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The combination of an innovative dry powder for inhalation and a standard cisplatin-based chemotherapy in view of therapeutic intensification against lung tumours

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ABSTRACT

Cisplatin is one of the most commonly used chemotherapy in lung cancer despite its high nephrotoxicity leading to an administration only every 3–4 weeks. This study is the first report of a preclinical investigation of therapeutic intensification combining a cisplatin dry powder for inhalation (CIS-DPI) with an intravenous (iv) cisplatin-based treatment. CIS-DPI with 50% cisplatin content (CIS-DPI-50) was developed using lipid excipients through scalable processes (high-speed and high-pressure homogenization and spray-drying). CIS-DPI-50 showed good aerodynamic performance (fine particle fraction of ~ 55% and a mass median aerodynamic particle size of ~ 2 μm) and a seven-fold increase and decrease in C_{max} in the lungs and in plasma, respectively, in comparison with an iv cisplatin solution (CIS-iv) in healthy mice. Finally, the addition of CIS-DPI-50 to the standard cisplatin/paclitaxel iv doublet increased the response rate (67% *vs* 50%), decreased the tumour growth and prolonged the median survival (31 *vs* 21 days), compared to the iv doublet in the M109 lung carcinoma model tending to demonstrate a therapeutic intensification of cisplatin.

1. Introduction

The survival rates of patients with lung cancer have significantly improved with platinum-based doublet chemotherapy and, more recently, with targeted therapies and immunotherapies. Despite therapeutic advances, lung cancer remains the world-leading cause of cancer-related deaths (approximately 2 million per year), due to innate or acquired tumour resistance to treatments [1].

Conventional chemotherapy combines a platinum salt, i.e. cisplatin or carboplatin, with another antineoplastic agent, which is most often paclitaxel or pemetrexed [2]. It remains very useful for treating non-small cell lung cancer (NSCLC, 85% of total cases [3]). These so-called platinum doublets are the first-line treatment, and are often in combination with other therapies, i.e., surgery, radiotherapy, and immunotherapy. It is used at nearly all stages of the disease (i.e. resectable stage II, resectable and unresectable stage III, and non-specifically altered stage IV) [3–6]. This is despite severe systemic toxicities that are due to chemotherapy's poor selectivity for tumour cells compared to normal cells and to the use of systemic routes of administration, i.e., mainly the intravenous (iv) route. The iv route results in a distribution of

Abbreviations: APSD, Aerodynamic particle size distribution; AUC, Area under the curve; BALFs, bronchoalveolar lavage fluids; BEV, Bronchiolar epithelial vacuolation; CIS-DPI, Cisplatin-based dry powder for inhalation; CIS-iv, iv cisplatin solution ; C_{max}, Maximum concentration ; DLT, Dose limiting toxicity; ETAAS, Electrothermal atomic absorption spectroscopy; FPD, Fine particle dose; FPF, Fine particle fraction; FPF_d, Fine particle fraction of the delivered dose; FPF_n, Fine particle fraction of the nominal dose; GSD, Geometric standard deviation; HCO, Hydrogenated castor oil; iv, Intravenous; k^i_{el}, Initial rate constant; k^t_{el}, Terminal elimination constant; LOD, Limit of detection; MMAD, Mass median aerodynamic diameter; MTD, Maximum tolerated dose; NGI, Next generation impactor; NSCLC, Non-small cell lung cancer; PBS, Phosphate-buffered saline; PEG, Polyethylene glycol; PK, Pharmacokinetic; RH, relative humidity; SEM, Standard error of the mean; $t^i_{1/2}$, Initial half-life; $t^t_{1/2}$, Terminal half-life; T_{max}, Time to reach the maximum concentration; TPGS, D-α-Tocopherol poly(ethylene glycol) 1000 succinate.

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chemotherapy to the entire organism before reaching the site of action, the cancerous cells. The lack of selectivity in chemotherapy will affect rapidly-dividing tissues such as bone marrow cells, the gastrointestinal mucosa, or the skin, causing myelosuppression, nausea and vomiting, and alopecia. In addition, chemotherapy can specifically alter other organs (depending on the anticancer drug) and induce, for example for cisplatin, nephrotoxicity and ototoxicity, with nephrotoxicity the dose-limiting toxicity (DLT) as it is cumulative and irreversible [7].

The severity of some of these toxicities therefore limits the administered dose. This DLT will directly determine the plasma concentrations and therefore the anticancer activity of chemotherapy that is dose-dependent [8]. In addition, the prevention of certain toxicities involves long rest phases (i.e., interruption of treatment) between the administrations to allow normal tissues to regenerate. Unfortunately this rest favours a repopulation of the surviving tumour cells and therefore a relapse of the disease [9]. In current clinical practice, chemotherapy is indicated through four to six cycles of iv administrations, all separated by a 3-week interruption period (to allow the organism to "recover" from the systemic toxicities induced before the next administration). During this interruption the tumour is no longer attacked and can restart its progression [9,10].

The addition of localized delivery to the systemic delivery could allow a therapeutic intensification due to a loco-regional diffusion of the drug close to the tumour, while fighting invasive and diffuse cancerous cells [11–13]. The pulmonary route as localized delivery for lung cancer therapy is promising as it may limit systemic exposure to chemotherapy and related severe systemic toxicities [13,14]. Pulmonary delivery allows high local drug concentrations and low systemic exposure, with a possibility to increase the frequency of administration of chemotherapy. Inhaled chemotherapy, by means of this high frequency of administration with a high therapeutic ratio, combined with conventional iv chemotherapy during the iv chemotherapy interruption, could potentially improve the therapeutic response against lung tumours with minimal systemic adverse effects. A high frequency of administration, i.e., several administrations per week, will only be feasible for as long as inhaled chemotherapy is well-tolerated in the lungs. The chemotherapy candidate and its formulation is therefore crucial to the development of this approach [13]. Cisplatin is a promising chemotherapy candidate due to its central position in lung cancer treatment and its good safety profile in the lungs, observed after nebulization to up to phase II [15]. However, in a phase I study, Wittgen *et al* have demonstrated that the use of a jet-nebulizer to administer a cisplatin solution in lung cancer patients was not adapted. Indeed, the targeted dose was not reached even after 6 h of nebulization [16]. Moreover, heavy equipment (i.e. negative pressure rooms, isolated cabin high-efficiency particulate air-HEPA filters) was needed to limit the environmental contamination and to protect the healthcare professionals. As an alternative to nebulizers, dry powder for inhalation (DPI) formulations are promising for inhaled chemotherapy as they allow a high chemotherapy dose to be deposited in the lungs, with a possible modulation of the drug release profiles by means of particle engineering. Controlling the cisplatin release in the lungs is relevant considering that (i) the high cisplatin doses possibly delivered as DPI in the lungs might be responsible for poor local tolerance and (ii) the dissolved fraction of cisplatin is rapidly absorbed into the systemic circulation [17]. Besides, DPI devices are activated by the patient's inspiratory flow only and can be disposable, which overcome the environmental contamination concerns encountered with nebulizers, allowing cisplatin administration outside the hospital care and therefore high frequency of administration [13].

Our research team recently initiated the development of DPI formulations based on solid-lipid microparticles of cisplatin that were characterized by interesting *in vitro* and *in vivo* properties [17,18]. Some of these formulations were composed of (i) tristearin as a highly hydrophobic lipid excipient with good aerodynamic properties and effective controlled-release properties and (ii) D-α-Tocopherol poly(ethylene glycol) 1000 succinate (TPGS) to avoid excessively fast elimination by the lung non-absorptive clearance mechanism. The use of TPGS also led to an improved PK profile, i.e., longer retention in the lungs, with limited systemic exposure to cisplatin [18]. Moreover, the controlled-release formulation administered at its maximum tolerated dose (MTD, i.e., 1 mg/kg cisplatin) in the M109 lung carcinoma orthotopic mouse model has shown a significantly higher survival rate in comparison to the untreated mice. This was not the case with uncoated cisplatin microparticles without lipid matrix administered at its MTD (i.e. 0.5 mg/kg), showing the limit of an immediate-release formulation due to its dose-limiting toxicity [19]. However, the controlled-release formulation at 1 mg/kg showed shorter median survivals than those obtained with iv cisplatin solution at 1 mg/kg. This result shows the importance of modulating the cisplatin release from microparticles to find the optimal balance between local tolerance and efficacy. Therefore, in this work, we present a new cisplatin DPI formulation with optimized controlled-release properties prepared with a view to therapeutic intensification in combination with conventional cisplatin-based iv chemotherapy. To the best of our knowledge, it is the first report of a consistent preclinical investigation of the concept of therapeutic intensification against lung tumours through the administration of the same active pharmaceutical ingredient, i.e., cisplatin, via both a localized and a systemic route of administration.

2. Materials and methods

2.1. Preparation and in vitro evaluation of cisplatin dry powders for inhalation

2.1.1. Preparation of CIS-DPI-33 and CIS-DPI-50

Cisplatin-based dry powders for inhalation were prepared following an adaptation of the protocol described by Levet *et al* [18]. Briefly, 5% w/v cisplatin (Umicore, Hanau-Wolfgang, Germany) was suspended in ethanol (Merck, Darmstadt, Germany). The cisplatin was reduced in size by means of, first, high-speed homogenization for 10 min at 24 000 rpm (CAT X620 homogenizer and T10 dispersing shaft, Ingenieurbüro, Staufen, Germany). It was then subject to high-pressure homogenization for 40 cycles at 1 000 bars in a closed configuration (EmulsiFlex-C3, Avestin Inc., Ottawa, Canada), with a heat exchanger placed ahead of the homogenizing valve maintained at 15 ± 1 °C. Samples of the reduced cisplatin were then analysed by means of the laser diffraction method (Malvern Mastersizer 3000 with a Hydro MV dispenser, Malvern Panalytical, Worcestershire, UK) described previously [18]. After the HPH process, particle volume mean diameter Dv[4;3] ranged from 0.7 to 1 μm. Hydrogenated castor oil (HCO) (Kolliwax, BASF, Ludwigshafen, Germany) and TPGS (Biomadys, France) were added to the pre-heated (55 ± 5 °C) cisplatin suspension to obtain a final concentration of (i) 50% w/w cisplatin and 50% w/w HCO/TPGS (99:1 w/w) mixture for CIS-DPI-50, and (ii) 33% w/w cisplatin and 67% w/w HCO/TPGS (99:1 w/w) mixture for CIS-DPI-33. The size-reduced cisplatin suspension (uncoated cisplatin DPI) and the cisplatin suspension mixtures were spray dried (Mini-Spray Dryer B-290, Büchi Labortechnik AG, Flawil, Switzerland) under the following operating parameters: feed rate 3.0 g/min, inlet temperature 70 °C, 0.7 mm nozzle, 1.5 mm nozzle cap, compressed air 800 L/min, and drying air flow 35 m^3/h, with relative humidity maintained at 50% (B-296 dehumidifier, Büchi Labortechnik AG). The powders were collected from a high-performance cyclone separator with a yield of ~ 60% and stored in a desiccator at room temperature. A single batch of each formulation was prepared and characterized for its cisplatin content (electrothermal atomic absorption spectroscopy, ETAAS), particle shape, and morphology (scanning electron microscopy by means of the method reported previously [18]), *in vitro* fine particle dose, and dissolution release profile (see below).

2.1.2. Determination of the fine particle dose and fraction using a fast screening impactor

The fine particle dose (FPD, particles below 5 μm) and its expression

as a fraction (fine particle fraction, FPF) of the delivered dose (FPF_d) and of the nominal dose (FPF_n) of CIS-DPI-33 and CIS-DPI-50 was determined using a fast screening impactor with a low resistance RS.01 Mod. 7 dry powder inhaler (RPC Plastiape, Osnago, Italy). The process used a size 3 HPMC capsule (VCaps, Capsugel-Lonza, Colmar, France) hand filled with 20.0 ± 0.5 mg powder as previously described [18]. Briefly, a pressure drop of 4 kPa in the device was reached with an aspiration flow rate of 100 L/min applied for 2.4 s (inhaled air volume of 4 L) and measured using a DFM3 flow meter (Copley Scientific, Nottingham, UK). This flow rate was obtained with an HCP5 air pump (Copley Scientific, Nottingham, UK) connected to a TPK critical flow controller (Copley Scientific, Nottingham, UK). The collection and solubilisation of cisplatin particles was performed by means of dimethylformamide (Sigma Aldrich, St-Louis, USA), and the platinum content of the different collection samples was determined by the validated ETAAS method.

2.1.3. Dissolution release profile

Dissolution properties of uncoated cisplatin microcrystals, CIS-DPI-50 and CIS-DPI-33 were established by means of the dissolution system for dose collection developed by Copley Scientific for dry powder for inhalation release profile studies, with a method adapted from the "paddle over disc" method previously described [20]. Briefly, uncoated cisplatin microcrystals, CIS-DPI-33 and CIS-DPI-50 were collected from stage 3 (about 3 mg cisplatin collected at a flow rate of 100 L/min for 2.4 s, aerodynamic diameter of particles between 2.18 and 3.42 μm) of a next generation impactor (NGI). A hydrophilic polycarbonate membrane with 0.4 μm pore size (Isopore®, Merck Millipore, Darmstadt, Germany) covered the collected powder. The cisplatin dissolution profile from CIS-DPI-33 and CIS-DPI-50 was evaluated in a paddle dissolution apparatus (Erweka DT6, ERWEKA GmbH, Heusenstamm, Hesse, Germany) filled with 400 mL of modified simulated lung fluid (mSLF, [20]). Dissolution testing was performed in accordance with sink conditions, at 37 ± 1 °C, pH 7.35 ± 0.05, with a paddle rotating speed of 50 ± 2 rpm, and the paddles placed at 25 ± 2 mm between the blade and the centre of the dose collector assembly. Volumes of 2.0 mL were sampled at pre-established times between 2 min and 24 h, replaced with mSLF, and analysed for their platinum content by the validated ETAAS method. At the end of the dissolution test, the dose collector assembly was opened into the dissolution vessel and incubated for an additional hour to determine the 100% cisplatin dissolution value. The test was performed in triplicate. Data are presented as the mean percentages of dissolved cisplatin *vs* time (means ± standard deviation – SD).

2.1.4. Production, characterization and stability of the selected cisplatin-based formulation

A total of three independent batches of CIS-DPI-50 (batch size of ~ 3 g) were produced following the procedures described in the previous section (2.1.1). Yields comprised between 55 and 65%. The three batches were characterized by means of the methods described previously in terms of cisplatin content (ETAAS), particle size distribution (laser diffraction), and residual solvent (thermogravimetric analysis) [18].

The aerodynamic performance of CIS-DPI-50 was evaluated according to European Pharmacopeia 10, Section 2.9.18 for dry powder inhalers. The delivered dose uniformity (DDU) and the aerodynamic particle size distribution (APSD) from an NGI (Copley Scientific, Nottingham, UK – Apparatus E) were determined using the low resistance RS.01 Mod. 7 dry powder inhaler (containing a size 3 HPMC VCaps capsule hand filled with 20.0 ± 0.5 mg powder). This was performed under the same conditions as in section 2.1.2.

The collected cisplatin masses were then plotted using Copley Inhaler Testing Data Analysis Software 1 (Copley Scientific, Nottingham, UK) to obtain the FPD and FPF (i.e. FPF_d and FPF_n), calculated as a percentage of the nominal dose, and the APSD parameters, including the mass median aerodynamic diameter (MMAD) and the geometric standard deviation (GSD). The mean delivered dose and DDU evaluation and the APSD were determined in 10 and 3 replicates per batch, respectively. Data are presented as the means of three independent batches ± standard error of the mean (SEM).

A stability study was conducted on one-batch of CIS-DPI-50 over 6 months (T0, T = 1 month, T = 3 months, and T = 6 months) of storage at 25 °C and 60% of relative humidity (RH). CIS-DPI-50 was contained in a size 3 HPMC VCaps capsule during storage and was characterized in terms of residual solvent (loss-on-drying), geometric particle size distribution (laser diffraction), and aerodynamic performance (NGI at 100 L/min, 2.4 s) through an RS.01 inhaler (20 mg powder per capsule). Data are represented for one batch as mean of three replicates ± SD.

2.2. In vivo experiments on mice

2.2.1. Mouse strains and housing conditions

Female 4–8 week-old CD1 mice (for pharmacokinetic studies) and female 6-week-old BALB/cAnNRj mice (16–20 g) (for efficacy studies) were purchased from Janvier Labs (Le Genest-Saint-Isle, France) and Charles River (Écully, France) and kept under conventional housing conditions (12 h/12 h night and day cycles, 22 ± 2 °C, 55 ± 10% relative humidity) and were given dry food and water *ad libitum*. Once entered in the studies, mice were weighed three times a week and were euthanized if their weight loss exceeded 20% when compared to the start of the experiment, or 15% when compared to the last weighing. All experiments were performed in accordance with EU Directive 2010/63/EU for animal experiments and were approved by the CEBEA (Comité d'Ethique et du Bien-Être Animal) of the ULB faculty of medicine under approval numbers 719 N, and CMMI-2017–01.

2.2.2. In vivo-evaluated formulations and administration

CIS-DPI-50 was delivered to mice by means of an endotracheal device (Dry Powder InsufflatorTM model DP4-M®, Penn-Century, Wyndmoor, PA, USA) following the protocol described elsewhere [17] but using isoflurane (3.5% isoflurane) for anaesthesia. To administer CIS-DPI-50 to mice, it was mandatory to dilute the formulation in an appropriate diluent, to deliver 1–2 mg of powder, as determined previously [21]. Therefore, CIS-DPI-50 was added to a previously optimized diluent powder for *in vivo* experimentation (i.e. a spray-dried mannitol/leucine 90:10 w/w powder) in a 2 mL glass vial following the so-called "sandwich method" to target 1% cisplatin for a dose of 0.5 mg/kg (efficacy study) and 2% cisplatin for a dose of 1 mg/kg (PK study) in a total mass of 250 mg (Table 1) [17]. The powders were blended using a Turbula 2C motion mixer (Bachofen AG, Uster, Switzerland), at 46.2 rpm for 4 h, as previously optimized by Levet *et al.*, [17]. The iv treatments (Table 1) were delivered by bolus injection through the previously vasodilated caudal vein. A sterile NaCl 0.9% solution was used for the saline solution delivered by iv route. CIS-iv is composed of 0.15 mg/mL of cisplatin diluted in sterile NaCl 0.9%. The paclitaxel solution, a Taxol-like solution [22], was prepared at 6 mg/mL using a mixture of Cremophor® El (BASF, Ludwigshafen, Germany) and absolute ethanol (50:50 v/v). The iv platinum doublet was made by mixing the solution of paclitaxel in the cisplatin solution to obtain final concentrations of 1 mg/mL and 0.15 mg/mL, respectively. These solutions were kept protected from light and prepared immediately before use.

Table 1
Summary of the treatments, routes of administration and doses.

Study	Treatment	Routes of administration	Dose
PK study	CIS-DPI-50 blend	Powders for endotracheal administration	1 mg/kg
Efficacy study	CIS-DPI-50 blend		0.5 mg/kg
	Paclitaxel/ cisplatin solution	Solutions for iv administration	Paclitaxel: 10 mg/kg Cisplatin: 1.5 mg/kg
	Negative control group		Saline: 0.9% NaCl

2.2.3. Pharmacokinetic profiles of CIS-DPI-50 and CIS-iv

CIS-DPI-50 blend was administered at 1 mg/kg cisplatin through endotracheal administration. Retro-orbital blood for plasma (heparinized tubes, centrifuged at 2 000 g for 10 min at 20 °C) and organs (lungs, liver, kidneys and spleen) were collected at nine timepoints comprised between 1 min and 48 h following the administration of CIS-DPI-50 blend (n = 5–6 mice per timepoint). Organs were washed in phosphate-buffered saline (PBS) and stored at −80 °C until platinum content analysis.

The pharmacokinetic (PK) profiles of cisplatin in the organs and plasma were established and PK curves of cisplatin and its adducts were graphed as platinum concentration *vs* time curves. PK parameters were calculated following a standard non-compartmental analysis and included the maximum platinum concentration (C_{max}), the time to reach C_{max} (t_{max}), and the area under the concentration–time curve (AUC). The initial and terminal elimination rate constants k^i_{el} and k^t_{el} were calculated from the log regression of platinum concentration *vs* time curves between the three first and the three last dosing timepoints, respectively. The initial and terminal half-life $t^i_{1/2}$ and $t^t_{1/2}$ were calculated as ln(2)/ k^i_{el} and ln(2)/ k^t_{el}, respectively. $AUC_{0-\infty}$ was estimated by means of the trapezoidal method from 1 min to 48 h for all organs and plasma.

To be able to compare the kinetic data obtained with other published data, PK parameters (C_{max}, $t^i_{1/2}$ and k^i_{el}) were generated with CIS-iv following the same procedure. Briefly, CIS-iv was administered at 1.5 mg/kg cisplatin though the tail vein. Sampling procedures (see above) were performed at 1-, 10- and 30-min post-administration and C_{max}, $t^i_{1/2}$ and k^i_{el} were calculated for all organs and plasma (n = 5 mice per timepoint).

2.2.4. In vivo efficacy study

The anti-tumour efficacy of endotracheal CIS-DPI-50 blend combined with a conventional iv platinum doublet combining cisplatin and paclitaxel was investigated on the M109 lung carcinoma orthotopic model in mice [22]. The M109 model is derived from the M109-HiFR-*luc2* cell line, i.e., the M109-HiFR cell transduced with the luciferase reporter gene *luc2* (pGL4.10[luc2] vector, Promega, Leiden, Netherlands). This model was obtained according to the orthotopic cell engraftment protocol described elsewhere [22]. On the third day post cell engraftment (i.e., day 3), mice were randomly allocated to one of the four groups of investigation. Treatments were administered from day 6 for two cycles of one week and were established as follows: (i) iv saline negative control untreated group (n = 11); (ii) iv solution delivering 1.5 mg/kg cisplatin and 10 mg/kg paclitaxel (as a Taxol-like solution [22]) (iv platinum doublet, n = 8), one administration per week (on Mondays) for 2 consecutive weeks; (iii) combination of the iv platinum doublet (on Mondays) with endotracheal CIS-DPI-50 at 0.5 mg/kg cisplatin (n = 15), three administrations per week (on Tuesdays, Thursdays, and Saturdays) for 2 consecutive weeks (Fig. 1).

Bioluminescence imaging of the whole mice was performed two times a week to follow *in vivo* tumour growth (primary tumour and metastases) and was expressed as the tumour growth % relative to day 3. Bioluminescence imaging was performed by means of a Photon Imager Optima (Biospace Lab, France) that dynamically counted the emitted photons for at least 25 min, with the mice under anaesthesia (4% and 2% isoflurane for initiation and maintenance, respectively) and after subcutaneous administration of 150 mg/kg of D-luciferin (Promega, Leiden, Netherlands) [23]. Image analysis was performed with M3Vision software (Biospace Lab). ROIs were drawn on the mice thorax, and signal intensities were quantified individually for a time laps of 5 min corresponding to the maximum signal intensity plateau. Kaplan-Meier survival curves were established by excluding deaths related to the endotracheal administration procedure, as previously reported [19,24,25]. Fig. 1 describes the *in vivo* procedure, from tumour grafting, randomization, scheme of treatment administration, and follow-up in terms of bioluminescence and survival analysis.

2.3. Platinum content

Platinum was used as a marker for cisplatin in simple matrix. For cisplatin content, *in vitro* aerodynamic and dissolution evaluations were determined using the validated ETAAS method as described [18]. Platinum content in complex matrices, such as plasma and organs, was determined using the validated ETAAS method as described [17]. The organs were digested in Suprapur® HNO_3 for three hours before platinum assay, as described previously [17]. The platinum assay in plasma was performed by means of an adaptation of a method described previously [17,26]. The limit of detection (LOD) and of quantification (LOQ) of the methods are indicated in the corresponding graphs and were 0.075 and 0.225 ng/mg in lungs and kidneys and 0.025 and 0.075 ng/μL in plasma. These analyses were performed using a SpectrAA 220Z atomic absorption spectrometer equipped with a GTA-96 graphite tube atomizer (Varian, Mulgrave, Australia).

2.4. Statistical analysis

All statistical tests were conducted using GraphPad PRISM® (7.0a) software. The Kaplan-Meier curve and log-rank test with an analysis of the p value by means of the Holm-Šídák method were used for the survival analysis. Two-way ANOVA with Bonferroni post-testing was used to analyse mean tumour growth over time. Results were considered as statistically significant (*) for $p < 0.05$, very significant (**) for $p < 0.01$, extremely significant for $p < 0.001$ (***), and extremely significant for $p < 0.0001$ (****). Results are presented as the mean value ± SEM, unless otherwise indicated.

3. Results and discussion

3.1. Selection of the CIS-DPI formulation

3.1.1. Selection of the cisplatin/lipid excipient ratios

The aim of this part was to develop a cisplatin dry powder containing lipid excipients to control the release of cisplatin using a scalable manufacturing process, biocompatible pharmaceutical-grade excipients, and low-toxicity solvents, corresponding to FDA recommendations [27].

As mentioned, the previous cisplatin DPI formulation developed by our group have demonstrated controlled-release properties, and has

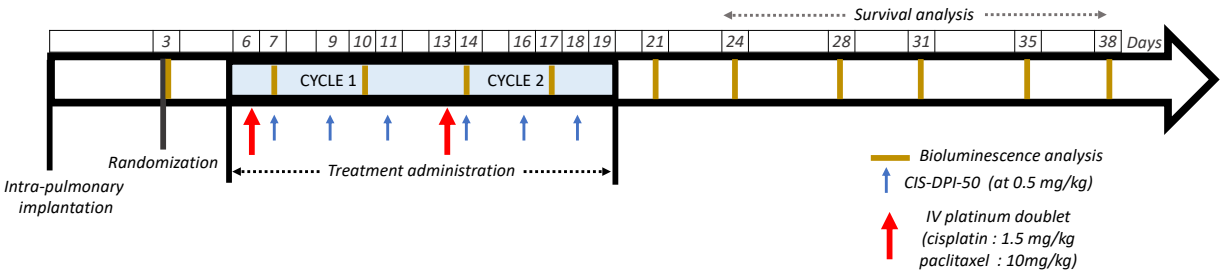


Fig. 1. Efficacy study scheme of administration and follow-up.

proven good lung targeting in mice [17,18]. However, to optimize its efficacy and facilitate its scale-up, both its composition and the manufacturing process were adapted. First, we preferred HCO over tristearin to slightly increase the hydrophilicity of the particles and therefore the cisplatin release rate over time after deposition in the lungs than observed with tristearin. HCO is a triglyceride mixture, mainly composed of trihydroxystearin, characterized by a higher melting temperature (85–88 °C) than tristearin (74.9 °C, [18]). This higher melting temperature allows the production of inhaled formulations as dry powder while reducing the risk of obtaining an oily and/or sticky phase that would negatively impact the aerodynamic properties of the DPI. Moreover, it is approved by authorities as an excipient for other routes of administration such as the oral and topical routes [27]. Furthermore, HCO has higher solubility in alcohols (including ethanol) than tristearin, which has allowed working with a higher solid concentration of HCO during spray-drying (4% w/w total solid *vs* 2% w/w previously [18]), reducing the manufacturing time.

Following similar preparation process, the minimum lipid content to coat the cisplatin microparticles sufficiently to control the cisplatin release was previously reported as 50% w/w [18]. Higher cisplatin to lipids ratio (i.e., 75:25) led to a high burst effect with low ability to control cisplatin release over time (data not shown). A sufficient proportion of HCO/TPGS in the CIS-DPI formulations to coat the cisplatin microparticles was considered critical to control the cisplatin release. HCO (i.e., mainly composed of trihydroxystearin) is less hydrophobic than tristearin. We therefore selected lipid (i.e., HCO/TPGS) proportions of 50% w/w related to cisplatin and above in the final powder, i.e., 50% and 67% w/w in CIS-DPI-50 and CIS-DPI-33, respectively. The selected TPGS content was 0.5% w/w. This content prolonged the retention of tristearin-based DPI formulations in the lungs after inhalation [17]. Higher TPGS content (i.e., 5%) in tristearin-based formulations led to no control of the cisplatin release *in vitro* (data not shown). This could be explained by the decreased hydrophobicity of the lipid coating in the presence of TPGS and the possible formation of hydrophilic pores in the lipid coating.

The presence of coating around cisplatin particles was confirmed in scanning electron microscopy (SEM). The CIS-DPI-50 and CIS-DPI-33 particles were roughly spherical, homogeneous particles that look very similar to the spray-dried HCO/TPGS (99:1) powder, indicating that the cisplatin had been effectively coated by the lipid matrix (Fig. 2). Conversely, the SEM images of uncoated-cisplatin particles obtained by Levet et al [18] (data not shown) showed more agglomerated and sticky particles in comparison to those obtained with CIS-DPI-33 and CIS-DPI-50.

The differentiation between CIS-DPI-33 and CIS-DPI-50 was performed by characterizing the FPD and FPF using a fast-screening impactor and on the basis of the *in vitro* cisplatin release over time in mSLF. As illustrated in Fig. 3.A, both of these formulations demonstrated similar and adequate FPF (60 ± 4% for CIS-DPI-50 and 58 ± 6% for CIS-DPI-33) but as predicted, lower FPD for CIS-DPI-33 (3.7 ± 0.5 mg, compared to 5.8 ± 0.9 mg for CIS-DPI-50) due to their different cisplatin contents (28 ± 5% and 49.5 ± 0.6%, respectively).

The dissolution profiles of CIS-DPI-50 and CIS-DPI-33 were different (Fig. 3B). After one hour, 44 ± 2% cisplatin was released from CIS-DPI-50 whereas release observed with CIS-DPI-33 was limited to 22 ± 1% (Fig. 3B). The cisplatin release increased gradually to reach ~ 50% after 24 h from CIS-DPI-50. In contrast, cisplatin release from CIS-DPI-33 was highly limited, reaching ~ 30% within 24 h. These results showed that a higher proportion of HCO, as in CIS-DPI-33, strongly limits the amount of initially dissolved cisplatin and slowed cisplatin release over 24 h. This may impair cisplatin total dose release into lung fluids from CIS-DPI-33 and therefore its local activity. It should be noted that dry powder formulations with lower amount of HCO were not selected in order to avoid much higher cisplatin burst release which might give lower local tolerance. About 80% cisplatin dissolution in 15 min was observed with uncoated-cisplatin microcrystal powder obtained by spray drying the cisplatin particles obtained after the HPH process (Fig. 3B).

Consequently, the results were in favour of CIS-DPI-50 better balancing a burst drug release that is high enough able to ensure rapid local activity with a controlled release able to attack the tumour sustainably with limited local toxicity. Moreover, CIS-DPI-50 was preferred over CIS-DPI-33 as it would deliver a higher FPD than CIS-DPI-33 from the same mass of powder delivered. Consequently, CIS-DPI-50 was selected to continue the *in vivo* investigation.

It should be noted that there are no pharmacopeia methods or guidelines describing *in vitro* dissolution tests of inhaled products. The main issue with those tests is the difficulty of reproducing *in vitro* the physiological conditions that the aerosol faces once it deposits and disperses in lung fluids. Many methods have been reported with many drawbacks, including difficult handling of the powder, leading to poor reproducibility and targeting [28,29]. In the case of CIS-DPI, this has been accentuated by particle agglomeration related to the highly lipophilic character of HCO, as noticed when the dissolution cup assembly was opened at the end of the dissolution test to determine the total amount of cisplatin introduced (i.e., 100% release) into the medium.

Therefore, the *in vitro* dissolution profiles for inhaled products must be interpreted with caution as they are used to compare different release patterns from formulations containing the same active ingredient. Dissolution tests for inhaled products are inadequate methods to investigate the release once deposited in the lungs. This is due to poor *in vitro in vivo* correlation [29]. Further development regarding the methodologies used and the composition of medium are thus highly awaited to try to solve these drawbacks.

3.1.2. Characterization of the selected CIS-DPI formulation

The cisplatin mean content of CIS-DPI-50 was 49.2 ± 0.3% w/w and was highly reproducible from batch to batch, with low SEM observed (49.5 ± 0.3%; 51.0 ± 0.9%; 47.2 ± 0.3%) (Table 2).

The loss on drying was limited (<0.2% w/w) and corresponded to the total residual content of ethanol and water. This result was a good prognostic for the long-term stability of the product during storage as stability could be impaired by high residual solvent content. As another indicator of potential good long-term stability, the more stable polymorph of HCO (β form) was preserved during spray drying as it was similar to the initial raw material, thus providing reassurance of its physicochemical stability during storage (see "X-ray powder diffraction of spray-dried *vs* raw HCO", Figure S1, supplementary data). These

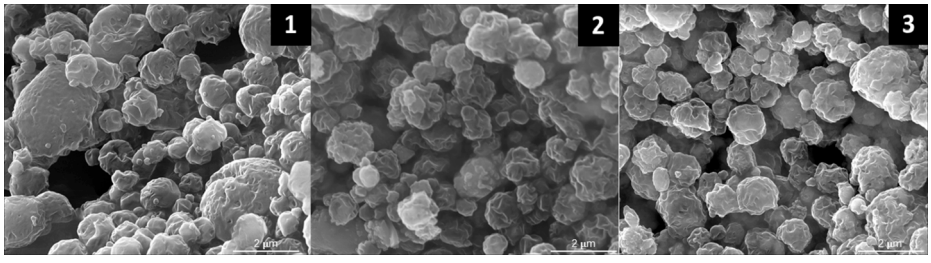


Fig. 2. Scanning electron microscopy micrographs of spray-dried HCO/TPGS (99:1) powder (1), CIS-DPI-33 (2) and CIS-DPI-50 (3) (magnification 30 000 x).

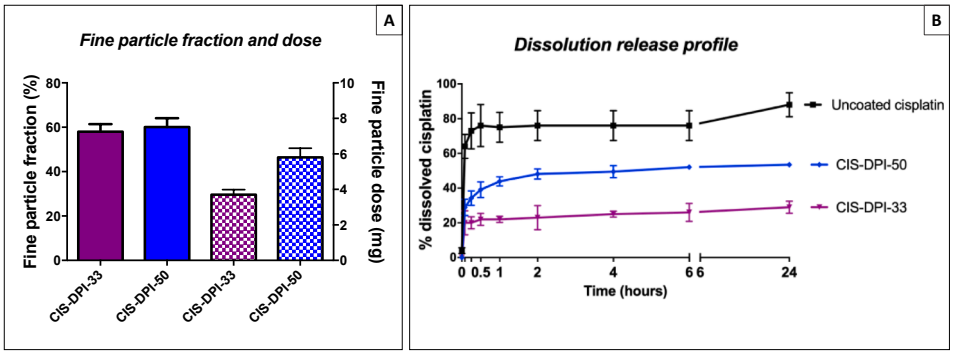


Fig. 3. FPF and FPD of CIS-DPI-33 and CIS-DPI-50. Impaction studies were performed using the fast screening impactor (100 L/min, 2.4 s) on 20 mg of powder hand-filled in an HPMC n°3 capsule in RS.01 inhaler (A). Dissolution profile of an inhalable fraction of uncoated cisplatin microcrystals, CIS-DPI-33 and CIS-DPI-50 (mean ± SEM, n = 3) selected from stage 3 (aerodynamic diameter comprised between 2.18 and 3.42 μm) of an NGI using an RS.01 inhaler (100 L/min, 2.4 s) (B).

observations were confirmed by a stability study that was conducted over 6 months at 25 °C and 60% RH. The results were promising with limited modifications of the physicochemical (residual solvent, geometric particle size distribution) and aerodynamic properties of the powder in normal storage conditions over 6 months, as demonstrated in Table 2 and in Fig. 4.

One of the most crucial parameters for DPI development is the particle size as it is directly correlated to the aerodynamic diameter, which dictates the deposition section in the respiratory tract [30]. The geometric particle size distribution was compatible with a deep lung deposition as the proportion of the particle size under 5 μm was 97 ± 3%, the Dv (50) was 1.9 ± 0.3 μm, and the Dv 90 was 3.4 ± 0.7 μm.

The delivered cisplatin dose for the three independent batches was 8.1 ± 0.2 mg cisplatin and corresponded to 82 ± 1% of the nominal dose, which indicated good aerosolization of the powder with limited powder retention in the device and the capsule. The delivered dose was considered as highly repeatable from batch to batch, as indicated by the low SEM observed (0.2 mg). Moreover, the DDU was very good, with relative standard deviation (RSD, %) < 3% within each batch on 10 replicates and corresponded to the European Pharmacopeia recommendations, with all the 10 assays per batch were comprised with the range 96–104% of the mean delivered dose) [31].

It is commonly considered that the particles must have an aerodynamic diameter ranging between 1 and 5 μm to deposit optimally in the lower respiratory tract [30]. This diameter is far more crucial in the development of anticancer drugs as it increases their potential to be deposited broadly in the sections from the bronchioles to the alveolar sacs (i.e. generations 4 to 23). Such deposition avoids two main potential limitations in the treatment. First, particle deposition in the upper airways (i.e. generations 0 to 3, aerodynamic diameter > 5 μm), such as the mouth, throat, trachea, or bronchi of the lower respiratory tract, can lead to local toxicities. Second, exhalation (with an aerodynamic diameter higher than 0.1–1 μm) can increase environmental contamination by the cytotoxic aerosol [13,14].

The APSD was in line with these recommendations and described a polydisperse aerosol as CIS-DPI-50 was retrieved at nearly all stages (i.e., from 0.07 to 10 μm, from the micro-orifice collector (MOC) to the pre-separator stage). This broad deposition was confirmed by the GSD obtained, i.e. 1.85 ± 0.02 μm, as it was higher than 1.22 μm (the commonly accepted threshold for considering the aerosol as monodisperse [32]). However, much higher proportion of the particles was comprised into stages 2 and 4, i.e., within 1 et 5 μm (Fig. 4). In contrast, the cisplatin doses collected from the induction port (i.e., the throat), the pre-separator and stage 1 were relatively low, i.e., about 16%.

Two main types of adverse reaction might be related to the fraction deposited in the oropharynx. First, this fraction, as part of the fraction deposited in the upper airways might induce local adverse reactions such as cough. These local reactions are expected to be immediate, i.e., within 15 min following the administration of CIS-DPI. Although any occurrence will be investigated in clinical trials, mainly in the first-in-humans study, potential local adverse reactions are expected to be limited because patients will be advised to rinse their mouth immediately after inhalation, which will help remove drug deposited in the mouth/throat. Secondly, any of the remaining oropharyngeal dose that is subsequently swallowed dose could potentially be absorbed via the digestive tract leading to systemic adverse reactions. However, these systemic reactions following administration of CIS-DPI are expected to be low as (i) the cisplatin dose administered to patients will be very low compared to the usual iv doses and (ii) the limited oral bioavailability factor of cisplatin, i.e., below 0.4 [33].

The MMAD was 2.2 ± 0.1 μm and therefore in the desired range for an anticipated good deposition in the lower airways (<5 μm) [34]. This good aerodynamic performance led to high FPD (5.25 ± 0.09 mg) and FPF values (FPF_d and FPF_n of 53.3 ± 0.9% and 74.4 ± 0.2%, respectively). This high drug deposition will be demonstrated in future clinical trials. In the case of nebulizers (i.e. the only device studied so far in clinical inhaled chemotherapy), it was estimated that 10–15% of the inhaled dose was deposited in the deep lungs in a phase I study with the same drug candidate [16]. The authors reported that despite three sessions of administration of about 20 min each per day, the DLT was not reached. This demonstrated the difficulty of reaching the therapeutical cisplatin dose using nebulizers [16].

Regarding the aerodynamic performance of CIS-DPI-50, it was concluded that the RS.01 DPI was suitable for this formulation. It assured a good aerosolization and dispersion and should allow good deposition in the lungs. The selected RS.01 inhaler was a low-resistance version that does not need a high inspiratory effort to reach an airflow that aerosolizes, disperses, and deposits the powder in the lower respiratory airways [14]. This is all highly important as lung cancer patients might have an impaired lung function due to the presence of the tumours, a tobacco-smoking history, or chronic obstructive pulmonary disease [14]. Our research group showed limited dependency on flow rates for lipid-based formulations [35], including with cisplatin [18], although this needs to be confirmed for CIS-DPI-50 delivered from the RS.01 inhaler.

Moreover, the RS.01 device is activated and controlled by the patient inspiratory flow, and very little drug is exhaled (i.e., 0.2% of the dose after the inhalation of a tobramycin based DPI by healthy volunteers [36]). This device was also chosen because it is inexpensive, simple to use, convenient, portable, and adapted for single use. All these points are required for the development of inhaled anti-cancer drugs, where an efficient and disposable DPI inhaler is required to limit environmental exposure [13].

Table 2
In vitro evaluation of CIS-DPI-50 in terms of platinum concentration (ETAAS), residual solvent (loss-on-drying), geometric particle size distribution (laser diffraction), delivered dose and uniformity (100 L/min, 2.4 s), and aerodynamic performance (NGI at 100 L/min, 2.4 s) through an RS.01 inhaler (20 mg powder per capsule). The data are presented as the mean values of three independent batches analysed in 3 or 10 (for DDU) replicates ± SEM. The stability study over 6 months of storage at 25 °C and 60% RH was also represented for one batch as mean of 3 replicates ± SD in terms of residual solvent, geometric particle size distribution, and aerodynamic performance.

Formulation	CIS-DPI-50 Production and characterization		Stability study over 6 months T0	T = 1 month	T = 3 months	T = 6 months
Theoretical composition (% w/w)	**Component** Cisplatin HCO TPGS			**Content** 50.0 49.5 0.5		
Cisplatin content (% w/w)	49.2 ± 0.3		46.7 ± 0.2			
Residual solvent content (% w/w)	0.15 ± 0.09		0.05 ± 0.05	0.05 ± 0.01	0.02 ± 0.00	0.02 ± 0.03
Geometric particle size distribution	**% < 5 µm**	97 ± 3	97.1 ± 0.3	94.4 ± 0.2	94.5 ± 0.3	100.00 ± 0.00
	Dv (10) (µm)	1.17 ± 0.04	1.10 ± 0.02	1.08 ± 0.02	1.06 ± 0.02	1.14 ± 0.01
	Dv (50) (µm)	1.9 ± 0.3	1.99 ± 0.06	1.99 ± 0.06	1.94 ± 0.05	1.89 ± 0.09
	Dv (90) (µm)	3.4 ± 0.7	3.90 ± 0.09	4.19 ± 0.09	4.16 ± 0.09	3.0 ± 0.1
	Dv [4;3] (µm)	2.2 ± 0.3	2.27 ± 0.06	2.40 ± 0.01	2.36 ± 0.05	2.01 ± 0.08
Delivered dose (mg) (mean ± SEM, n = 3 batches analysed in ten replicates)	8.1 ± 0.2		–			
Delivered dose uniformity (min and max in % of the mean delivered dose, ten assays per batch)	96–104%		–			
Aerodynamic performance (NGI, 100 L/min)	**FPF_d (%)**	74.4 ± 0.2	81.0 ± 0.6	82.3 ± 0.7	82.1 ± 0.6	81 ± 2
	FPF_n (%)	53.3 ± 0.9	60 ± 1	61 ± 1	55 ± 1	55.1 ± 0.7
	FPD (mg)	5.25 ± 0.09	5.6 ± 0.1	5.7 ± 0.1	5.1 ± 0.1	5.15 ± 0.07
	MMAD (µm)	2.2 ± 0.1	1.98 ± 0.03	1.97 ± 0.01	2.00 ± 0.03	2.06 ± 0.03
	GSD (µm)	1.85 ± 0.02	1.79 ± 0.01	1.77 ± 0.02	1.75 ± 0.04	1.79 ± 0.01

3.2. Pharmacokinetic profiles after cisplatin administration

As mentioned above, it is difficult to mimic the lung physiological conditions *in vitro* and its particular anatomical specificities (i.e., huge deposition surface and rapid drug absorption). Moreover, the *in vitro* dissolution experiments could not consider the non-absorptive clearance mechanisms such as the mucociliary escalator and alveolar macrophages uptake. It was therefore useful to investigate the *in vivo* PK profile of the selected formulation in healthy mice. This was assessed to evaluate the fate of cisplatin (followed by the quantification of platinum element) in plasma and the main organs once it was deposited in the lungs. This study was crucial to determine the combination regimen, of repeated CIS-DPI-50 administrations with iv cisplatin-based treatment, that would offer the best compromise between enough lung (and therefore tumour) exposure to cisplatin and the related potential local and systemic toxicities.

The selection of CIS-DPI-50 dose to be combined to iv cisplatin-based chemotherapy was crucial. This dose will be different if CIS-DPI-50 is administered as a monotherapy or in combination with other treatments as the combination can intensify both the efficacy and the adverse effects and in particular DLTs. For inhaled chemotherapy tested in clinical trials until now, DLT is usually related to lung toxicities [13]. Consequently, the selection of the dose was conducted on the basis of the evaluation of the pulmonary tolerance of CIS-DPI-50 at 0.5 and 1 mg/kg [37]. The results from that study demonstrated that CIS-DPI-50 at both doses as well as its excipient were tolerated. Consequently, a dose of 1 mg/kg was selected for the PK study (Table 1).

Fig. 5.A presented the PK curve of platinum lung concentrations obtained over time after the administration of CIS-DPI-50 at 1.0 mg/kg cisplatin and CIS-iv at 1.5 mg/kg cisplatin. As the bioanalytic method was not selective to cisplatin but to platinum (as commonly referred to in clinical trials for platinum salts including cisplatin [15,16]), lung platinum concentrations measured over time with CIS-DPI-50 and CIS-iv consisted of unbound (i.e., dissolved) active cisplatin, inactive platinum adducts, and bound cisplatin as particles not yet dissolved in lung fluids. The PK profile of CIS-iv is known and has been investigated in depth and reported in healthy mice [17]. However, the protocol slightly differed from our study protocol (e.g., timepoints, cisplatin dose, blood sampling). We therefore determined exposure to platinum following CIS-iv at 1.5 mg/kg cisplatin (its MTD [19]) following the protocol of the present study. Only the three first timepoints were evaluated, i.e., 1 min, 10 min, and 30 min, which allowed us to determine the C_{max}, k^i_{el}, and $t_{1/2}{}^i$.

As anticipated, platinum C_{max} in the lungs was obtained immediately following the administration of CIS-DPI-50 and CIS-iv, at the first timepoint (i.e., 1 min), which corresponded to the time required to euthanize the mice and collect the lungs. The C_{max} in the lungs for CIS-DPI-50 was 19 ± 2 ng/mg, corresponding to 26 ± 3% cisplatin recovered from the actual delivered dose. The C_{max} of CIS-iv in the lungs was nearly four-fold lower than for CIS-DPI-50 (i.e., 5 ± 1 ng/mg, Table 3), despite the higher cisplatin dose delivered (i.e., 1 *vs* 1.5 mg/kg, respectively). The platinum concentration in the lungs first decreased to 6 ± 2 ng/mg 30 min after the administration of CIS-DPI-50, with a k^i_{el} and $t^i_{1/2}$ of 0.036 min^{-1} and 19 min, whereas following CIS-iv administration, k^i_{el} and $t^i_{1/2}$ were 0.023 min^{-1} and 30 min (Table 3). Levet *et al.* reported a more rapid elimination from the lungs, with a k^i_{el} and $t^i_{1/2}$ of 0.26 min^{-1} and 2.6 min following the administration of cisplatin microparticles without lipid excipients [17]. The initial clearance profile could be related to the clearance of cisplatin from the lungs, mainly by absorption to the systemic compartment. This is because cisplatin is a small molecule, which could facilitate its diffusion though the lung epithelium once released from microparticles in lung fluids as observed previously with endotracheal delivery of cisplatin solution and an immediate-release formulation [17]. Therefore, the retention time of cisplatin in the lungs consecutive to a single CIS-DPI-50 administration was estimated to be around 2.2 h (seven times the $t^i_{1/2}$). This retention time was>7-fold higher than cisplatin microparticles without lipid excipients (18.2 min) [17]. These parameters clearly indicated a delay in the lung clearance of cisplatin due to the presence of the lipid matrix and the PEGylated excipient (TPGS). Between 2 and 8 h, the platinum concentrations in the lungs remained constant around 2 ng/mg, i.e., 2-fold higher than the lung Cmax of CIS-iv). These concentrations then continued to decrease to 0.94 ± 0.4 ng/mg after 48 h (k^t_{el} and $t^t_{1/2}$ of 0.00029 h^{-1} and 39 h). This terminal clearance profile could be attributed more to the clearance of platinum adducts from the tissue [17], which can remain for months following administrations [38]. The $AUC_{0-\infty}$ in the lungs was 4 611 ±

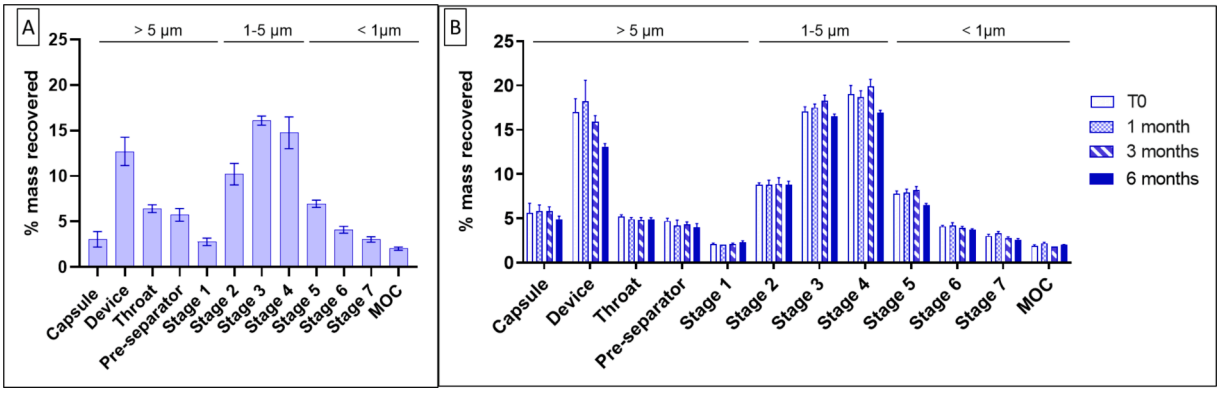


Fig. 4. Deposition profiles of the CIS-DPI-50 formulation in an NGI using a RS.01 inhaler (10 mg cisplatin per capsule, 100 L/min, 2.4 s). The cut-off diameters at 100 L/min were 6.12, 3.42, 2.18, 1.31, 0.72, 0.40, 0.24 and 0.07 µm for stages 1, 2, 3, 4, 5, 7 and the micro-orifice collector (MOC), respectively. The data are presented as the mean ± SEM of the three batches analysed in triplicate (A) and as the mean ± SD of one batch analysed in triplicate, after its production (T0), and after 1 month, 3 months and 6 months of storage at 25 °C and 60% RH.

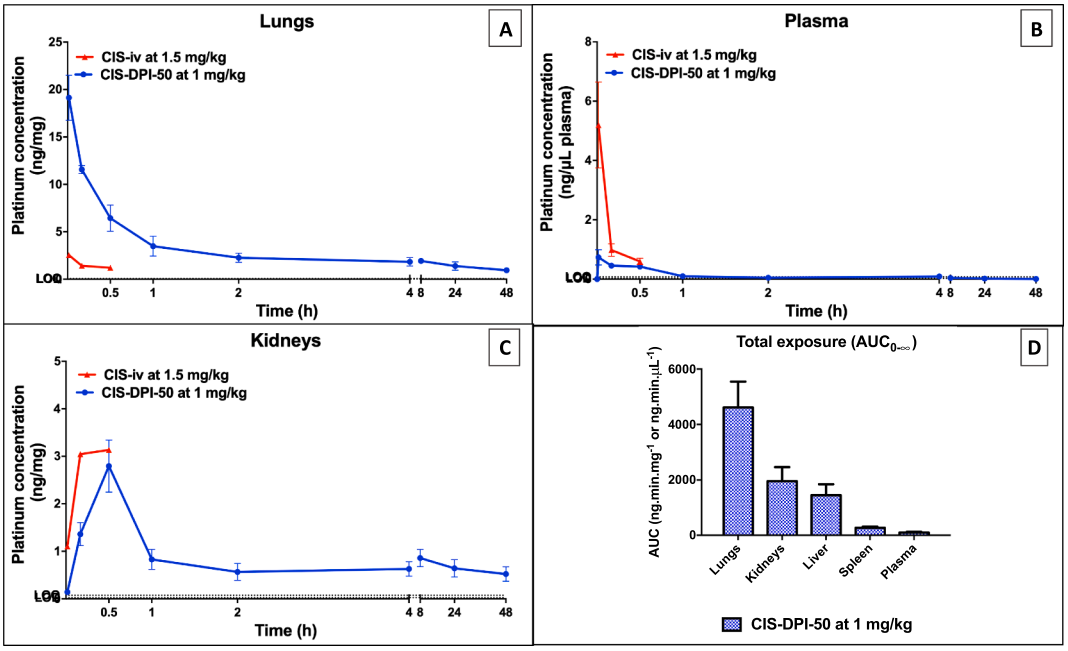


Fig. 5. PK study of CIS-DPI-50 and CIS-iv (1 and 1.5 mg/kg, respectively) in healthy mice. Platinum concentration was evaluated in the lungs (A), plasma (B) and kidneys (C). Total AUC for different organs of CIS-DPI-50 (D). All the results are expressed as means ± SEM (n = 5–6 per timepoint).

Table 3
CIS-DPI-50 and CIS-iv pharmacokinetics.

Pharmacokinetics	CIS-DPI-50			CIS-iv		
	Lungs	Plasma	Kidneys	Lungs	Plasma	Kidneys
C_{max} (ng/mg – ng/µL)	19 ± 2	0.7 ± 0.6	2.8 ± 0.6	2.6 ± 0.8	5 ± 1	3.1 ± 0.2
T_{max} (min)	1	1	30	1	1	30
$AUC_{0-\infty}$ (ng.min.mg^{-1})	4611 ± 932	95 ± 30	1952 ± 510	–	–	–
K^i_{el} (min^{-1})	0.036			0.023	0.066	0.03
$t^i_{1/2}$ (min)	19			30	10	23
K^t_{el} (h^{-1})	0.00029					
$t^t_{1/2}$ (h)	39					

932 ng.min.mg^{-1}, which also demonstrated the prolonged retention of CIS-DPI-50 within the lungs ($AUC_{0-\infty}$ of 1 462 ng.min.mg^{-1} for microparticles without lipid coating [17]). As anticipated, the use of HCO instead of tristearin decreased the lung AUC ($AUC_{0-\infty}$ of 6 072 ng.min.mg^{-1} for a cisplatin-based tristearin-containing DPI formulation [17]), tending to indicate a more rapid release profile (and thus a shorter lung retention) of cisplatin with a HCO-based DPI.

As expected, the C_{max} in plasma following CIS-DPI-50 administration was more than six-fold lower than for CIS-iv (0.73 ± 0.6 *vs* 5 ± 1 ng/µL, respectively, Table 3. Moreover these results were in line with observations during a PK study conducted in sheep using CIS-DPI-50 and CIS-iv [39]. Indeed, a 10-fold reduction in plasma C_{max} and AUC was

observed following the administration of CIS-DPI-50 compared to CIS-iv at the same dose [39]. Overall, these data confirmed the advantage of CIS-DPI-50 in terms of targeting the lungs compared to iv cisplatin solution, with a seven-fold increase and decrease in the C_{max} in lungs and in plasma, respectively.

As expected, total exposure of untargeted organs to platinum after CIS-DPI-50 delivery was limited compared to the lungs, with lower $AUC_{0-\infty}$ values (Fig. 5.D). These values demonstrated the PK advantage of CIS-DPI-50, compared to iv cisplatin solution [17], for a lung-targeting therapeutic approach. These data were also compatible with the data obtained in sheep, with significantly increased platinum concentration in the lungs and significant lower exposure in other organs 1 h following administration [39].

Interestingly, the results observed on renal exposure were in the same range as those previously reported with the tristearin-based cisplatin DPI formulation. Renal C_{max} and $AUC_{0-\infty}$ values (Table 3) were higher than with a cisplatin nebulized solution and an immediate-release cisplatin DPI formulation [17]. Renal excretion though a non-linear and saturable process is the main elimination process for cisplatin and its metabolites [40]. This seems to be the most plausible explanation of this renal exposure for inhaled cisplatin dry powders, with prolonged lung retention leading to prolonged renal exposure to cisplatin and derivatives (all quantified as platinum in the study). Despite the fact that nephrotoxicity of cisplatin and its metabolites is directly related to their concentrations, the presence of platinum in the kidneys after administration does not necessarily imply renal toxicity. This is because not all metabolites or platinum adducts induce renal impairment [40]. Still, observations on renal exposure following CIS-DPI-50 and cisplatin iv administration, in particular the C_{max} values (about 3 ng/mg, Table 3), were the basis for the construction of the combination regimen (Fig. 1). This was done to avoid any accumulative renal toxicities related to a more frequent treatment administration, as cisplatin nephrotoxicity is dose-duration-frequency dependent [41].

Therefore, in the administration scheme used for the combination regimen, endotracheal CIS-DPI-50 administrations were staggered from CIS-iv administration by 24 h. This was done to be sure to avoid adding more cisplatin to the lung and the kidney from adding endotracheal CIS-DPI-50 to CIS-iv (Fig. 1), as reported previously [37]. Indeed, the co-administration of CIS-DPI-50 and CIS-iv, the same day at their MTD has led to irreversible renal injury [37]. In addition to his adjustment, results from that study have demonstrated that the co-administration of CIS-iv at 2 mg/kg and CIS-DPI-50, even 24 h later has led to cumulative renal toxicities. Therefore, CIS-iv dose was reduced by 25% (i.e. 1.5 mg/kg), as commonly used in clinical trials [37]. Therefore, this optimized regimen was selected for the efficacy study and is represented in (Fig. 1).

3.3. *Efficacy evaluation on the M109 lung carcinoma orthotopic model in mice*

To evaluate the add-on value of CIS-DPI-50 when combined with a conventional platinum doublet chemotherapy based on cisplatin and paclitaxel for lung cancer treatment, three groups were compared: the untreated negative control group, the iv platinum doublet used at the MTD (i.e. cisplatin at 1.5 mg/kg and paclitaxel at 10 mg/kg), and the combination of CIS-DPI-50 at 0.5 mg/kg with the platinum doublet iv solution (Table 1). The scheme of administration for each of them followed the rationale previously explained, with a delay of 24 h between the iv doublet solution administration once a week and the endotracheal CIS-DPI-50 three times a week, both for 2 consecutive weeks (=2 cycles) (Fig. 1). The efficacy investigation was performed in terms of tumour growth (according to the bioluminescence values) and survival rates *vs* time. Preliminary studies had investigated the previous CIS-DPI formulation made with tristearin at 0.5 mg/kg or iv cisplatin solution at 1.5 mg/kg used once a week for 2 consecutive weeks for iv and three times a week for 2 consecutive weeks for inhalation at day 6 post-tumour graft. Both had significantly prolonged survival in comparison to the negative control group ($p < 0.05$, $p < 0.01$, respectively) and had similar activity in the M109 model ($p > 0.05$, log rank) (supplementary data, Figure S2). This demonstrated the importance of modulating the cisplatin release from microparticles to find the optimal balance between tolerance and efficacy, with better results with the selected CIS-DPI-50 formulation.

Tumour growth results indicated an exponential increase in untreated control mice (Fig. 6, A and B). This illustrated the M109 lung carcinoma model aggressiveness. Significant reduction in tumour growth was observed with the iv platinum doublet as from day 21 ($p < 0.001$, two-way ANOVA), confirming the responsiveness of the M109 to chemotherapy. The tumour growth tended to be lower in the CIS-DPI-50 combination group compared to the iv platinum doublet (Fig. 6), although no significant difference was found ($p > 0.05$). The antitumour response to the combination was more rapid than with the iv platinum doublet. A significant separation of the negative control tumour growth curve (exponential growth from day 3 to the end of the study) was observed from day 17 with the CIS-DPI-50 combination group ($p < 0.05$) *vs* from day 20 with the iv platinum doublet (Fig. 6). Furthermore, according to the curve profiles, treatment with the combination group tended to demonstrate slower tumour re-growth than with the iv platinum doublet (Fig. 6.D).

A responder was defined as a mouse with tumour growth < 150% in comparison to the 100% value measured at day 3. As anticipated, mice treated with the iv platinum doublet controlled the tumour growth, as 50% (4/8) responded to the doublet (Fig. 6.B and 7). The addition of CIS-DPI-50 to iv platinum doublet tended to increase the peak responder rate to 67% (6/9). As demonstrated in Fig. 7, a peak in terms of the percentage of responders was observed for the combination group. Moreover, the response tended to be more sustained in the combination group *vs* the iv platinum doublet, with a shift of the % responders curves to the right (Fig. 7).

In addition, a survival analysis was performed to assess the treatment effectiveness in terms of survival rates in days post tumour engraftment. This analysis matched the tumour growth. It must be noted that six mice from the combination group died immediately following the endotracheal administration and are not included in the survival analysis, as previously observed for this type of procedure in animal studies [19,24]. Indeed, the endotracheal technique of administration is invasive as it requires anaesthesia, endotracheal intubation, and the delivery of repetitive puffs of air tidal volume per administration. Moreover, these mice are more weakened than healthy mice as they bear tumours and experience up to six repeated endotracheal administrations.

The median survival times were significantly prolonged by 5 days following the iv platinum doublet when compared to the negative control (26 *vs* 21 days, $p < 0.01$, log-rank test, Fig. 8). The combination of CIS-DPI-50 and iv platinum doublet prolonged the median survival significantly by 10 days when compared to the negative control group ($p < 0.0001$, Fig. 8). However, despite the fact that the survival was increased by 5 more days (26 *vs* 31) for the combination group in comparison with the iv platinum doublet, no significant difference was noticed between these two treated groups ($p > 0.05$, Fig. 8). This could be explained by the rapid tumour re-growth once the treatment was stopped (Figs. 6 and 7) leading to a limited differentiation of the two survival curves.

It was therefore interesting to analyse the number of mice alive at specific days. At 24 days following the graft, all the negative control groups had died, whereas 62% (5/8) of mice among the iv platinum doublet group were alive and 100% (9/9) of the combination group iv platinum doublet and CIS-DPI-50 group were still alive. On day 28, 25% (2/8) of the mice were alive following the administration of the iv platinum doublet whereas 50% (4/8) were still alive among the mice treated with the combination of iv platinum doublet and CIS-DPI-50. This led to a 20% increase in terms of the median survival rate when compared to the iv conventional treatment.

Taking all these results together, adding CIS-DPI-50 to the

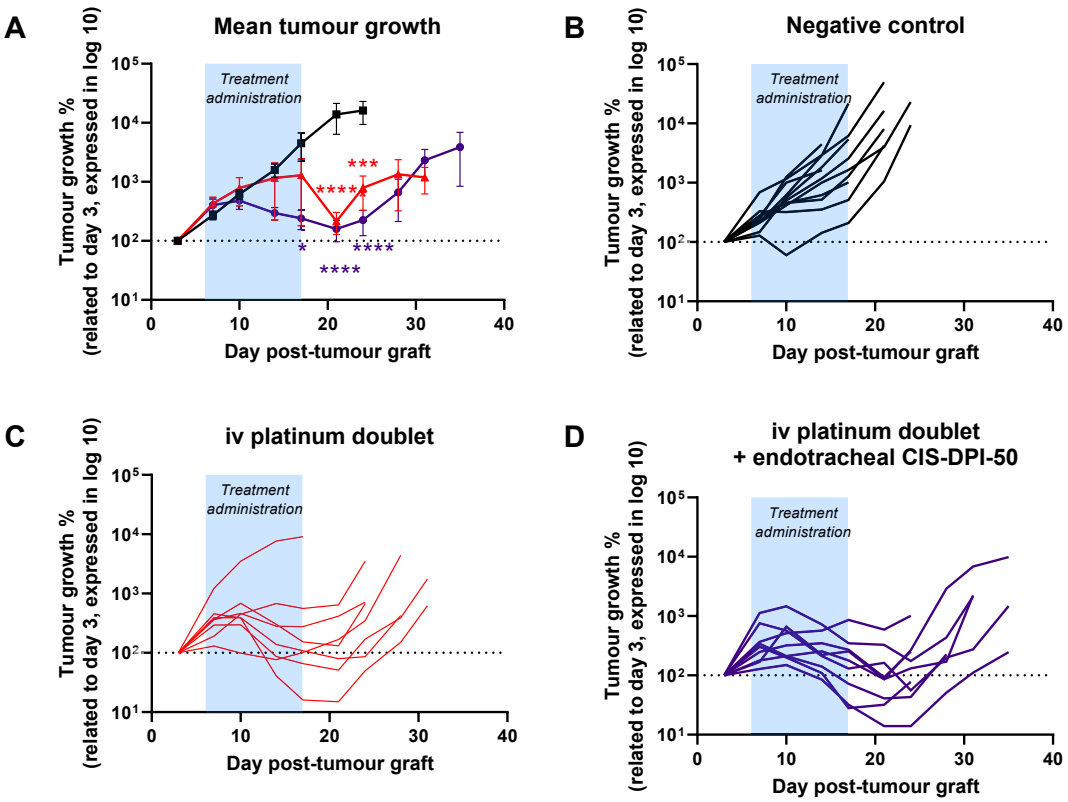


Fig. 6. Tumour growth according to bioluminescence imaging and expressed as the mean ± SEM (A) and per subject of the negative control group (B, n = 11), the group treated with iv platinum doublet (C, n = 8), and the combination of CIS-DPI-50 and iv platinum doublet (D, n = 9) following the regimen described in Fig. 1. * $p < 0.05$, **** $p < 0.0001$ between the negative control and either the iv platinum group groups (red asterisks) or the combination group (purple asterisks). No significant difference was found between the iv platinum group groups and the combination group ($p > 0.05$, two-way ANOVA with Tukey-multiple comparison test).

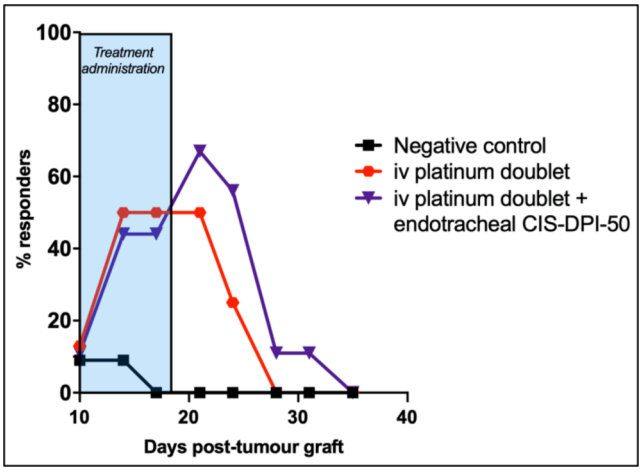
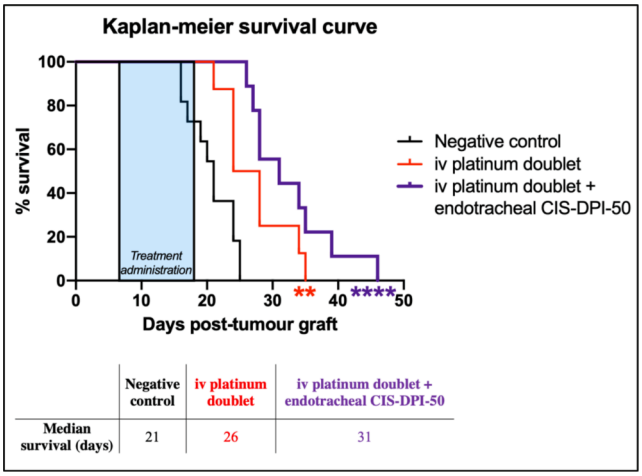


Fig. 7. Response rate rate *vs* time. A responder was defined as having tumour growth values < 150% in comparison to the 100% value measured at day 3. Mice were left untreated (negative control, n = 11), treated with the iv platinum doublet (n = 8), or with the combination of CIS-DPI-50 and the iv platinum doublet (n = 9) following the regimen described in Fig. 1.



	Negative control	iv platinum doublet	iv platinum doublet + endotracheal CIS-DPI-50
Median survival (days)	21	26	31

Fig. 8. Kaplan-Meier survival curves and median survivals of M109 mice. Mice were left untreated (negative control, n = 11), treated with the iv platinum doublet (n = 8), or with the combination of CIS-DPI-50 and the iv platinum doublet (n = 9) following the regimen described in Fig. 1. **** $p < 0.0001$, **$p < 0.01$ between the group and the negative control (log-rank test).

conventional platinum doublet chemotherapy tended to increase the response rate, helped to better control the tumour growth, and favoured a prolonged survival rate. These results were in line with results previously described for NSCLC patients, for whom an increased treatment effectiveness in terms of tumour size reduction, survival, and recurrence of the disease was correlated to higher platinum concentrations [8]. Moreover, the study conducted in NSCLC patients by Zarogoulidis *et al.* on inhaled carboplatin demonstrated a statistically significantly higher survival for the group that received inhaled carboplatin and the conventional iv doublet in comparison to the conventional iv doublet alone [42]. As previously described, this would be related to a higher concentration of the chemotherapeutic agent in the tumour site, lymph nodes, and systemic circulation [43].

These trends confirmed that the promising strategy of adding a loco-

regionalized treatment during the off-cycles to conventional iv chemotherapy favours a reduction in the increase of the tumour and improves the median survival rates. This was despite the limitations of the preclinical investigation in mice regarding the CIS-DPI endotracheal administration procedure and the model aggressiveness which have not led the possibility to investigate the real impact of the frequency of CIS-DPI treatment administrations as it will be the in human patients (potential daily CIS-DPI administration *vs* a single administration every 3–4 weeks for CIS-iv).

4. Conclusions

This study succeeded in developing a promising cisplatin-based dry powder formulation for inhalation that was manufactured using GRAS excipients by means of a scalable process. This CIS-DPI formulation was characterized by good aerodynamic performance with the use of a low-resistance inhaler device that offered good aerosolization and potential low contamination of the environment by the exhaled cytotoxic aerosol. Cisplatin distribution and elimination pharmacokinetics following its administration in the mouse lungs helped in the construction of a therapeutic regimen where its administration was combined with iv cisplatin treatments.

The selected scheme of administration (i.e. 24 h delay between the iv and the first endotracheal administration) and the safer dose of CIS-DPI-50 (i.e. 0.5 mg/kg) with the iv cisplatin solution at its MTD (i.e. 1.5 mg/kg) was previously reported to not impair renal tolerance, and was selected for the efficacy study. Achieving this result was challenging as the dose-limiting toxicity of cisplatin is cumulative and irreversible nephrotoxicity due to saturable concentration mechanisms. This approach was taken to be able to evaluate the feasibility of its administration as a locoregional treatment during chemotherapy off-cycles, without decreasing systemic tolerance as these off-cycles are applied to allow normal tissue to recover. This combined regimen showed positive trends in the efficacy study on the aggressive M109 lung carcinoma orthotopic model, with decreased tumour growth and recurrence of the disease resulting in an increased survival rate. Therefore, the selected cisplatin-based dry powder formulation for inhalation could be proposed as an add-on treatment to intensify the therapeutic response of cisplatin-based doublets in lung cancer therapy. The next step of the development would be to perform a complete preclinical toxicity program following ICH guidelines on the formulation and the selected excipients (i.e., as the excipients are not approved in an inhaled formulation), in parallel to the scaling-up of the manufacturing process, prior to a first-in-human study.

Declaration of Competing Interest

K. Amighi, N. Wauthoz, and R. Rosière are inventors of patents related to some technologies described in the paper and are co-founders of InhaTarget Therapeutics. R. Rosière is also the CSO of InhaTarget Therapeutics and a scientific collaborator of the Unit of Pharmaceutics and Biopharmaceutics. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejpb.2021.04.018.

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