGFR) signalling by FRS2α knockdown exerted potent effects on proliferation of cancer cell lines. A, human breast cancer cells expressing FGFR2 IIIb and human foetal osteoblastic cells expressing FGFR2 IIIc were treated with alofanib for 1 h and then incubated with FGF1 for 72 h. Cell growth was assessed. In each case, values represent the means ± standard deviation for two experiments ($P < 0.001$, compared with negative control). B, SUM 52PE and hFOB cells were incubated with/without FGF1 and treated with alofanib for 1 h. Cell lysates were immunoblotted for FRS2α. FGF1 significantly increased proliferation of the hFOB and SUM 52PE cells in untreated control groups ($P < 0.0001$).

3.2. Inhibition of FGF signalling by alofanib suppressed the angiogenesis

To assess the inhibitory activity of alofanib on tumour angiogenesis, we first evaluated its antiangiogenic function *in vitro*. As shown in Fig. 5, alofanib dose dependently decreased FGF2-induced cell viability in HUVECs with the GI50 value of 11 nmol/l, and FGF2-triggered proliferation of SVEC-4-10 with the GI50 value of 58 nmol/l. For comparison, the *in vitro* cellular activities of two other targeted agents (brivanib and bevacizumab) were also examined. Alofanib was found to be most potent inhibitor of FGF2-induced angiogenesis. Overall, brivanib, a VEGFR/FGFR inhibitor (IC50 value of 289 nmol/l), and anti-VEGF monoclonal antibody bevacizumab (GI50 value was not reached) were considerably less potent.

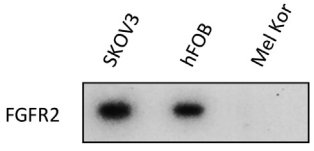

Fig. 2. Fibroblast growth factor receptor 2 (FGFR2) expression was increased in hFOB and SKOV3 cell lines in comparison with Mel Kor cell line.

Given the importance of vascular endothelial cell motility in the process of angiogenesis, we further evaluated the potential effect of alofanib on SVEC-4-10 endothelial cell migration *in vitro*. Results showed that treatment with alofanib (10–100 nmol/l) remarkably impaired the chemotactic motility of SVEC-4-10 cells (Fig. 6). Alofanib induced a substantial decrease in capillary-like tubular structures number and length relative to vehicle and bevacizumab *in vitro*.

We next evaluated the activity of alofanib in comparison with vehicle or bevacizumab on FGF- or VEGF-stimulated SVEC-4-10 endothelial cell

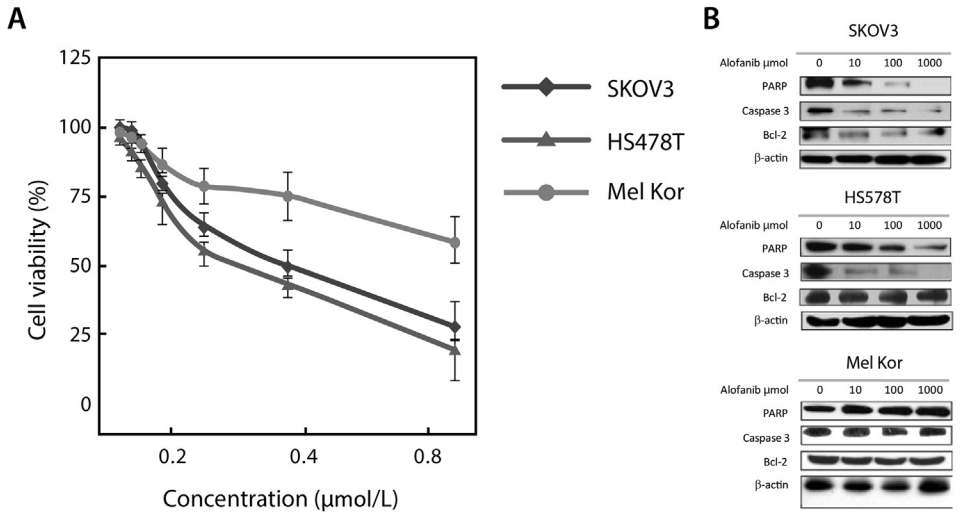

Fig. 4. The mechanism of *in vitro* growth inhibition by alofanib is cell line-dependent. A, ovarian cancer SKOV3 cells, breast cancer HS478T cells and melanoma Mel Kor cells were exposed to alofanib and then calculated. Data are presented as means (±standard error) from three different experiments. B, Cells were treated with alofanib (10, 100, and 1000 nM) for 72 h and whole-cell lysates were immunoblotted with cleaved caspase 3, cleaved PARB, and Bcl-2 antibodies.

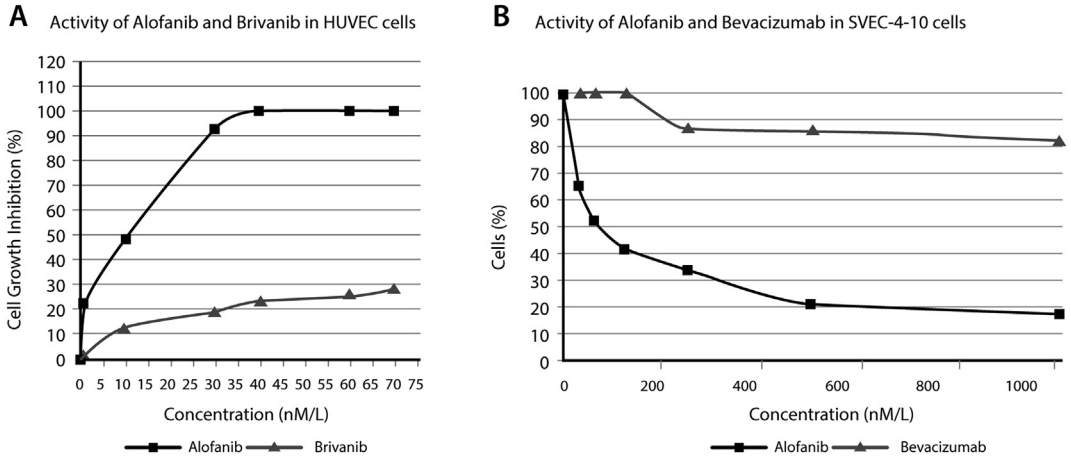

Fig. 5. Alofanib inhibits fibroblast growth factor 2 (FGF2)-induced proliferation of human and mouse endothelial cells. Dose–response effect of alofanib was evaluated in HUVEC endothelial cells in comparison with brivanib (A), and in SVEC-4-10 endothelial cells in comparison with bevacizumab (B). Cells were treated with different concentrations of alofanib, brivanib and bevacizumab for 6 h followed by stimulation with 25 ng/ml FGF2. Cell growth inhibition (A) and number of cells (B) were assessed ($P < 0.001$, compared with negative and positive control). Basic FGF significantly increased proliferation of the HUVEC and SVEC-4-10 endothelial cells in untreated control groups ($P < 0.0001$).

proliferation as well as formation of tubular structures *in vivo*. In Matrigel assay, the number of endothelial cells, capillary-like tubular structures and microvessels was much less in alofanib groups than that of the vehicle and bevacizumab groups (Fig. 7, all $P < 0.001$). Alofanib significantly decrease a number of mature vessels. Bevacizumab demonstrated a dramatic inhibitory effect on VEGF-induced endothelial cell proliferation and microvessels formation when compared with vehicle ($P < 0.001$). FGFR2 inhibitor alofanib had no significant activity in VEGF-stimulated angiogenesis (all $P > 0.05$).

3.3. Alofanib in vivo antitumour activity is associated with FGFR2 expression

To evaluate the activity of alofanib *in vivo*, immunocompromised mice were used for xenotransplantation of FGFR2 high-expressing triple-negative breast cancer cells (SUM 52PE), FGFR2 low-expressing triple-negative breast cancer cells (HS578T), and FGFR2-negative lung cancer cells (NCI-H226). In each study, 20 animals with measurable tumours were selected on day 10 and randomized into treatment groups (alofanib 30 mg/kg, gavage, daily, $N = 10$) or vehicle (water, gavage, daily,

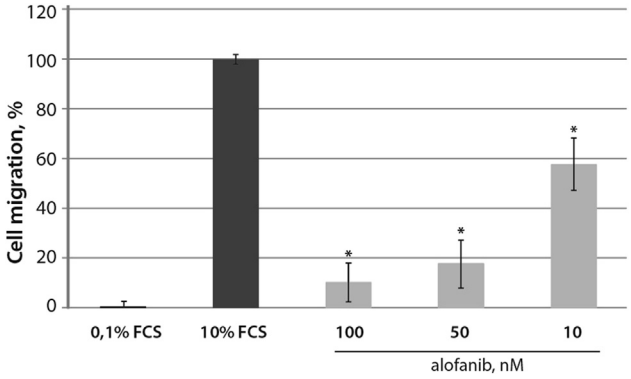

Fig. 6. Alofanib dose dependently inhibited SVEC-4-10 endothelial cells migration. SVEC-4-10 cells were cultured in Dulbecco's Modified Eagle's Medium and were treated the next day with compound (10–100 nM) for 24 h. Cell migration (%) was assessed. The number of cells treated with vehicle without alofanib is 100%. Columns, mean (N = 5) and bars, standard error. *P < 0.05 different from control.

N = 10). Alofanib significantly inhibited aggressive growth of SUM 52PE tumour xenograft (P < 0.0001). At study on day 31, mean tumour volumes (±standard error of the mean [SEM]) were 2712.2 ± 37 mm^3 in the vehicle group and 1080.7 ± 49 mm^3 in the study group. In addition, animals of the study group received alofanib from day 31 to 40 with disease stabilization (no differences in tumour volume between days 31 and 40, P = 0.167). The tumour growth curve (Fig. 8A) shows a nearly exponential increase in median tumour volume up to day 31 in the vehicle group and a high rate of slow growing tumours up to day 40 in the alofanib group. In the HS578T xenograft study, differences in tumour volume between study and control groups were weak at day 31 (mean tumour volumes ± SEM, 703 ± 89.1 versus 1053 ± 179.8 mm^3, respectively, P < 0.001) and at day 40 (1104 ± 162.2 versus 1592 ± 335 mm^3, P = 0.01, Fig. 8B). There was no significant impact of alofanib on FGFR2-negative NCI-H226 lung cancer growth (day 31, 1114 ± 280.6 versus 1053 ± 259.7 mm^3, P = 0.619, Fig. 8C). No group mean body weight losses or clinical manifestations of toxicity were observed during all xenograft studies.

4. Discussion

There is compelling evidence for deregulated FGF/FGFR2 signalling in the pathogenesis of many cancers that originate from different tissue types. This report provides the first pharmacologic profile of the alofanib, a selective allosteric inhibitor of FGFR2 with potential as a targeted antitumour therapy.

In the cancer cell lines tested here, incubation with alofanib resulted in potent inhibition of SUM 52PE and hFOB proliferation. SUM 52PE breast cancer cells are dependent on FGFR2 signalling to activate signal transduction pathways involving FRS2, MAPK and

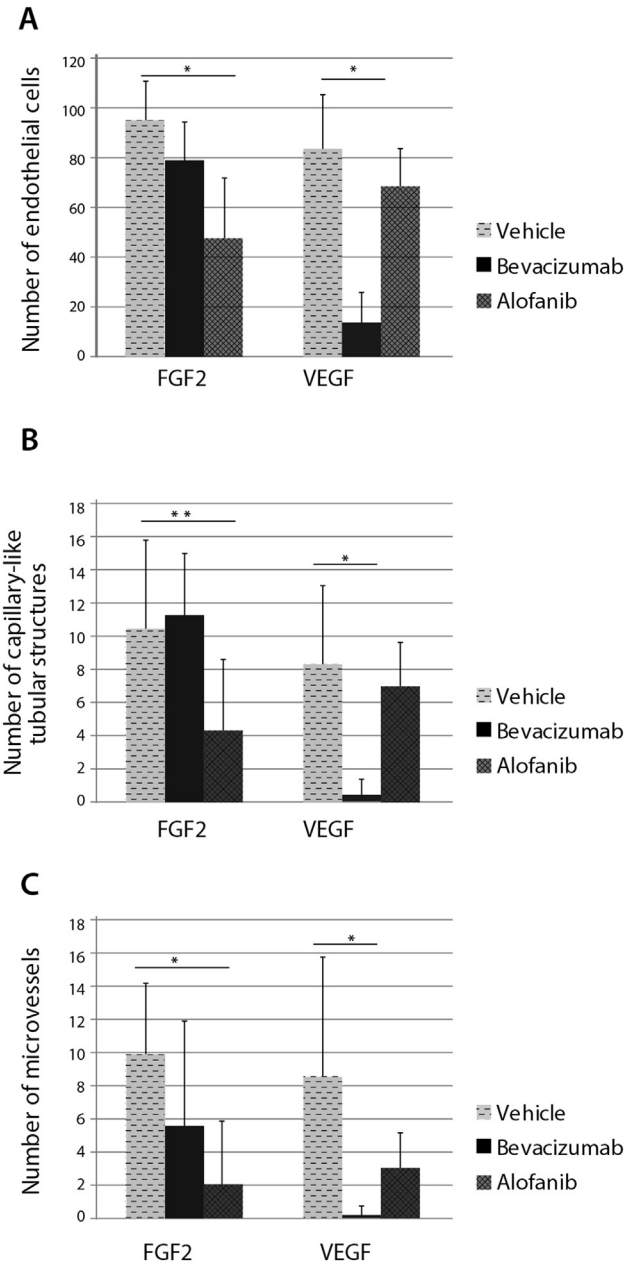

Fig. 7. Alofanib inhibits angiogenesis in mouse models. Angiogenesis was induced by injection of Matrigel containing either fibroblast growth factor 2 (FGF2) or vascular endothelial growth factor (VEGF) into immunodeficient mice. Alofanib and bevacizumab were given intraperitoneally at 15 and 10 mg/kg/d (0, 3 and 6 d), respectively. On day 7 after implantation, Matrigel plugs were recovered and were embedded in paraffin for haematoxylin and eosin staining, histological and immunohistochemical (rabbit anti-CD31/PECAM-1 antibodies, Abcam; EnVision™+ kit, Dako) analysis. Columns, mean from five animals in each group; bars, standard error. **P < 0.01, significantly different from FGF2- and bevacizumab-positive control and *P < 0.01, significantly different from VEGF-positive control.

phosphoinositide 3-kinase (PI3K) [15]. Published data support a key role for FRS2 signalling in activating both the MAPK to induce cell proliferation and differentiation and also PI3K to activate cell survival

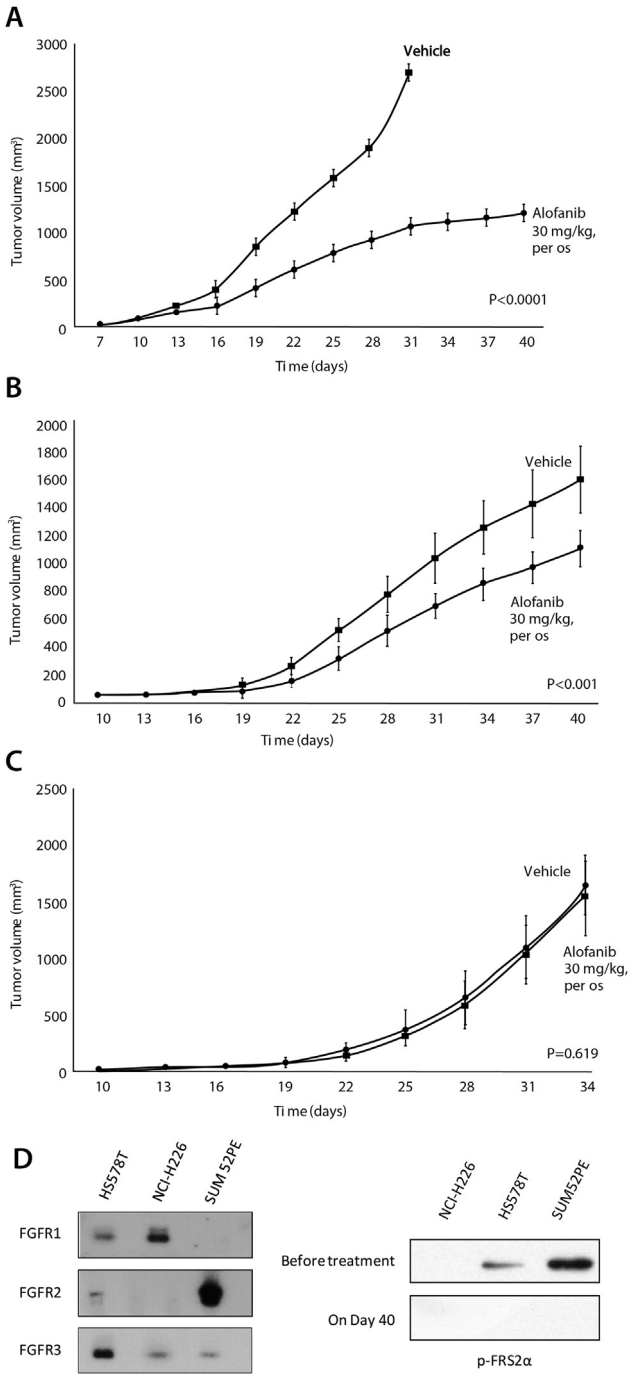

Fig. 8. The mechanism of *in vivo* growth inhibition by alofanib is fibroblast growth factor receptor 2 (FGFR2) dependent. A, FGFR2 high-expressing SUM 52PE xenografts were established and mice were dosed for 40 d. B, FGFR2 low-expressing HS578T xenografts were established and mice were dosed for 40 d. C. FGFR2-negative NCI-H226 xenografts were established and mice were dosed for 34 d. Mean tumour volumes (±standard error of the mean) are plotted. D, Immunoblot analysis of FGFR1–3 protein expression in cancer cells (left). Example of inhibition of FRS2α in xenograft tumours (right).

pathways [33]. Moreover, SUM 52PE is a cancer cell line that expresses isoform IIIb of FGFR2, and hFOB is an osteoblast cell line that expresses FGFR2 IIIc. Tumour-specific isoform switch of the FGFR2 underlies the malignant phenotypes of cancer [4,34]. Additionally, FGFR2 IIIb and FGFR2 IIIc themselves are alternatively spliced, and some variants result in shortening of the C-terminal domain of the receptor, which attenuates receptor internalization and degradation and results in enhanced signalling through the FRS2 [5]. Consistent with these data, alofanib shows potent inhibition of both cellular proliferation and FRS2α phosphorylation in SUM 52PE and hFOB cells. Increased expression of FGFR and FRS2α was associated with decreased progression-free survival among patients with metastatic cancer treated with targeted therapy as demonstrated [13].

Across other cancer cell lines tested, alofanib inhibited FGF-dependent proliferation of SKOV3 ovarian cancer cells and HS578T triple-negative breast cancer cells. SKOV3 and HS578T are tumour cell lines with known irregulations in FGFR2 expression. Conversely, FGFR2-negative melanoma cell line Mel Kor did not show antiproliferative sensitivity to alofanib. Furthermore, alofanib induced apoptosis in sensitive FGFR2-positive cells and not the resistant Mel Kor cells.

FGFs can also activate FGFRs on endothelial cells directly or stimulate angiogenesis indirectly by inducing the release of angiogenic factors from other cell types [17]. A second important mechanism of the action of alofanib is an inhibition of tumour angiogenesis. Alofanib inhibited FGF-stimulated proliferation of human and mouse endothelial cells with GI50 values between 11 and 58 nmol/l. Our data show that alofanib was at least 26-fold more potent than brivanib and bevacizumab. In human *in vitro* angiogenesis assay, alofanib reduced endothelial cell migration up to 60%. Treatment with alofanib ablated experimental FGF-induced angiogenesis *in vivo* and significantly reduced endothelial cell migration, capillary-like tubular structure and mature vessel formation in comparison with bevacizumab. These findings suggest that bevacizumab could impact on VEGF-induced angiogenesis and has no activity in FGF-induced angiogenesis. Recently, several studies showed that increased activity of FGFR or EGFR pathways is associated with resistance against VEGF inhibitors (Tsimafeyeu *et al.*, 2013c) [3,23].

The *in vivo* data presented here are consistent with alofanib being a predominantly FGFR-selective small-molecular inhibitor. In preclinical xenograft models, alofanib significantly inhibited aggressive growth of FGFR2 high-expressing SUM 52PE triple-negative breast cancer and had moderate activity in the FGFR2 low-expressing HS578T triple-negative breast cancer. Treatment with alofanib did not result in the FGFR2-negative NCI-H226 lung cancer growth. Accordingly, we believe that alofanib is capable of clinically testing an FGFR tumour-driven hypothesis, aiming to select patients based on deregulated tumour FGFR2 expression.

In summary, alofanib is a small-molecule allosteric inhibitor of FGFR2 that shows promising antiangiogenic and antitumour activities in preclinical studies.

Authors' contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Conflict of interest statement

I. Tsimafeyeu, M. Byakhov and S. Tjulandin have ownership interest in Ruspharmtech LLC.

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