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VEGF neutralizing aerosol therapy in primary pulmonary adenocarcinoma with K-ras activating-mutations

Virginie Hervé^{1,2,3}, Nathalie Rabbe^{4,5}, Laurent Guilleminault^{3,6,7}, Flora Paul^{3,6}, Laurene Schlick^{4,5}, Nicolas Azzopardi⁸, Michael Duruisseaux⁴, Delphine Fouquet^{1,2,3}, Jérôme Montharu⁹, Françoise Redini¹⁰, Gilles Paintaud¹¹, Etienne Lemarié^{3,6}, Jacques Cadranel^{4,5}, Marie Wislez^{4,5,†}, and Nathalie Heuzé-Vourc'h^{3,6,*,†}

¹Université François Rabelais, UMR, Tours, France; ²INSERM, UMR, Tours, France; ³Centre d'Etude des Pathologies Respiratoires, UMR, Tours, France; ⁴Sorbonne Universités, UPMC Univ Paris, Theraoscan, Paris, France; ⁵AP-HP, Hôpital Tenon, Service de Pneumologie, F-75970, Paris, France; ⁶Université François Rabelais, EA, Tours, France; ⁷Service de Pneumologie et Explorations Fonctionnelles Respiratoires, Hôpital Bretonneau, Tours, France; ⁸Université François-Rabelais de Tours, CNRS, GICC UMR, Tours, France; ⁹Université François Rabelais, PST Animaleries, Tours, France; ¹⁰INSERM, UMR957, Nantes, France; ¹¹Université François-Rabelais de Tours, CNRS, GICC UMR, CHRU de Tours, Laboratoire de pharmacologie-Toxicologie, Tours, France

[†]Nathalie Heuzé-Vourc'h and Marie Wislez equally contributed to the study design and are co-senior authors of this article.

Keywords: Non-small cell lung cancer, lung adenocarcinoma, anti-VEGF, monoclonal antibody, aerosol, K-ras mutation, anti-angiogenic agent, airways delivery, pharmacokinetics

K-ras mutations promote angiogenesis in lung cancer and contribute to the drug resistance of cancer cells. It is not clear whether K-ras mutated adenocarcinomas are sensitive to anti-angiogenic therapy with monoclonal antibodies (mAbs) that target vascular endothelial growth factor (VEGF). Anti-angiogenic mAbs are usually delivered systemically, but only a small proportion reaches the lung after intravenous injection. We investigated the relevance of a non-invasive pulmonary route for the delivery of anti-VEGF mAbs in the mouse K-ras^{LA1} model. We found that pulmonary delivery of these mAbs significantly reduced the number of tumor lesions and inhibited malignant progression. The antitumor effect involves the VEGFR2-dependent inhibition of blood vessel growth, which impairs tumor proliferation. Pharmacokinetic analysis of aerosolized anti-VEGF showed its low rate of passage into the bloodstream, suggesting that this delivery route is associated with reduced systemic side effects. Our findings highlight the value of the aerosol route for administration of anti-angiogenic mAbs in pulmonary adenocarcinoma with K-ras activating-mutations.

Introduction

Lung cancer remains the leading cause of cancer-related deaths worldwide, and non-small cell lung cancer (NSCLC) accounts for 85% of lung cancers. Over the past two decades, the treatment of NSCLC has radically changed with the introduction of targeted drugs, e.g., small molecule tyrosine kinase inhibitors and monoclonal antibodies (mAbs), and the selection of patients based on tumor histology and genotyping. This has led to substantial clinical benefits and improved overall survival rates. Notably, adenocarcinoma subtypes with epidermal growth factor receptor mutations and ELM4-ALK translocations have benefited from improvements in the management of advanced NSCLC.^{1–3} However, new therapeutic options are required for adenocarcinoma with V-Ki-ras2 Kirsten ras sarcoma (K-ras) activating mutations, which are encountered in 25% of adenocarcinomas. Indeed, these cancers are associated with poor survival and rarely respond to chemotherapy and some targeted therapies.⁴ Clinical trials with

MAPK inhibitors in this population are ongoing. It is unclear whether K-ras mutated lung adenocarcinomas are sensitive to anti-angiogenic therapies. Indeed, K-ras mutations promote endothelial cell-dependent tumor angiogenesis, mainly via the upregulation of vascular endothelial growth factor (VEGF) and interleukin (IL)-8 and through the repression of the anti-angiogenic protein thrombospondin-1.^{5,6} In a mouse model of lung adenocarcinomas mutated for K-ras, the neutralization of CXCR2, the mouse homolog of IL-8 receptor (CXCR2), blocks angiogenesis and impairs the formation of early lung lesions.⁶

Among the various angiogenesis inhibitors tested recently in NSCLC, bevacizumab (Avastin; Genentech/Roche) is the first molecule to target tumor angiogenesis and is the only mAb approved for use in non-squamous subtype NSCLC. Its addition to chemotherapy regimens results in a significant improvement in overall survival.⁷ Bevacizumab is a recombinant humanized IgG1 directed against soluble human VEGF-A (also called VEGF),⁸ a crucial regulator of angiogenesis and differentiation

*Correspondence to: Nathalie Heuzé-Vourc'h; Email: nathalie.vourc'h@med.univ-tours.fr

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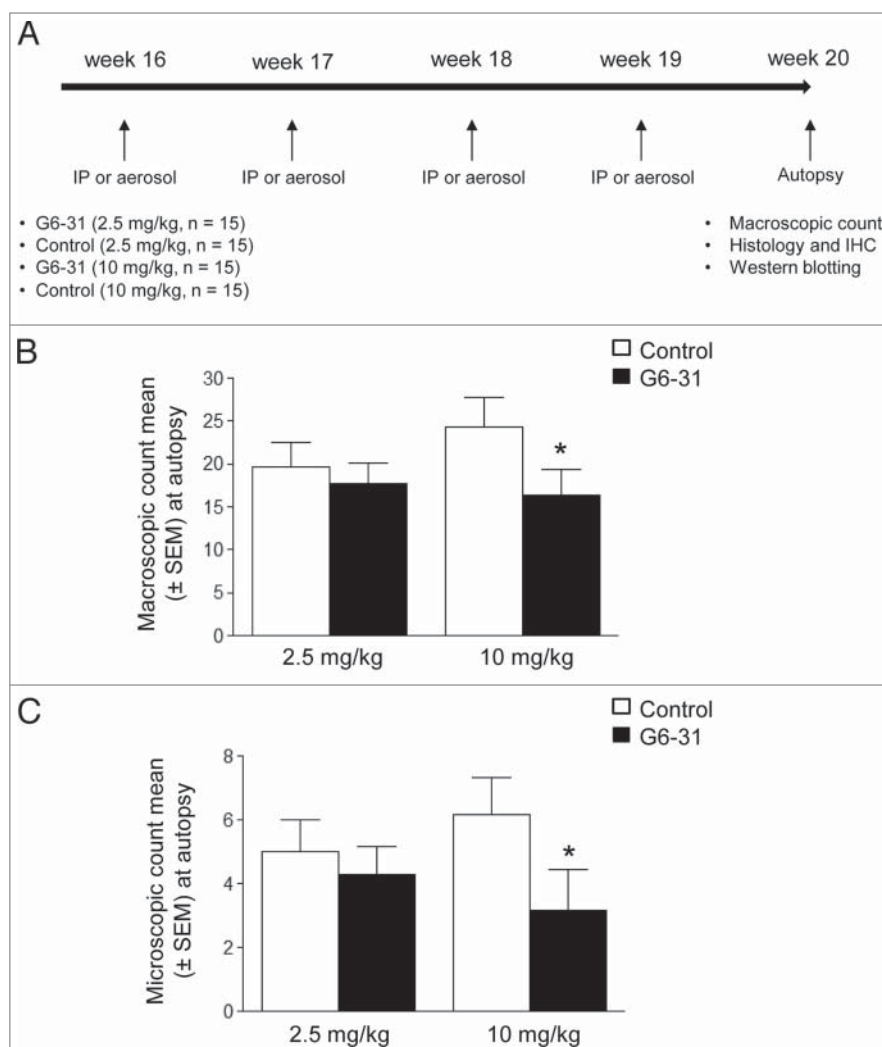


Figure 1. Assessment of G6-31 activity against lung lesions when administrated intraperitoneally. (A) Experimental procedure; Four month-old K-ras^{LA1} mice received G6-31 or an isotype control, administrated by i.p injection or aerosol, once a week for 4 wk. At the time of death visible lesions were counted on the whole lungs. (B) Quantification of visible nodules per mouse (n = 15 mice per group; 2.5 mg/kg and 10 mg/kg; *P < 0.05 Mann-Whitney test). Results are expressed as the mean ± SEM of nodules. (C) Quantification of lung lesions on H&E stained sections from control and G6-31 treated group (n = 15 mice per group; 2.5 mg/kg and 10 mg/kg; P < 0.05 Mann-Whitney test). Results are expressed as the mean ± SEM of the number of lesions.

of progenitor endothelial cells that interacts with two related tyrosine kinase receptors VEGFR-1 and VEGFR-2.^{9,10}

The airways appear to be a promising alternative to the intravenous (i.v.) route for the delivery of mAbs into the lungs to treat lung cancer. Although the i.v. route is conventionally used for their administration, the assessment of the partitioning of mAbs from the blood into the lungs shows that only a small portion of injected mAbs reach the lungs.^{11,12} Thus, the i.v. route may not be optimal to deliver mAbs to the lungs to achieve a therapeutic concentration at this target site. In theory, airways offer several advantages: loco-regional delivery allows the direct administration of the mAbs to the target and limits their passage into the bloodstream, thereby reducing systemic side effects. However, several issues should be considered before administering mAbs through the airways. We

showed previously that the pharmacological properties of mAbs are preserved after aerosolization, and that aerosolized mAbs are well tolerated and result in a therapeutic response in animal models.¹³ Moreover, passage in the bloodstream was low.¹⁴ The aims of this study were to determine whether adenocarcinomas with K-ras mutations benefit from anti-VEGF therapies and whether pulmonary delivery is an effective method of administration.

We treated a mouse model of K-ras-induced lung adenocarcinoma (K-ras^{LA1})¹⁵ with a cross-species anti-VEGF mAb (G6-31) and analyzed the anti-tumor efficacy when administrated systemically or locally into the lungs. We also studied the molecular pathways associated with the antitumor effect and the pharmacokinetics (PK) of the pulmonary and systemic routes to examine the value of the pulmonary route for the local delivery of mAbs.

Results

Systemic administration of anti-VEGF mAb reduces tumor burden in lungs from K-ras^{LA1} mice

G6-31 is a murine IgG2a mAb that binds with high affinity to both human and murine VEGF-A,⁵ and high expression of VEGF-A is associated with tumor progression in K-ras mutated NSCLC (Fig. S1). In K-ras^{LA1} mice, VEGF is expressed in papillary tumor cell subtypes from large adenocarcinomas (> 0.5 mm in diameter), but not in tumor cells from atypical alveolar hyperplasia and adenomas (Fig. S1A-B-C).

We sought to evaluate the efficacy of anti-VEGF mAb against K-ras mutated adenocarcinoma. We treated K-ras^{LA1} mice intraperitoneally once a week for 4 wk with G6-31 or with an isotype control either at 2.5 or 10 mg/kg (Fig. 1A).

Assessment of the total number of visible lung lesions, after the animals were killed, showed that mice treated with G6-31 at 10 mg/kg had significantly fewer lung lesions (P = 0.02) than control mice (Fig. 1B; Fig. S2). Treatment with 2.5 mg/kg of G6-31 did not significantly affect the number of lung lesions. Accordingly, the microscopic count of lesions on lung tissue sections showed significantly fewer lesions (P = 0.02) in mice treated with G6-31 at 10 mg/kg than in control mice (Fig. 1C).

Local administration of anti-VEGF mAb into the lungs is well tolerated and reduces tumor burden in the lungs of K-ras^{LA1} mice

We first confirmed that G6-31 inhibited VEGF-A-mediated VEGFR2 phosphorylation whether it was nebulized or not with MicroSprayer® Aerosolizer (Fig. S3A).

We then determined whether aerosol delivery of G6–31 was well tolerated in 16 wk-old wild-type mice with a genetic background similar to that of K-ras^{LA1} mice. We treated mice orotracheally with the antibody (10 mg/kg) once a week for 4 wk (Fig. 1A). This treatment was not associated with any secondary effects or changes of behavior and we found no lesions or pulmonary hemorrhage on lungs (Fig. S3B) and no lesions on other organs (kidneys, liver and spleen; data not shown).

The number of lung macroscopic lesions in K-ras^{LA1} mice that received G6–31 at either dose by pulmonary route was significantly lower than in control mice (2.5 mg/kg dose, $P = 0.0010$; 10 mg/kg dose, $P = 0.0013$). Accordingly, the number of lung microscopic lesions in K-ras^{LA1} mice treated with G6–31 at 10 mg/kg by the pulmonary route was significantly lower than in control mice ($P = 0.04$) (Fig. 2A–B; Fig. S2). Altogether, these results highlight the effectiveness of the pulmonary route for the delivered of mAb.

Anti-VEGF mAb reduces adenocarcinoma lesions

We performed histological analysis to examine the effect of anti-VEGF therapy on malignant progression. Mice treated with G6–31 (10 mg/kg) delivered by intra-peritoneal (i.p.) injection or aerosol had fewer adenocarcinoma lesions than control mice ($P = 0.008$), whereas the number of atypical alveolar hyperplasia and adenoma was not modified by the treatment (Fig. 3A). These results are in agreement with the restricted expression of VEGF to adenocarcinomas in K-ras^{LA1} mice that we observed by immunohistochemistry (Fig. S1).

Anti-VEGF mAb inhibits angiogenesis

We used immunohistochemistry to analyze microvascular density in the tumor because G6–31 achieves tumor regression by blocking angiogenesis.^{16–19} Microvascular density, as assessed by Von Willebrand Factor (vWF) immunostaining (Fig. 3B), was lower in G6–31 treated mice than in control mice (Fig. 3C). G6–31 mostly affected the microvascular density of large vascular structures (i.e., those with a diameter $> 10 \mu\text{m}^{20,21}$) when it was delivered by i.p. injection (Table 1), whereas it mostly affected small vessels (mostly capillaries with a $d < 10 \mu\text{m}^{20,21}$) when it was administered by aerosol (Table 1).

The effect of anti-angiogenic therapies on tumor oxygenation and hypoxia is still unclear and may depend on the tumor model.^{22–26} Thus, we analyzed the expression of the oxygen-sensitive regulatory subunit hypoxia-inducible factor (HIF)-1 α , as a marker of tumor hypoxia, in K-ras^{LA1} mice treated with G6–31 (Fig. S4; Table S1). Western blotting showed that there was no significant difference in abundance of HIF-1 α in whole lung extracts from mice treated with G6–31 (either by i.p. or pulmonary administration) and control mice (Table S1).

Anti-VEGF mAb impairs tumor cell proliferation

We analyzed tumor cell proliferation and apoptosis in the lung of animals treated with G6–31 by i.p. or aerosol, to explore the mechanisms associated with reduced tumor burden. The mean proliferative index measured by proliferating cell nuclear antigen (PCNA) expression was significantly lower in G6–31-

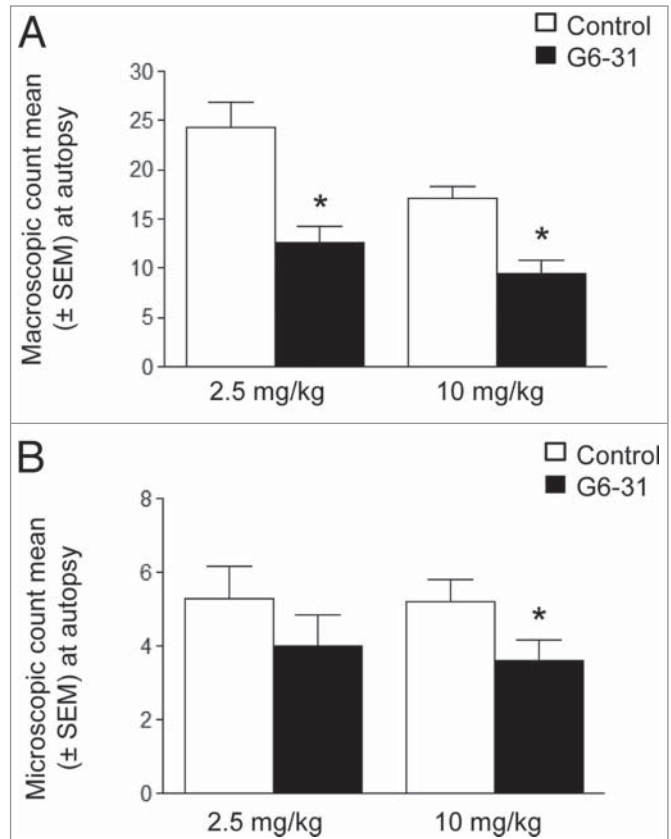


Figure 2. Assessment of aerosolized G6–31 activity against lung lesions. (A) Quantification of visible nodules per mouse ($n = 15$ mice per group; 2.5 mg/kg and 10 mg/kg; $*P < 0.05$ Mann-Whitney test). Results are expressed as the mean $\pm$ SEM of nodules. (B) Quantification of lung lesions on H&E stained sections from control and G6–31 treated group ($n = 15$ mice per group; 2.5 mg/kg and 10 mg/kg; $P < 0.05$ Mann-Whitney test). Results are expressed as the mean $\pm$ SEM of the number of lesions.

treated animals (10 mg/kg) than in control animals, regardless of the route of administration (Fig. 4A; Table 2). However, immunohistochemistry showed that the abundance of cleaved caspase 3 (CC3), which is a marker of apoptosis, was significantly higher in tumors from mice treated intraperitoneally than in those from control mice. This was not the case for G6–31 administrated via the airways, suggesting different mechanisms of action of G6–31 depending on the delivery route ($P < 0.0001$; Figure 4B and C).

VEGF neutralization impairs VEGFR2 activation

VEGFR2 is believed to predominantly contribute to VEGF-mediated tumor angiogenesis; therefore, we analyzed members of its signaling pathway by western blotting in whole lung lysates in response to G6–31 treatment by aerosol or i.p. injection. Neutralization of VEGF by G6–31 impaired VEGFR2 phosphorylation (Y1175), indicating that the VEGFR2 pathway is inhibited in G6–31 treated mice²⁷ ($P = 0.0008$ for i.p. and $P = 0.012$ for aerosol; Figure 5; Table 3). Phosphorylation of AKT (Ser 473)

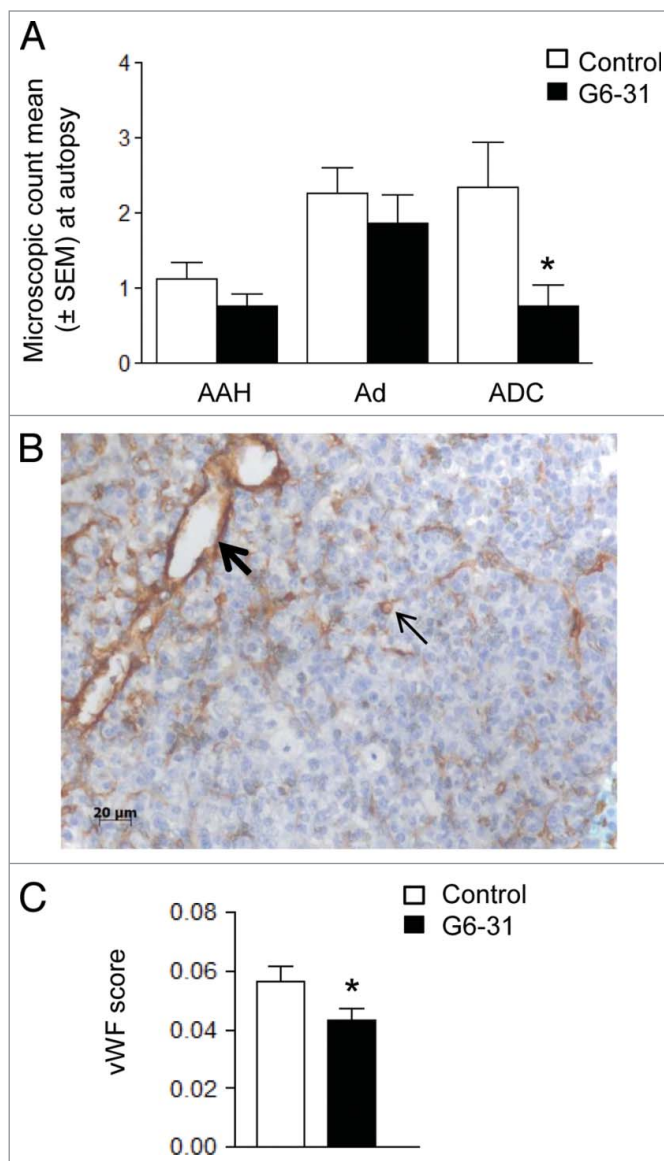


Figure 3. Effect of G6-31 at 10 mg/kg administered by i.p. injection or aerosol on K-ras^{LA1} lung tumors according to each lesion type and effect on microvascular density. **(A)** Quantification of AAH (atypical alveolar hyperplasia), Ad (adenoma) and ADC (adenocarcinoma), on H&E stained lung sections from control and G6-31-treated (10 mg/kg dose) mice (n = 30 mice per group; *P < 0.05 Mann-Whitney test). Results are expressed as the mean ± SEM of the number of each lesion type. **(B)** Representative image of vWF immunostaining in the lung of K-ras^{LA1} mice. Bold arrow shows large vessel while standard arrow shows small vessel. **(C)** Quantification of the microvascular density, from vWF immunostaining in control and G6-31-treated (10 mg/kg dose) mice (n = 30 mice per group; *P < 0.05 Mann-Whitney test). Results are expressed as the mean ± SEM.

was significantly lower in mice treated with G6-31 administrated by aerosol than in control mice ($P = 0.0018$; Figure 5; Table 3). Phosphorylation of PI-3K (Y458) and ERK (T202/Y204) signaling factors downstream of VEGFR2,^{27,28} were unaffected by the treatment (Fig. 5; Table 3).

Table 1. Effect of G6-31 (10 mg/kg) on microvascular density in K-ras^{LA1} lung tumors, assessed from vWF immunostaining. Quantification of the microvascular density (MVD) of small (< 10 μ m) and large (> 10 μ m) vascular structures (n = 30 mice per group; control and G6-31 10 mg/kg; *P < 0.05 Mann-Whitney test)

	vWF% (± SEM)	
	I.P. G6-31 / Placebo	Aerosol G6-31 / Placebo
Small vessel (< 10 μ m)	+ 9.85 (± 20.05) $P = 0.96$	− 44.21 (± 7.67) $P = 0.018$
Large vessel (> 10 μ m)	− 43.10 (± 12.09) $P = 0.0096$	− 11.50 (± 14.69) $P = 0.27$

Altogether, these results show that anti-VEGF mAb delivered by the airways or by i.p. injection reduces tumor burden through similar mechanisms.

Aerosolized anti-VEGF mAb passes slowly and poorly into the systemic circulation

We developed a quantitative ELISA to analyze the passage of anti-VEGF mAb into the circulation after pulmonary delivery in K-ras^{LA1} mice treated with a unique dose of antibody (10 mg/kg) (Fig. S5). Only a small amount of G6-31 enters the bloodstream after pulmonary delivery (Fig. S6). The maximum serum concentration was observed about 6 h after G6-31 aerosolization and was about 100-fold lower than that after systemic delivery (mean of 1.25 μ g/mL for the highest values of the pulmonary route vs. 150 μ g/mL for the systemic delivery) (Fig. 6A). We first used a non-compartmental analysis to describe the PK parameters of aerosolized G6-31. However, this approach was invalidated because the *MRT* was higher in the i.v. group than in the pulmonary group, and consequently the mean absorption time was negative, which is impossible given that pulmonary administration should lead to a longer persistence of the drug in the organism (Table S2). This bias can be most likely due to the nonlinear profile of G6-31 PK as observed in Figure 6A. Thus, we used a compartmental analysis as presented in Figure 6B. This model was able to describe the concentration-time profiles for G6-31 following i.v. and pulmonary administration with a good fit between the observed and predicted values (Fig. 6A). The estimated PK parameter values of the compartmental approach are summarized in Table 4. The estimated bio-available fraction was 5.1%. This value is consistent with the bio-available fraction already estimated for other mAbs.¹⁴ We used a Michaelis–Menten equation to quantify the saturable component of the elimination of G6-31; the maximum elimination rate V_{max} was 12.8 d^{−1} and a Michaelis constant k_M was 0.45 μ g·mL^{−1} (Fig. S7).

Discussion

The first aim of this study was to evaluate whether K-ras mutated lung tumors benefit from an anti-angiogenic drug given as a monotherapy. We showed that VEGF neutralization with a

mAb (G6-31, IgG2a,k) markedly inhibits lung tumorigenesis. It reduced the number of K-ras mutated adenocarcinomatous lesions in a mouse model of lung adenocarcinomas in which VEGF is highly expressed.²⁹ We found that the anti-VEGF mAb mediated its antitumor activity partly through the alteration of VEGF-dependent angiogenic mechanisms, as shown by the inhibition of VEGFR2 signaling, and the regression of blood vessels. The VEGF/VEGFR2 pathway plays a central role in angiogenesis and regulates endothelial cell migration, proliferation, permeability and survival.^{27,30,31} Both VEGF and VEGFR2 are commonly expressed in NSCLC³² and are important components that promote tumor growth, progression and metastasis.³³ In agreement with these findings, we found that VEGF neutralization by G6-31 impairs cell proliferation in the lungs of K-ras^{LA1} mice.

Table 2. Quantification of the level of PCNA (detected by western blotting) by densitometric analysis in mice treated with G6-31 by i.p. or aerosol (15 mice per group, 10 mg/kg) and in control mice (15 mice per group) (**P* < 0.05 for both, Mann-Whitney test). Results are expressed as the mean ratio of PCNA normalized to β-actin and relative to the control group ± SEM

	Densitometry - Mean% (± SEM)	
	I.P. G6-31 / Placebo	Aerosol G6-31 / Placebo
PCNA/β actin	– 46.80 (± 7.70) <i>P</i> = 0.0065	– 31.49 (± 8.97) <i>P</i> = 0.009

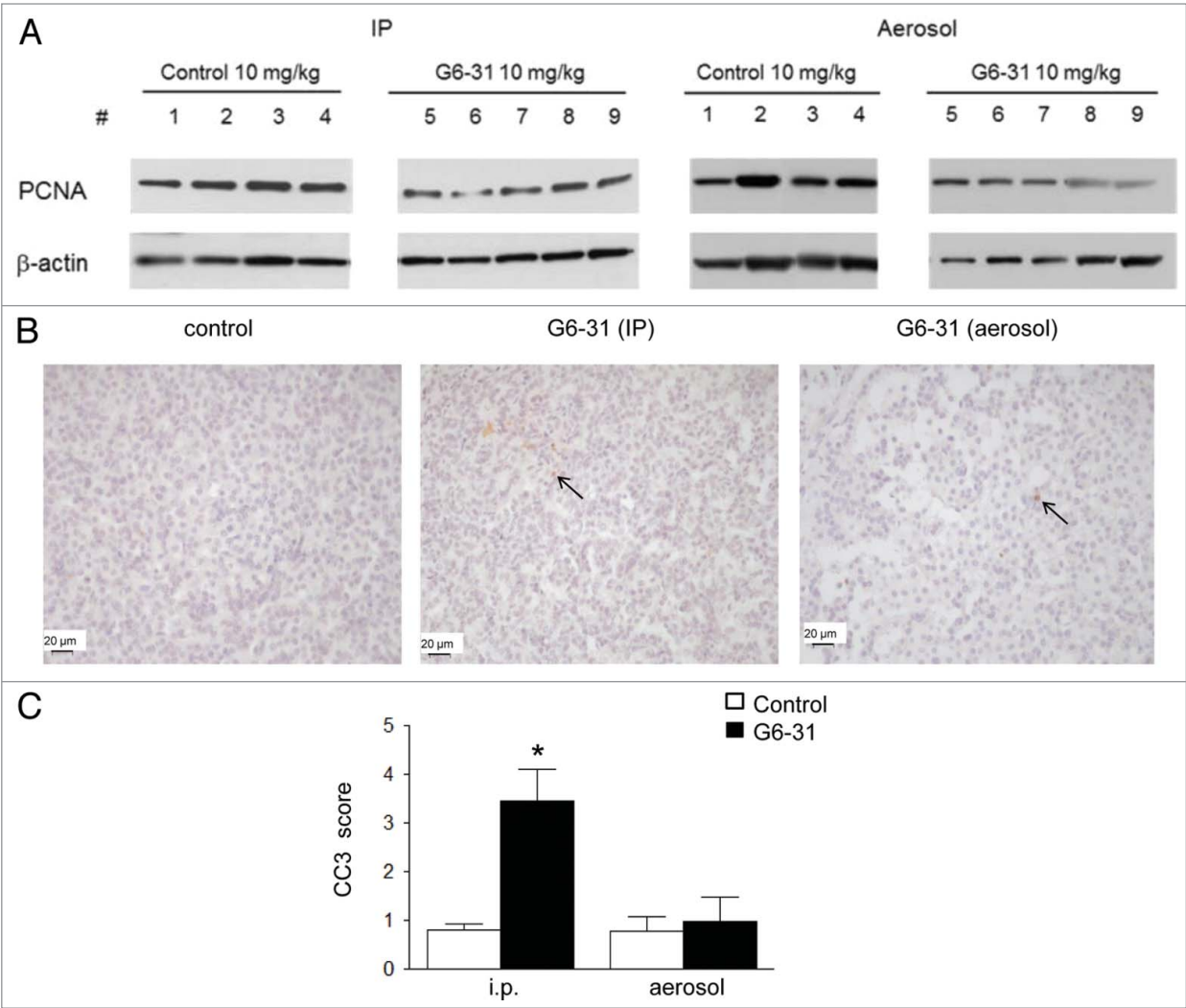


Figure 4. (A) Detection of PCNA by western blotting in whole lung protein extracts from control mice (mice 1–4) and G6-31-treated (10 mg/kg) mice (mice 5–9). Pulmonary delivery is on the right and i.p. delivery is on the left. (B) CC-3 immunostaining. Representative images of the control (left) and G6-31 (10 mg/kg dose) delivered either by i.p. (middle) or by pulmonary route (right). Brown cells are positive for CC-3 (arrows show some representative cells). The slices were counterstained with hematoxylin (× 400 magnification). Quantification of apoptosis in tumor lesions of the right lung of K-ras LA1 mice (n = 15 mice per group; **P* < 0.05 Mann-Whitney test). Results are expressed as the mean staining scores of CC-3 positive cells relative to the control group ± SEM.

But the antitumor efficacy of G6-31 in K-ras^{LA1} mice may not depend only on the angiogenic properties of the VEGF/VEGFR pathway since autocrine or paracrine VEGF promotes cancer cell survival.³¹

As described herein, we found an induction of apoptosis after anti-VEGF mAb treatment. The reason why CC3, a marker of apoptosis, was increased only in the tumor lesions after a systemic treatment with G6-31 has not been clarified. This discrepancy may be due to a concentration-dependent effect of the anti-VEGF mAb, which is undoubtedly more concentrated within the lungs following pulmonary delivery than following systemic delivery.^{34,35} Similar differential apoptotic effects were previously reported for Taxol.³⁶

Intratumoral hypoxia regulates VEGF levels and is often encountered in NSCLC.³⁷ We analyzed HIF-1 α as a molecular marker of tumor hypoxia and did not find any change to HIF-1 α expression by western blotting with whole lung protein extract, or by IHC on tumor area sections. This suggests that the intrinsic tumor cell characteristics of K-ras mutated NSCLC do not lead to tumor adaptation or evasion of anti-angiogenic agents through therapy-induced hypoxia.³⁸⁻⁴⁰ These findings support the rationale for targeting angiogenesis alone in NSCLC and particularly in K-ras mutated adenocarcinomas. Moreover, neutralizing VEGF may be doubly relevant in NSCLC with K-ras mutations, allowing the blockade of both VEGF-VEGFR1 and -VEGFR2 molecular pathways. Recently, VEGF-VEGFR1 signaling pathway has been shown to contribute to tumor survival and metastasis.^{31,41}

We also showed here that the local delivery of G6-31 through the pulmonary route achieved an antitumor response in K-ras^{LA1} mice. The pulmonary delivery of G6-31 was well tolerated and significantly reduced the number of tumor lesions. Neutralization of VEGF by aerosolized G6-31 inhibited tumor proliferation,

Table 3. Quantification of the level of phosphorylated VEGFR2 and total VEGF-2 by densitometric analysis in mice treated with G6-31 by i.p. injection or aerosol (15 mice per group, 10 mg/kg) and in control mice (15 mice per group) (**P* < 0.05, Mann-Whitney test). Results are expressed as the mean ratio of phospho-VEGFR2 Y1175 to total VEGFR2 relative to that of the control group $\pm$ SEM. The same method of quantification was applied to Phospho-PI3K, β -actin, Phospho-AKT, total AKT, phospho-ERK and total ERK. Results are expressed as the mean ratio of Phospho-PI3K to β -actin relative to that of the control group $\pm$ SEM, of Phospho-AKT to total AKT relative to that of the control group $\pm$ SEM and of Phospho-ERK to total ERK relative to that of the control group $\pm$ SEM

Densitometry - Mean% ($\pm$ SEM)		
	I.P. G6-31 / Placebo	Aerosol G6-31 / Placebo
Phospho-VEGFR2/ VEGFR2	- 63.81 ($\pm$ 5.09) <i>P</i> = 0.0008	- 68.80 ($\pm$ 11.09) <i>P</i> = 0.0127
Phospho-PI3K/ β actin	- 30.14 ($\pm$ 13.31) <i>P</i> = 0.72	- 5.13 ($\pm$ 3.24) <i>P</i> = 0.28
Phospho-AKT / AKT	+ 34,23 ($\pm$ 13.34) <i>P</i> = 0.10	- 44,48 ($\pm$ 4.19) <i>P</i> = 0.0018
Phospho-ERK / ERK	+ 1.29 ($\pm$ 20.31) <i>P</i> = 0.90	+ 3.33 ($\pm$ 3.40) <i>P</i> = 0.28

angiogenesis and blocked VEGF/VEGFR2 molecular pathways. Although the systemic delivery of G6-31 gave similar results, the effects on blood vessels differed according to the route of administration. G6-31 delivered through the pulmonary route correlated with a significant reduction in the number of small vessels, whereas systemic injection mostly affected large vessels. Moreover, downstream mediators of VEGF/VEGFR2 (PI3K/AKT), and apoptosis were also differently modulated according to the delivery route, suggesting the involvement of several signaling pathways in the pharmacodynamics of G6-31 dependent on the route of administration.

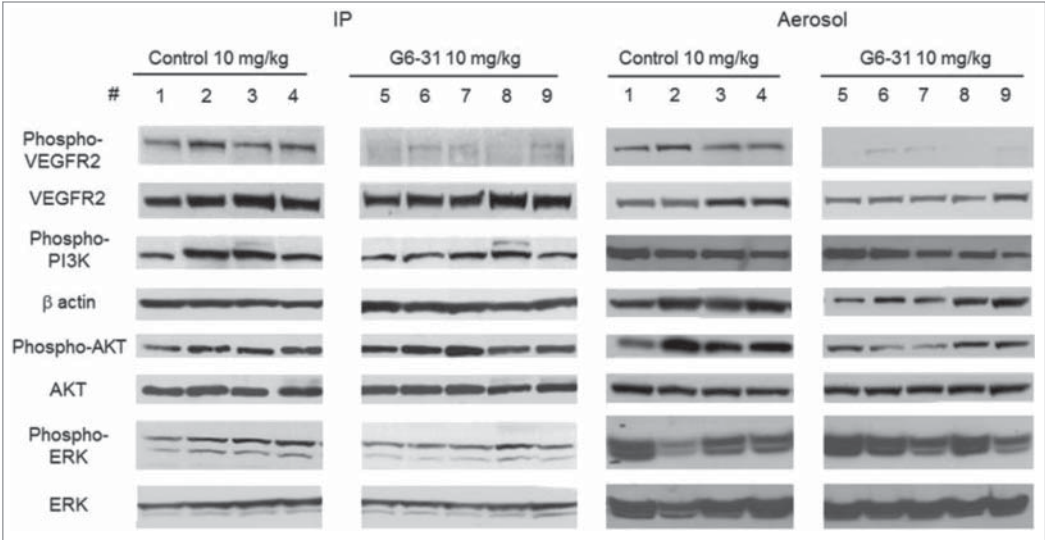


Figure 5. Blockade of VEGFR2 signaling pathway. Phospho-VEGFR2 Y1175, total VEGFR2, Phospho-PI3K, β -actin, Phospho-AKT, total AKT, Phospho-ERK and total ERK were analyzed by western blotting in whole lung protein extracts from control mice (mice 1-4) and from mice treated with G6-31 at 10 mg/kg (mice 5-9). Pulmonary delivery is on the right and i.p. delivery is on the left.

The PK study of anti-VEGF mAb revealed significant differences between the local and systemic routes. Non-compartmental and compartmental analyses estimated a low value of bio-availability of G6-31 in serum after pulmonary delivery, in agreement with previous results.¹⁴ This poor passage into the bloodstream after pulmonary delivery is advantageous because of the pulmonary localization of the multiple aberrant foci. We found that the non-compartmental analysis was not relevant for estimating the PK parameters, and we used a compartmental approach to take into account the nonlinear

elimination of G6–31. Passage into the circulation may be related to FcRn-mediated active transport and lymphatic drainage, as described previously.^{14,42,43} A linear elimination of mAbs targeting soluble antigens is generally observed,⁴⁴ whereas those targeting membrane-bound antigen often display nonlinear kinetics mainly due to target-mediated drug disposition. We found that the kinetics of G6–31 elimination were nonlinear, and its estimated parameters were similar to mAbs with target mediated elimination. This may be explained by the high affinity of G6–31 for its target antigen VEGF-A.¹⁸ By reducing systemic exposure, the pulmonary route may reduce systemic adverse side effects. However, the low passage of the drug into the bloodstream may also limit the therapeutic effect of anti-VEGF mAb on extra-pulmonary metastasis.

In conclusion, our results show the antitumor efficacy of G6–31 in K-ras^{LA1} visualized by a blood vessel regression and a decrease in tumor growth rate. This antitumor effect occurs through inhibition of angiogenic and non-angiogenic mechanisms related to the VEGF/VEGFR pathway.³¹ Our findings highlight the clinical value of anti-VEGF inhibitors for patients with K-ras mutated lung adenocarcinoma. They also emphasize the relevance of using local delivery of mAbs through the pulmonary route to treat lung adenocarcinomas.

Material and Methods

Antibodies

Murine anti-human/mouse VEGF-A G6–31 (batch PUR21821, 8 mg/mL), was kindly provided by Genentech (MTA OR-209 778). IgG2a,k from murine myeloma that was used as a control isotype antibody, was purchased from Sigma Aldrich (St Quentin Fallavier, France). For immunohistochemistry, we purchased rabbit polyclonal anti human/mouse CC3 (Asp175) (Cell Signaling Technology, Ozyme, St Quentin en Yvelines, France), anti-human/mouse vWF (DakoCytomation, Trappes, France), anti-human/mouse VEGF (SC-507, Santa Cruz,

Table 4. Estimated values of compartmental pharmacokinetic parameters. Abbreviations: *F*, bio available fraction; *k_{ar}*, absorption rate; *V₁*, central volume of distribution; *k₁₀*, systemic elimination and *k₁₂*, *k₂₁* distribution rates. *V_{max}*, maximum elimination rate; and *k_M*, Michaelis constant

Parameter	Value (CV%)
<i>F</i> (%)	5.1 (–)
<i>k_a</i> (day ^{–1})	1.59 (18)
<i>k₁₀</i> (day ^{–1})	0.2 (13)
<i>V₁</i> (mL)	2 (13)
<i>k₁₂</i> (day ^{–1})	0.46 (–)
<i>k₂₁</i> (day ^{–1})	0.94 (60)
<i>V_{max}</i> (day ^{–1})	12.8 (23)
<i>k_M</i> (μg/mL)	0.46 (13)
<i>a</i>	0.026
<i>b</i>	0.15

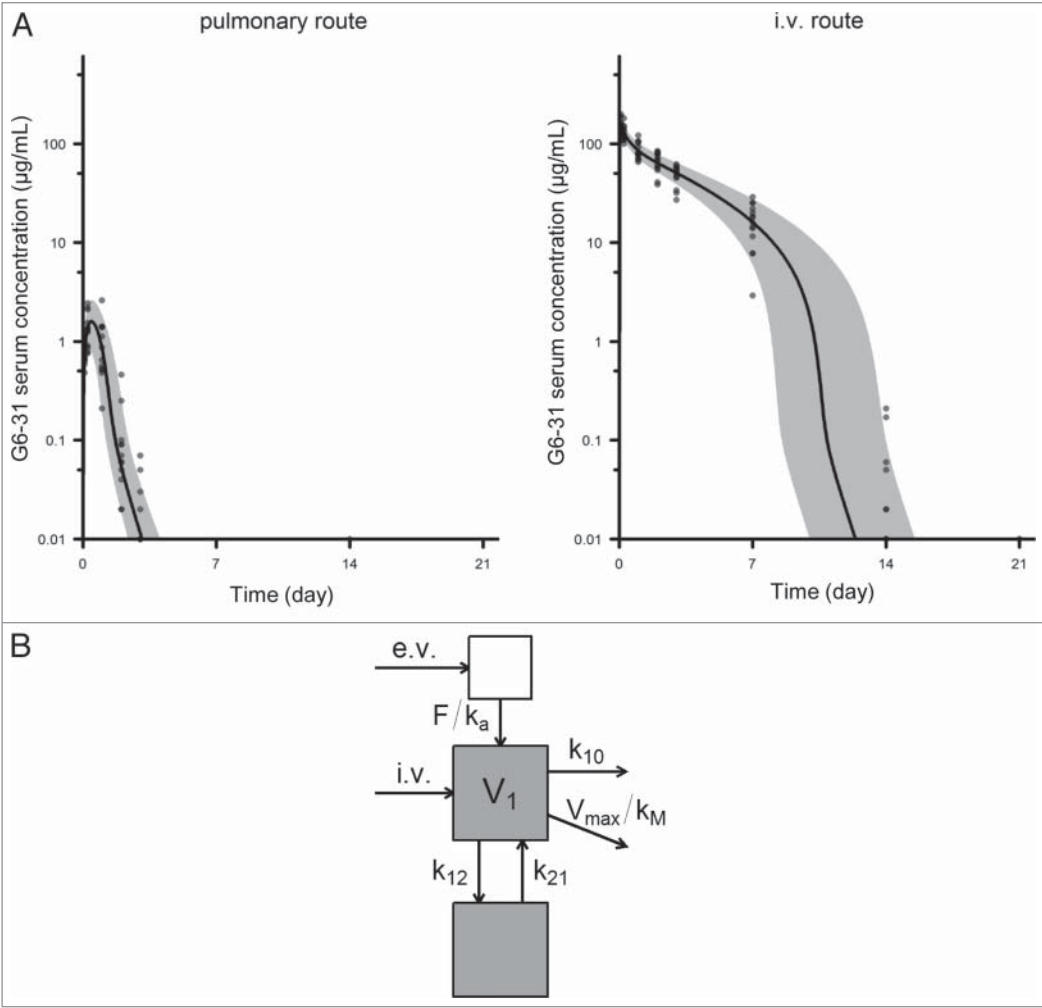


Figure 6. Pharmacokinetics of G6–31. (A) Serum concentration of G6–31 mAb was measured by ELISA in the serum collected at different time points from mice that received a single dose of the mAb (10 mg/kg) either by the pulmonary route (left, *n* = 13) or intravenously (right, *n* = 15). Gray area and solid line show the 5th-95th and 50th percentiles of the model-predicted time-concentration profiles, respectively. (B) Schematic representation of the pharmacokinetic compartmental model of G6–31.

Tebu-Bio SA, Le Perray-en-Yvelines, France), rat mAbs against F4/80 (Interchim, Montluçon, France) and mouse anti-HIF1 α [H1 α 67] - ChIP Grade (ab1) (Abcam, Paris, France).

For western blotting, we used the following primary antibodies: rabbit monoclonal anti-human/mouse phospho-VEGF receptor 2 (Tyr 1175), anti-human/mouse VEGF receptor 2, anti-human/mouse phospho-AKT (Ser 473), rabbit polyclonal anti-human/mouse AKT, rabbit polyclonal anti-mouse phospho-ERK1/2 (T202/Y204), rabbit polyclonal anti-human/mouse ERK1/2 and anti-mouse phospho-PI3K (Tyr 458)/p55 (Tyr 199) (Cell Signaling Technology), rabbit polyclonal anti-human/mouse active CC3 and rabbit monoclonal anti-human/mouse HIF-1 α from Novus Biologicals (Interchim, France), rabbit polyclonal anti-human/mouse PCNA (proliferating cell nuclear antigen) and rabbit polyclonal anti-human/mouse β -actin from Sigma Aldrich. Secondary antibodies for western blotting were peroxidase-conjugated Affini-Pure goat anti-rabbit IgG (H⁺L) and peroxidase-conjugated Affini-Pure goat anti-mouse IgG (H⁺L) from Jackson ImmunoResearch (Interchim).

Cell culture

Human umbilical vein endothelial cells (HUVEC) were grown according to the manufacturer's instructions (PromoCell GmbH, Heidelberg, Germany). HUVECs were seeded in 6-well plates and maintained up to 80% confluence. Native or aerosolized G6-31 at 1, 5 or 10 nM in 1X PBS was pre-incubated with 0.5 nM of human VEGF-A (Cell Signaling) for 60 min, and then added to the cells. After 7 min incubation at 37 °C, cells were lysed in RIPA buffer and VEGFR 2 phosphorylation (Phospho-VEGFR 2 Tyr 1175 and total VEGFR Ab from Cell Signaling) was analyzed by western blotting.

Animal experiments

Animals

K-ras^{LA1} or wild-type mice with the same genetic background (C57BL/6N) were housed and handled according to the guidelines from the European Animal Care and Use Committee (agreement #2010/11), and body weight was measured weekly. K-ras^{LA1} is a murine model of lung adenocarcinoma with K-ras mutations.¹⁵ The K-ras^{LA1} mice were provided by the NCI Mouse Models of Human Cancers Consortium (MMHCC) (NCI Mouse Repository/NIH, Rockville, MD). These mice carry a latent *K-ras* allele with two copies of exon 1: one wild-type copy and one copy with the G12D mutation. The latent allele is stochastically activated in cells through homologous recombination, which results in the deletion of the wild-type copy of exon 1 and the expression of an oncogenic form of the *K-ras* gene. Substantial epithelial changes were observed at 4–8 wk, and included atypical adenomatous hyperplasia and small adenomas, followed by adenomas with papillary or atypical features, and finally well-differentiated adenocarcinomas at 6–8 mo.^{6,15} All animals were censored for survival based either on mortality or pre-determined morbidity criteria for euthanasia. Mice were monitored three times a week by an investigator who

was blind to the data and were euthanized if they showed signs of respiratory distress which included hunching, ruffled fur, labored breathing, low body temperature, lack of mobility, lethargy, > 20% decrease in body weight, or abdominal distension, according to the standards of the Institutional Animal Care and Use Committee.

Tolerance of aerosolized G6-31 in normal mice

Sixteen week-old wild-type mice were anesthetized with anesthetic gases and received, either G6-31 (10 mg/kg) or NaCl 0.9%, by the pulmonary route (aerosol) administered with a MicroSprayer[®] Aerosolizer - Model IA-1C - connected to a FMJ-250 High Pressure Syringe (Penn Century, Philadelphia, PA). The mice received this treatment once a week for 4 wk (n = 15 per group). At the end of the experiment, mice were killed and lungs, liver, spleen and kidneys were collected for histological and biochemical studies.

Antitumor efficacy of repeated doses of G6-31 when administrated by the intra-peritoneal or aerosol route

Sixteen week-old K-ras^{LA1} mice received either G6-31 (2.5 or 10 mg/kg) or a control isotype mouse IgG2a,k (2.5 or 10 mg/kg), once a week for 4 wk (n = 15 per group). G6-31 or IgG2a,k were administrated by i.p. injection or pulmonary route. Visible surface lung lesions were counted during autopsy by investigators who were blind to the treatment group. After perfusion with NaCl 0.9%, one lung was snap-frozen to extract protein and the other one was fixed in 4% formaldehyde and embedded in paraffin for histological and immunohistochemistry studies as described previously.⁶

Collection of blood for pharmacokinetics from mice that received G6-31 by the intravenous or aerosol route

A PK study was conducted in 16-wk-old K-ras^{LA1} mice. At this age, multiple aberrant lung foci have already developed.^{6,15} A unique dose of G6-31 (10 mg/kg) was administrated by either the i.v. or the pulmonary route (n = 15 per group). Blood samples (100–200 μ L) were collected submandibularly, at different times after G6-31 delivery: 0, 2, 6, 24, 48, 72 h, 7, 14, 21 and 28 d. Concentrations of G6-31 in sera were measured by quantitative ELISA and PK of G6-31 was studied by non-compartmental and compartmental approaches.

Histological analysis

All lesions were identified by histological analysis on hematoxylin and eosin (H&E) staining. According to the histological criteria established by Johnson et al.,¹⁵ two investigators blind to the treatment group counted atypical adenomatous hyperplasias, adenomas and adenocarcinomas from one H&E section per lung for each mouse at $\times 20$ magnification. Results were given as microscopic lesion count (mean $\pm$ SEM) for all lesions and for each histological subtype.

Immunohistochemical analysis

4- μ m sections were deparaffinized, rehydrated, and washed with PBS pH7.4. The VEGF, CC3, F4/80 and HIF1 α antigens were retrieved with 0.01 M citrate buffer (pH 6) (DakoCytomation) for 30 min in a steamer. Slides were exposed for 10 min to protease 1 (Ventana, Roche Diagnostics, Meylan, France) to detect vWF. Samples were blocked for endogenous activity in 3% hydrogen peroxide-PBS, avidin/biotin blocking kit (Invitrogen, Life Technologies, Saint-Aubin, France), and Protein Block serum-free (Dakocytomation), and were then incubated with the primary antibody overnight at 4 °C. Standard avidin-biotin immunoperoxidase methods, with diaminobenzine as the chromogen, were used for detection. The primary antibody was omitted as a negative control.

We used a quantitative approach to score CC3 and F4/80 staining. We counted positive intra-lesional cells for the whole tumor section and expressed this number as the number of positive cells per tumor-section area. Tissues were visualized at $\times$ 20 magnification to score all antigens, except for CC3, which was scored from tissue visualized at $\times$ 40 magnification.

For the quantification of microvascular density, we evaluated the total number of vWF positive sections in the whole tumor section and discriminated small ($< 10 \mu$ m) from large ($> 10 \mu$ m) vessels.^{20,21} Microvascular density was expressed as the percentage of vessels per tumor-section area.

Western blot analysis

Lysates from lung tissue samples were separated by SDS-PAGE and transferred onto a polyvinylidene fluoride nitrocellulose membrane (Invitrogen). After saturation, membranes were immunoblotted, overnight at 4 °C or for 2 h at room temperature, with primary antibodies in TBS containing 5% nonfat dry milk or 5% bovine serum albumin (BSA) for Cell Signaling antibodies. The membranes were washed and then incubated with secondary antibody for 1 h. Antibody binding was detected with an enhanced chemiluminescence kit according to the manufacturer's instructions (Super Signal West Pico Chemiluminescent Substrate, Pierce, Illkirch, France). The films were subsequently scanned, and band intensity was quantified with densitometry software (Image J, NIH).

Measurement of G6-31 concentrations by a quantitative ELISA

The concentration of G6-31 antibodies in sera was determined by a quantitative ELISA, especially developed for this study. Microplates (NuncMaxisorpimmunoplate, Thermo Scientific, Courtaboeuf, France) were coated with 7.5 ng of recombinant human VEGF-A (Ozyme, St Quentin Yvelines, France), diluted in 100 μ L of 1 M carbonate buffer pH 9.6 and were incubated overnight at 4 °C. After three washes with PBS pH 7.4 containing 0.05% Tween 20, the plates were incubated for 90 min at 37 °C with 1% of BSA (Sigma, St Quentin Fallavier, France) prepared in PBS to block unsaturated binding sites (200 μ L/well). After one wash, serum samples and standards (dilutions of G6-31) were distributed (100 μ L/well) in the wells and incubated for 60 min at 37 °C. Serum samples were initially

diluted from 1:25 to 1:32000 in PBS containing 1% BSA (PBS-BSA). Standard dilutions of G6-31 were serially diluted from 125 to 0.25 ng/mL in PBS-BSA. After five washes, a peroxidase-conjugated rat anti-mouse IgG2a polyclonal antibody (BD Biosciences, Le Pont de Claix, France) was added (final dilution 1:10000, 100 μ L/well) and incubated for 60 min at 37 °C. Peroxidase activity was detected with 100 μ L of freshly prepared SigmaFastTM OPD (Sigma, St Quentin Fallavier, France) substrate added to every well. After a 10 min incubation at room temperature, the reaction was stopped by the addition of 50 μ L/well of 4 N H₂SO₄. After incubation for 30 min at room temperature, absorbance was measured at 490 nm with an automated microplate reader (Versa Max, Molecular Devices, Saint-Gregoire, France).

Serum G6-31 concentration was calculated from the linear part of the standard curve. The lower limit of quantification (LLOQ) was 0.114 ng/mL and the lower limit of detection (LLD) was 0.069 ng/mL. Correction was made for serum dilution and G6-31 concentration was expressed in μ g/mL of serum. For each microtiter plate, the assay included antigen, sera and enzyme conjugate controls.

Pharmacokinetic analysis

The non-compartmental approach describes the PK profiles in terms of area under the concentration-time curve ($AUC_{0 \rightarrow \infty}$ in $\text{mg} \cdot \text{L}^{-1} \cdot \text{day}$), area under the first moment concentration-time curve ($AUMC$ in $\text{mg} \cdot \text{L}^{-1} \cdot \text{day}^2$), mean residence time (MRT in day), elimination half-life ($t_{1/2}$ in day), mean absorption time (MAT in day) and bio-available fraction (F in%). This analysis was performed using R (version 3.0.1, Vienna, Austria).⁴⁵

The compartmental approach consisted of population PK modeling, with Monolix (version 4.2.2), LixsoftOrsay, France.⁴⁶ This method describes the concentrations measured after both routes of administration with the same model, optimizes the estimation of common parameters, and allows the calculation of the fraction absorbed after pulmonary delivery (F). Analysis of the data revealed that G6-31 elimination is accelerated at low concentrations. One- and two-compartment models with first-order or saturable elimination from the central compartment were tested to describe this nonlinearity. A Michaelis-Menten equation with maximum elimination rate (V_{max}) and the Michaelis-Menten constant (k_M) was used to describe the saturable component of the elimination of G6-31. The other PK parameters were the central volume of distribution (V_I) and systemic elimination (k_{10}) and distribution (k_{12} , k_{21}) rates. A first-order absorption rate (k_a) was also used for the pulmonary route.

Statistical analysis

The differences between groups (mean $\pm$ SEM) were compared with the non-parametric Mann-Whitney test. P values < 0.05 (*) were considered statistically significant (GraphPad Prism 5.0 software).

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher’s website.

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