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Aerodynamical, Immunological and Pharmacological Properties of the Anticancer Antibody Cetuximab Following Nebulization

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Abstract:

Purpose Despite an increasing interest in the use of inhalation for local delivery of molecules for respiratory diseases and systemic disorders, methods to deliver therapy through airways has received little attention for lung cancer treatment. However, inhalation of anticancer drugs is an attractive alternative route to systemic administration which results in limited concentration of the medication in the lungs, and triggers whole-body toxicity. In this study, we investigated the feasibility of nebulization for therapeutic antibodies, a new class of fully-approved anticancer drugs in oncology medicine.
Materials and methods Cetuximab, a chimeric IgG1 targeting the epidermal growth factor receptor (EGFR), was nebulized using three types of delivery devices: a jet nebulizer PARI LC+®, a mesh nebulizer AeronebPro® and an ultrasonic nebulizer SYST'AM® LS290. Aerosol size distribution was measured using a cascade impactor and aerosol droplets were observed under optical microscopy. The immunological and pharmacological properties of cetuximab were evaluated following nebulization using A431 cells.
Results The aerosol particle clouds generated with the three nebulizers displayed similar aerodynamical characteristics, but the IgG formed aggregates in liquid phase following nebulization with both the jet and ultrasonic devices. Flow cytometry analyses and assays of EGFR-phosphorylation and cell growth inhibitions on A431 demonstrated that both the mesh and the jet nebulizers preserved the binding affinity to EGFR and the inhibitory activities of cetuximab.
Conclusions Altogether, our results indicate that cetuximab resists the physical constraints of nebulization. Thus, airway delivery represents a promising alternative to systemic administration for local delivery of therapeutic antibodies in lung cancer treatment.

Keywords: aerosol - aggregation - anticancer antibody - cetuximab - lung cancer - nebulizer

Introduction

The lung is the most common site of metastasis from primary neoplasia, and lung cancer is the leading cause of cancer-related deaths worldwide. Despite the availability of new drugs that target cancer cells and more efficient chemotherapeutics, the outcome for the treatment of lung cancer has not changed dramatically and the cure rate remains one of the lowest among all malignancies (1). The delivery of an inadequate drug concentration to the tumor site after intravenous or oral administration is a potential reason for the limited efficacy of new therapeutics.

Inhalation is an attractive delivery route for anticancer drugs. It offers several potential advantages over systemic and oral routes, including loco-regional delivery to the lungs providing high drug concentration, avoidance of the first-pass metabolic degradation in the liver and reduction of systemic side effects (2, 3). This route has been used for a long time to administer local-acting agents in respiratory diseases (*i.e.* asthma, infections). Its use is also being adopted for the delivery of systemic-acting therapeutics whether they are small molecules or macromolecules (3-6). As a hallmark of success, the first inhaled insulin powder, Exubera®, has recently been approved in Europe and US for the treatment of adult patients with type 1 or type 2 diabetes (7).

Although preclinical studies have shown significant antitumor activity of aerosolized drugs (8-12), and phase-I clinical trials demonstrated the feasibility of nebulization and the safety of anticancer therapeutics delivered through the airways in humans (13-16), inhalation remains minimally explored as a method of drug administration for lung cancer treatment. Aerosolization has been tested on few anticancer drugs. Among new anticancer molecules,

therapeutic antibodies have emerged as a new class of successful drugs for hematopoietic malignant neoplasia and solid tumours, becoming a major strategy for therapy in oncology and representing half of the anticancer molecules in development in industry. Currently nine naked or conjugated antibodies have been approved by the Food and Drug Administration (FDA) and the European MEdicines Agency (EMEA) for human cancer treatments and numerous antibodies are in late clinical trial phases (17, 18).

Therapeutic monoclonal antibodies (mAb) are typically of the IgG1 subclass and correspond to large glycoproteins with a molecular mass around 150 kDa. Unlike synthetic small molecules for which lung metabolism is minimal, proteins are highly susceptible to hydrolysis occurring inside cells that engulf foreign particles, such as macrophages, within the lung (3). Previous studies delineated the potential of solid aerosols for pulmonary delivery of antibodies (19-21) and showed that manipulations of aerosol characteristics allow control of Ig release and prevent phagocytosis. However, spray-drying of pure proteins in aqueous solutions often resulted in the production of aggregates and/or loss of therapeutic activity requiring changes in drug formulation.

Although nebulization of large proteins into airways was widely debated, recent data demonstrated that proteins could be efficiently administered through airways as liquid aerosols when conjugated to the constant Fc region of IgG. The high bioavailability of those Fc-conjugated macromolecules was achieved *via* exploitation of the FcRn transport/protection pathway of antibody (4, 22, 23). Thus, nebulization that allows direct utilization of drugs in solution, such as anticancer antibodies, might represent an alternative to dry-powder inhalers for local delivery of mAb in lung cancer treatment.

Because the nebulized anticancer antibody needs to be fully-active when delivered to patients to be efficient, this paper attempts to determine whether mAb resists the physical constraints of nebulization. Herein, we investigated the impact of three different types of nebulizers on aggregation, and the immunological and pharmacological properties of cetuximab, a chimeric IgG1 targeting EGFR, which is currently being tested in i.v. from in clinical trials for lung cancer treatment (24-26).

Material and Methods

Cell culture and antibodies

The A431 human epidermoid carcinoma cells and the human bronchioalveaolar carcinoma cell line A549 were obtained from ATCC (American Type Culture Collection). The cells were grown at 37 °C in an atmosphere of 5% CO_2 in Roswell Park Memorial Institute Medium (RPMI-1640) containing 10% fetal bovine serum (FBS), 100U/mL penicillin, 100µg/mL streptomycin.

Cetuximab and rituximab (control irrelevant antibody) were purchased from Merck KGaA (Darmstadt, Germany) and Roche (Fontenay-sous-Bois, France) respectively. Cetuximab and rituximab were supplied at a concentration of 2 mg/mL and 10 mg/mL respectively, as neutral aqueous solutions (sodium phosphate buffer).

Polyclonal antibodies anti-total EGFR (SC-03) and anti-phospho EGFR (pY1068) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and from Merck KGaA (Darmstadt, Germany) respectively. FITC-conjugated $F(ab')_2$ anti-human IgG and peroxidase-conjugated anti-rabbit IgG were respectively purchased from Jackson Immuno Research (Cambridgeshire, UK) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Nebulization and characterization of aerosol particles

Cetuximab was nebulized with three different devices: (1) a jet nebulizer, PARI LC+® (PARI, Germany) using an air compressor that breaks the medication into small breathable particles, (2) an ultrasonic nebulizer, SYST'AM® LS290 (SYST'AM, France) applying high frequency vibration through medication solutions, creating standing waves that generate aerosol and (3) a mesh nebulizer, AeronebPro® (Aerogen, USA) using a vibrational element with a micro-pumping action to create aerosol particles.

Briefly, 9 mL of cetuximab were loaded in each nebulizer (in triplicate) and samples were collected in liquid phase with an impinger (Ace Glass Inc., Vineland, USA) operating at an air flow rate of 12.6 L/min. The total period of operation did not overtake 20 minutes. A BCA (BiCinchoninicAcid) Protein Assay Kit (Perbio Sciences, France) was used to control cetuximab concentration following nebulization, and volumes of nebulized cetuximab were adapted for cell assays. Following nebulization, cetuximab solutions were observed under an optical microscope (Axiovert 25, Zeiss).The number of aggregates per milliliter was calculated using a Malassez heamatocytometer and their sizes estimated.

Particle size distribution was measured using an IMPAQ-GS-1E cascade impactor (California Measurements, USA) under a flow rate of 1 L/min. The IMPAQ-GS-1E cascade impactor has been calibrated by the manufacturer at 1 L/min. This air flow rate determines cut off diameters of each stage. The IMPAQ-GS-1E cascade impactor has been used by other authors with the same air flow rate. The advantage of the low flow cascade impactor is to limit the influence of the addition of air to measure the particle size (27, 28). The deposited aerosol on each impactor stage was resuspended in PBS containing 1% SDS. A BCA Protein Assay Kit was then used to determine the mass of antibody deposited on each stage. MMAD and the estimated breathable fraction (namely FPF for fine particle fraction (<5 µm)) were determined using data of cascade impaction of three independent experiments.

Recognition of EGF by flow cytometry

A549 and A431 cells were harvested by trypsinization, washed with phosphate-buffered saline (PBS) and incubated in PBS containing 2 % of FBS (FACS buffer) with native or nebulized cetuximab (2 µg/mL and 10 µg/mL for A549 and A431 respectively) for 1 hour at 4°C. Rituximab (2 µg/mL or 10 µg/mL) was used as isotype-matched nonbinding antibody. Subsequently, cells were washed twice with FACS buffer and incubated at 4°C in the dark for

30 minutes with FITC-F(ab')2 anti-human IgG diluted in FACS buffer. After two additional washing steps, cells were resuspended in 0.5 mL of FACS buffer containing 7-amino actinomycin D (2 µg/mL), which was used to exclude dead cells. Cells were analyzed on an EPICS XL flow cytometer using Expo32 software (Beckman Coulter).

Evaluation of affinity to EGFR by flow cytometry

A431 cells ($1.5\ 10^5$) were harvested using trypsin and incubated with unlabeled native or nebulized cetuximab (5.10^{-6} to 5.10^{-2} mg/mL) for 30 minutes at 4°C. Then, cells were incubated for 30 minutes at 4°C with FITC-cetuximab (5 µg/mL) or with the isotype-matched nonbinding antibody FITC-rituximab (5 µg/mL). After two washes with PBS, cells were resuspended in 0.45 mL of PBS and analyzed on an EPICS XL flow cytometer using Expo32 software (Beckman Coulter).

Growth inhibition assay

Cells were plated in a 96-well culture plate at a density of 5,000 cells/well and allowed to grow overnight in appropriate maintenance medium. Twenty-four hours after plating, RPMI-1640 was added to the cells with or without cetuximab (50 µg/mL of native or nebulized antibody). Then, they were incubated for another 48 hours, harvested and counted using a Malassez heamatocytometer. The relative growth was calculated as the ratio of the median control value. Experiments were repeated six times in triplicate with the different types of nebulized cetuximab.

Phosphorylation inhibition assay

A431 cells were seeded on a 6-well plate at a density of 4.10^5 cells/well and allowed to grow overnight in appropriate maintenance cell culture medium. The medium was then replaced

with RPMI-1640 with or without cetuximab (50 µg/mL of native or nebulized antibody). After 24 hours incubation, cells were treated with 10ng/mL recombinant EGF for 10 minutes, washed with ice-cold PBS and lyzed with 200 µL of RIPA buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 % NP40, 1 % SDS, 50 mM NaF, 1 mM sodium orthovanadate, 1 X Protease Inhibitor Cocktail (Sigma, France)). Protein lysates were collected and immediately boiled at 100°C for 7 minutes. After 15 minutes centrifugation at 12,000 xg, supernatants containing cell proteins were recovered and protein concentration was determined using a BCA Protein Assay.

Protein extracts were separated on a 7.5 % SDS-PAGE and then transferred on PVDF (polyvinyldene difluoride) membranes by electroblotting. The membranes were incubated with 5 % nonfat dry milk diluted in TBS-T (10 mM Tris [pH 7.4], 150 mM NaCl, 0.05 % Tween 20) for 1 hour to block nonspecific binding, and were then incubated with either the anti-phospho EGFR IgG or the anti-total EGFR antibody (to control loading equal amounts) under the conditions recommended by the manufacturers. The blots were washed with TBS-T three times and incubated with the appropriate secondary antibody conjugated to HRP for 1 hour. After three additional washes, the membranes were developed using an enhanced chemiluminescence plus detection system (Amersham Biosciences, Buckinghamshire, UK).

Statistical analysis

For growth inhibition assays, the results were expressed as medians and the difference between groups was compared using a Kruskal and Wallis non parametric test. A p value inferior or equal to 0.05 was considered for statistical significance.

Results

To analyze the feasibility of nebulizing cetuximab, we used three devices with different technological approaches (a jet nebulizer PARI LC+®, a mesh nebulizer AeronebPro® and an ultrasonic nebulizer SYST'AM® LS290) and then evaluated the aerodynamical characteristics of cetuximab aerosols, protein aggregation, and the immunological and pharmacological properties of nebulized cetuximab.

Aerosol characteristics

Because precipitates were observed in the liquid phase recovered from Impinger after the generation of aerosol particles with some nebulizers, solutions were observed with an optical microscope. Whereas solutions obtained after nebulization with the AeronebPro® device remained comparable to the native medication, both the jet and the ultrasonic nebulizers resulted in the formation of insoluble particles (Figure 1). As revealed by a BCA protein assay performed on the solid fraction, the insoluble particles contained proteins (data not shown). This result indicates that immunoglobulin aggregates were formed in the liquid phase after nebulization of cetuximab with both the ultrasonic and jet devices. As shown in Table 1, the SYST'AM® LS290 induced the formation of more and larger aggregates than the PARI LC+®. However, the increased temperature in the medication compartment (reaching approximately 30°C) after 20 minutes of operation that was observed with the SYST'AM® LS290 was not responsible for the numerous aggregates formation since incubation of cetuximab solution during 20 minutes at 37°C in a water bath, did not result in the generation of insoluble particles.

To determine particle size, cetuximab aerosols were drawn through a ten-stage cascade impactor. Because insoluble aggregates were observed following resuspension of cetuximab

from each stage in PBS in some cases, SDS 1 % was added in the saline solution to dissolve the aggregates and allow the quantification of antibody on each stage. Although small differences were observed in the particle size and FPF with the three nebulizers (Table 2), all MMAD and FPF ranged from 1.6 to 2.7 µm and 58 to 84 % respectively, which are well suited particle sizes for pulmonary deposition within the respiratory zone (2).

To determine the biological impact of nebulizers on anticancer antibodies, we analyzed both the immunological and pharmacological properties of aerosolized-cetuximab in cell assays. In all the experiments, cetuximab was recovered as a liquid solution with Impinger following nebulization with the various devices. The effects observed with the SYST'AM® LS290-generated cetuximab aerosol on cells were variable, either increased or reduced compared to the native cetuximab. This discrepancy is most likely attributed to the presence of aggregates, and thus resulted in the disqualification of the ultrasonic device for further analysis.

Evaluation of aerosolized cetuximab affinity to EGFR

Cetuximab is a chimeric antibody that binds to EGFR, a type I transmembrane receptor overexpressed in numerous human tumors including ovary or lung carcinomas (29). To determine whether nebulization modified the binding of cetuximab to EGFR, flow cytometry analysis was performed on two cell lines known to express this receptor: A549, a non small cell lung cancer line and A431, cells derived from an epidermoid carcinoma (30).

As shown in figure 2, the histogram profiles of cells are unchanged with comparable means of fluorescencewhether the cells are labeled with native or aerosolized cetuximab, indicating that nebulization with either the mesh or the jet device did not alter mAb binding to EGFR. To gain insight into the impact of nebulization on the immunological properties of cetuximab, the

binding of cetuximab to EGFR was assessed by competition assay on A431 cells using a FITC-labeled cetuximab. Figure 3 showed that native and aerosolized cetuximab display similar means of fluorescence, with IC50 values comprised between 1.65 10^{-4} and 1.9 10^{-4} mg/mL.

Capacity of aerosolized cetuximab to interfere with EGFR transduction pathway

In agreement with previous reports, we found that non-small cell lung cancer cell lines (A549, H460) (data not shown) displayed a limited response to cetuximab *in vitro* (30). Thus, the nebulization effect on the pharmacological properties of cetuximab was analyzed using the highly sensitive A431 cells.

Cetuximab binding to EGFR is expected to block ligand-receptor interaction, therefore preventing receptor phosphorylation and blocking signaling pathways (31, 32).

When A431 were incubated with recombinant EGF (10 ng/mL) for 10 minutes, EGFR phosphorylation was increased (data not shown). Pre-incubation of cells with native cetuximab (50 µg/mL) limited EGFR phosphorylation by subsequent addition of EGF (figure 4). Moreover, both AeronebPro®- PARI LC+®-nebulized cetuximab led to a decrease in EGFR phosphorylation after ligand stimulation that was comparable to the native antibody.

Ability of aerosolized cetuximab to inhibit tumor cell growth

As previously shown, the stimulation of EGFR by its natural ligands enhances cell proliferation, and cell growth is potently inhibited by EGFR inhibitors such as cetuximab (32). In this study, we investigated the anti-proliferative effect of native and aerosolized cetuximab in A431 by counting cells after 48 hours treatment with the antibody. As shown in figure 5, native cetuximab induced a 45 to 50 % growth inhibition in A431 cells and

cetuximab nebulized with either AeronebPro® or PARI LC+® were similarly potent in suppressing A431 proliferation.

Discussion

In the present study, we analyzed the feasibility of nebulizing fully-active therapeutic antibodies, a class of drugs undergoing major development in medical oncology. As a model, we chose cetuximab, an anti-EGFR chimeric IgG1 which prevents the binding of EGFR natural ligands, therefore preventing receptor activation and blocking signaling pathways leading to tumor cell growth and survival (33).

At the present time, two types of devices have been tested for macromolecule administration in the conducting airways: dry-powder inhalers that deliver solid aerosols and nebulizers resulting in the administration of drug droplets (4, 6, 19-21). Despite the approval of dry-powder inhalers for administration of locally-acting drugs in asthma and chronic obstructive pulmonary diseases, these devices have important limitations for antibody aerosolization. Anticancer antibodies, whether they are naked or conjugated (with the exception of trastuzumab (Herceptin®) and gemtuzumab ozogamycin (Mylotarg®)), are generally supplied as liquids, but spray-drying of pure proteins in aqueous solutions often produces aggregates and/or loss of activity. Although the addition of disaccharides or surfactant in the medium can stabilize immunoglobulin during spray-drying, novel drug formulation must be established and evaluated (19-21, 34).

The use of nebulizers to administer anticancer antibodies is attractive since these devices offer the benefit of direct utilization of drug in solutions. However, delivery of peptides and proteins as liquid aerosols into the lungs has been largely discussed because these molecules are easily hydrolyzed within the conducting airways when they are delivered as liquid aerosols (3). Recently, a new paradigm for the pulmonary delivery of high molecular weight proteins with Fc domains emerged suggesting that it may not be the case for antibodies. Indeed, studies showed that FcRn, a transport (transcytosis) and protective pathway for

immunoglobulins, is responsible for efficient delivery and absorption through the lungs. In addition, large proteins conjugated to the Fc domain of IgG contained in aerosol droplets have a longer half-life (4, 22, 23). Because FcRn is expressed by epithelial cells of the upper and central airways and alveolar macrophages, this receptor might protect therapeutic mAbs administered as liquid aerosols from intracellular degradation, favor direct uptake of the drug by tumor cells derived from epithelial cells, or transport it across the lung epithelial barrier into the pulmonary bloodstream that supplies tumor cells. Although nebulizers might be a promising alternative to dry-powder inhalers for the pulmonary delivery of mAb, the impact of nebulization on anticancer antibody activity remains to be fully explored.

The most important parameter to determine the successful delivery of inhaled drug into the lungs is the aerodynamical characteristics of the aerosol. Particles between 1-3µm are preferably deposited in the alveolar tissue, while particles greater than 6µm are generally swallowed rather than inhaled. To generate cetuximab aerosols, we tested three devices with different processes of nebulization. Particle size demonstrated that the three devices formed droplets of cetuximab with an aerodynamical diameter comprised between 1.6 to 2.7µm, a range of size appropriate for efficient deposition at their site of action in central (2-5µm) and peripheral airways (<2µm) (2). However, the cetuximab droplets produced with AeronebPro® were slightly larger (2.4-2.7µm) than with the other devices. Interestingly, Bitonti and Dumont suggest that fine particle aerosols targeting proteins to the alveolar space is not crucial for efficient lung deposition and absorption of large proteins (such as erythropoietin) conjugated to the Fc domain of IgG, because FcRn, the appropriate carrier-mediated transport system, is mainly expressed by the epithelial cells of the upper/central airways (4). Thus, the droplet size of AeronebPro®-cetuximab may become a major asset to allow IgG transport/protection by FcRn. Further experiments will be required to define FcRn expression

in lung cancer and determine the role of this IgG transporter on lung tumor uptake (intratumoral distribution) of therapeutic antibodies.

As observed, the jet and ultrasonic nebulizers produced numerous macroscopic aggregates of cetuximab in solution that were more abundant and larger with the latter device. This physical instability reaction is usually induced by a variety of physical factors, such as temperature, ionic strength, agitation, surface/interface adsorption, or simply time. In agreement with Steckel *et al.*, a moderate rise in temperature (compared to other ultrasonic nebulizers) was measured in the medication compartment with SYST'AM® LS290 (35). However, it is not responsible for the aggregation formation since no insoluble particles were observed after incubation of native cetuximab in the same conditions in a water bath. Previous studies showed that recirculation of the aerosol droplets into the reservoir exerts high shear stress on the drugs, leading to protein denaturation and aggregation (34, 36). In contrast to the AeronebPro®, both the jet and the ultrasonic nebulizers have a recirculation system that might explain aggregate formation.

Surprisingly, the formation of aggregates in cetuximab solution following nebulization with the SYST'AM® LS290 and PARI-LC+® did not correlate with an increase in aerosol particle size, as observed in the impaction results. These results suggest that the intramolecular interactions of hydrophobic regions of partially folded, unfolded or denatured IgGs that lead to aggregation formation is prevalent and favored in liquid phase (37). Impaction and rapid drying of droplets on the stages reduced aggregation formation in the impactor. Because the experiments were assessed at room temperature and relative humidity which does not mimic the 100% relative humidity and 37°C environment of airways, the results may not be predictive of the aerosol particle behavior inside the pulmonary tract.

Insoluble aggregates are often constituted of denatured molecules and associated with no or reduced biological activity (6, 38). The experiments assessed on cells with the SYST'AM® LS290-nebulized cetuximab resulted in unreliable results that were most likely associated with the variability in the amount of aggregates added to the cells. However, the slightly higher percentage of fluorescence inhibition observed in the competition assays with SYST'AM® LS290-nebulized cetuximab following filtration on a 0.2µm membrane compared to the same concentration of unfiltrated antibody suggests that cetuximab-binding to EGFR might be less efficient if the antibody is aggregated (data not shown). Analysis of PARI LC+®-aerosolized cetuximab binding to EGFR and anticancer activity demonstrated no significant difference compared to the native or AeronebPro®-nebulized cetuximab, indicating that the antibody retained its immunological and pharmacological characteristics following nebulization with the jet device. However, our results did not rule out a potential loss of activity of the cetuximab contained in the aggregates since the biological effects observed might only be due to the soluble fraction of cetuximab in the solution.

As previously described with recombinant human proteins (*i.e.* erythropoietin-α), protein denaturation, aggregation or micelle formation is also characterized by an altered immunogenicity, resulting in the generation of antibodies induced by the breakdown of immune tolerance existing normally to self-antigens (38). The mechanisms leading to the breakdown tolerance are not elucidated but mainly result in the production of binding antibodies that can either have no consequences for the patient or diminish the therapeutic potential of the drugs. Therefore, the use of appropriate nebulizers is of great importance with respect to anticancer antibody stability because formation of antibody aggregates may increase the drug immunogenicity.

Although airways are an ideal route of administration for the local treatment of lung cancer, aerosol drug delivery and deposition within the lung remain challenging. In the conditions we tested, the mesh nebulizer AeronebPro® that has no recirculation system seemed to be the best device to deliver an anticancer antibody retaining immunological and pharmacological properties into the lungs, while creating droplets susceptible to deposition primarily in the upper/central conducting airways where FcRn is mainly express. Whereas our findings support the feasibility of nebulizing therapeutic antibodies, structural analysis of the molecules following nebulization would be useful to fully validate this concept. Further studies are also required to compare the antitumor activity and side effects of mAbs delivered via airways or systemic routes in animal models.

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Figures caption

Figure 1: Aggregates in cetuximab solution after nebulization.

Aggregates were observed under an optical microscope (on the left cetuximab aerosolized with the PARI LC+® and with the SYST'AM® LS290 on the right).

Figure 2: Binding of cetuximab to EGFR following nebulization.

A431 ***(A)*** or A549 ***(B)*** were respectively incubated with 10µg/mL or 2µg/mL cetuximab or matched control isotype. Subsequently, followed by incubation with FITC-F(ab')2 anti-human IgG and then analyzed by flow cytometry. Isotype control antibody histograms are indicated as gray line and cetuximab treated samples as black line. Mean fluorescence is indicated in the upper corner of the histograms.

Figure 3: Evaluation of aerosolized cetuximab affinity to EGFR.

A431 cells were incubated with FITC-cetuximab (5µg/mL) or with matched control FITC-rituximab and then, with unlabeled cetuximab (5.10^{-6} to 5.10^{-2} mg/mL). Cells were analyzed by flow cytometry. Results of cells treated with cetuximab are indicated as dashed line, with cetuximab nebulized by AeronebPro and PARI LC+ as solid and dotted line respectively.

Figure 4: Aerosolized cetuximab inhibits EGFR phosphorylation.

4.10^5 A431 were seeded on a 6-well plate and treated with cetuximab (50µg/mL) for 24h. Cells were then incubated with 10ng/mL recombinant EGF for 10 minutes and lysed. Immunoblots of protein lysates were analyzed for phosphorylated and total EGFR. Results are representative of one experiment out of at least three independent experiments showing similar profile.

Figure 5: Effect of cetuximab on A431 cells proliferation.

Cells (5.000 cells per well in a 96-well plate) were treated for 48h with cetuximab (50µg/mL) and then counted using a Malassez cell. Results are expressed as median of the percents of relative growth and are representative of six independent experiments. ● $p<0.025$ *vs.* control

Figure 1

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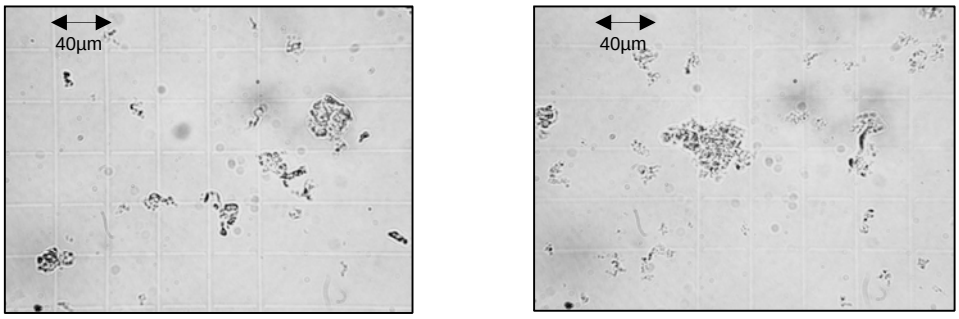

Figure 1 : Aggregates in cetuximab solution after nebulization.

Table 1
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Table 1: Quantity of cetuximab aggregates in solution after nebulization.

Experiment	1	2	3
	Aggregates per mL		
Native cetuximab	0 - 0	- 0	
Cetuximab AeronebPro	0 - 1 000	- 2 000	
Cetuximab PARI LC+	380 000 - 490 000	- 610 000	
Cetuximab SYST'AM	840 000 - 990 000	- 1 510 000	

Nebulizations were performed as described in Material and Methods. Aggregates were counted under an optical microscope using a Malassez cell. The results are expressed as number of aggregates per mL, counted in at least three independent experiments.

Table 2

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Table 2: Aerodynamical characteristics of cetuximab aerosols.

	MMAD (µm)	Fine Particule Fraction (%)
AeronebPro	2.4	74
	2.7	58
	2.6	58
PARI LC+	2.3	77
	1.7	82
	1.7	82
SYST'AM	2	74
	1.6	84
	1.7	80

Figure 2

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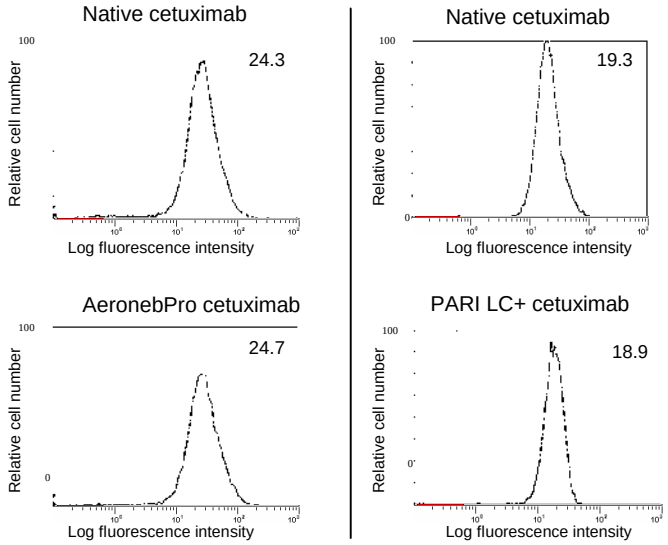
A. A431



B. A549

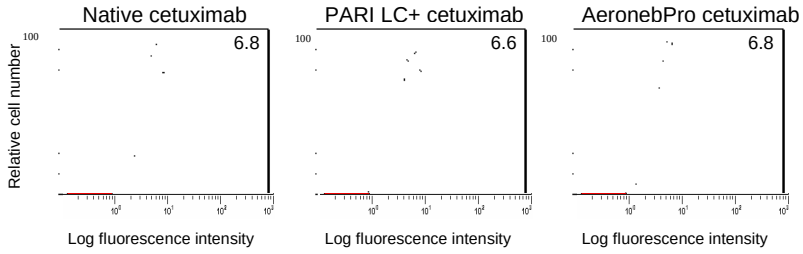


Figure 2 0 Binding of cetuximab to EGFR following nebulization.

0

Figure 3

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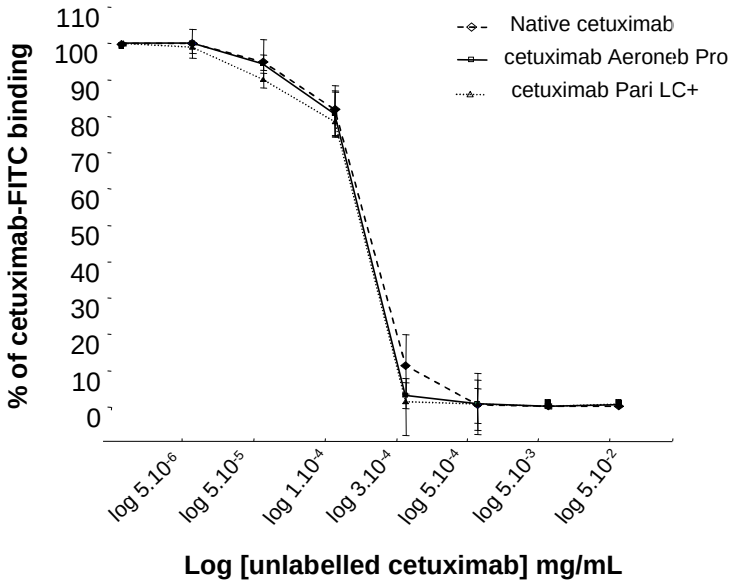
A -



B -

	IC50 (mg/mL)
Native cetuximab	$1.9\ 10^{-4}$
Cetuximab AeronebPro	$1.7\ 10^{-4}$
Cetuximab PARI LC+	$1.65\ 10^{-4}$

Figure 3 : Evaluation of cetuximab affinity following nebulization.

Figure 4

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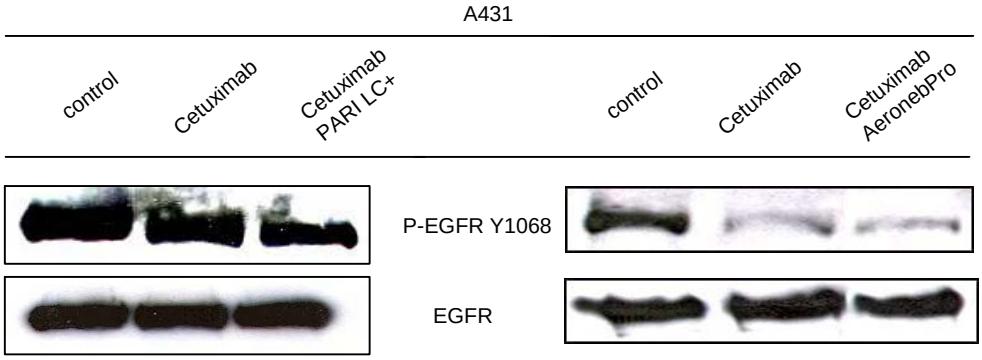

Figure 4 : Aerosolized cetuximab inhibits EGFR phosphorylation.

Figure 5

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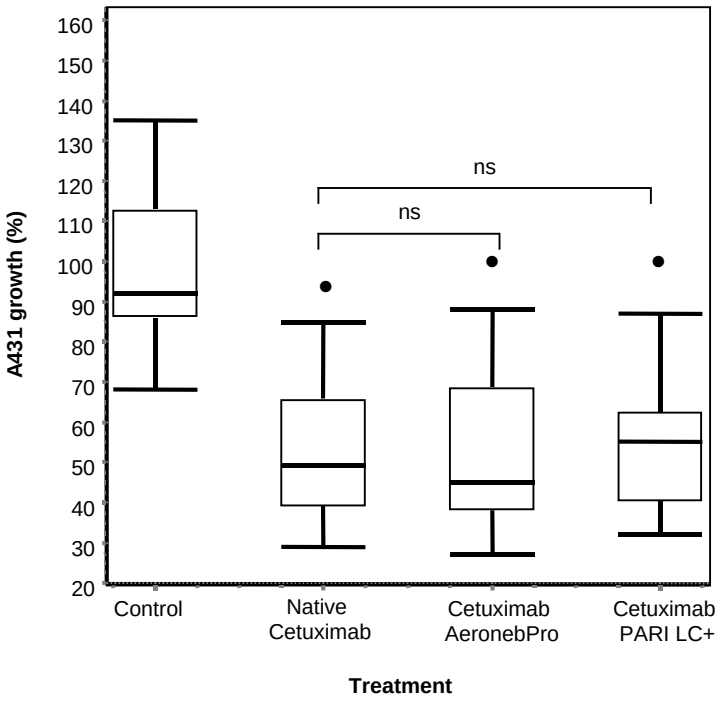

Figure 5 : Effect of cetuximab on A431 cell proliferation.