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Pulmonary and renal tolerance of cisplatin-based regimens combining intravenous and endotracheal routes for lung cancer treatment in mice

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ABSTRACT

Despite recent advances, platinum-based chemotherapy (partially composed of cisplatin, CIS) remains the backbone of non-small-cell lung cancer treatment. As CIS presents a cumulative and dose-limiting nephrotoxicity, it is currently administered with an interruption phase of 3–4 weeks between treatment cycles. During these periods, the patient recovers from the treatment side effects but so does the tumour. Our strategy is to increase the treatment frequency by delivering a cisplatin controlled-release dry powder for inhalation (CIS-DPI) formulation during these off-cycles to expose the tumour environment for longer to CIS, increasing its effectiveness. This is promising as long as the pulmonary and renal toxicities remain acceptable. The aim of the present investigation was to evaluate the pulmonary and renal tolerance of CIS-DPI (three times per cycle) and CIS using the intravenous (IV) route (CIS-IV) (one time per cycle) as monotherapies and to optimize their combination in terms of dose and schedule. At the maximum tolerated dose (MTD), combining CIS-DPI and CIS-IV impaired the pulmonary and the renal tolerance. Therefore, pulmonary tolerance was improved when the CIS-IV dose was decreased by 25% (to 1.5 mg/kg) while maintaining the MTD for CIS-DPI. In addition to this dose adjustment, a delay of 24 h between CIS-DPI and CIS-IV administrations limited the acute kidney injury.

1. Introduction

Despite recent advances in new therapies (i.e. targeted therapy and immunotherapy), lung cancer remains the most frequent and deadliest cancer worldwide (18.4% of all cancer deaths in 2018) (Bray et al., 2018). The 5-year relative survival rate is strongly related to the stage at diagnosis and varies from 57.4% for localized stages to 5.2% for distant stages in the USA (2009–2015) (National Cancer Institute, 2020). As 57% of newly diagnosed patients already have distant metastasis, the overall 5-year relative survival remains low at 19.4% (USA, 2009–2015) (National Cancer Institute, 2020).

To treat non-small cell lung cancer (NSCLC, 85% of lung cancers), platinum-based chemotherapy remains the backbone of care. It combines a platinum compound (mostly cisplatin (CIS) or carboplatin) with another anti-cancer drug (e.g. paclitaxel, gemcitabine or pemetrexed). Platinum-based chemotherapy is highly recommended as an adjuvant therapy after surgery for resectable stage II and III cancers, and in combination with radiotherapy (and more recently also with immunotherapy) for unresectable stage III cancers (Duma et al., 2019; Passiglia et al., 2020). For patients with advanced diseases and with no specific genomic alterations at the tumour level, immunotherapy is the first-line therapy as monotherapy or in association with platinum-based chemotherapy (Hanna et al., 2020; Majem et al., 2019).

Although platinum-based chemotherapy drugs are highly effective, they lack selectivity for tumour cells. Systemic distribution of the drugs through the intravenous (IV) route leads to heavy side effects (e.g. hair loss, digestive disorders) (American Cancer Society, 2020). Among them, some are dose-limiting such as the dose-dependent and cumulative nephrotoxicity for CIS (Sakaida et al., 2016). This leads to administering CIS in well-spaced cycles (every 3–4 weeks, for a maximum of 4–6 cycles) with massive hydration (at least 2 h before and 6 h after the 6 h-8 h treatment administration) (Hayati et al., 2016; "NCCN Guidelines - NSCLC- Version 3.2020," 2020; Sakaida et al., 2016). A significant

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Abbreviations

AKI	Acute kidney injury
AM	Alveolar macrophages
AUC	Area under the curve
BALF	Bronchoalveolar lavage fluid
Bw	Body weight
CIS-DPI	Cisplatin controlled-release dry powder for inhalation
CIS	Cisplatin
COH	Hydrogenated castor oil
C_{max}	Maximum concentration
DLT	Dose limiting toxicity
DPI	Dry powder for inhalation
ET	Endotracheal
FMA	Formaldehyde
HE	Haematoxylin Eosin
IP	Intraperitoneal
IV	Intravenous
KIM-1	Kidney injury molecule 1
L	Lymphocytes
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
MTD	Maximum tolerated dose
NGAL	Neutrophil-gelatinase associated lipocalin
NSCLC	Non-small-cell lung cancer
PAS	Periodic acid schiff
PBS	Phosphate buffer saline
PMN	Polymorphonuclear neutrophils
REC	Recovery
TPGS	d-α-Tocopherol polyethylene glycol 1000 succinate

correlation has been observed between the pulmonary tumour platinum concentration and the treatment effectiveness in terms of tumour size reduction, survival and recurrence of the disease for NSCLC patients (Kim et al., 2012). Consequently, while the resting period encountered with platinum-based chemotherapy gives an opportunity to the patient to recover, it also allows the surviving cancer cells to proliferate and invade the lung or other tissues (lymph nodes or distant organs) as these cells are no longer exposed to the treatment (Kim and Tannock, 2005).

Regarding its pharmacokinetic advantages, the pulmonary route has been widely investigated for the treatment of different pulmonary diseases (Newman, 2018). Delivering a drug into its target site leads to a rapid onset of action and provides a high drug concentration locally while reducing its systemic exposure, thus improving the therapeutic ratio. This improvement is mostly needed for cytotoxic drugs as this gives the opportunity to reduce the administered dose, which interestingly would also reduce the systemic side effects (Newman, 2018). In addition to these advantages, the pulmonary delivery of cytotoxic drugs allows them to reach the lymphatic circulation (Zarogoulidis et al., 2013), which could increase the treatment efficacy.

Administering CIS directly to the lungs several times during the resting period encountered with platinum-based chemotherapy would intensify the tumour exposure to cytotoxic drug. This is both an opportunity to benefit from the advantages of the pulmonary route and a challenge to overcome its limitations. Its first limitation is that a high CIS dose deposited repetitively in the lungs can impair the pulmonary tolerance. The second one is that the solubilized part may be eliminated from the lungs by absorption into the blood and be concentrated by proximal tubules, inducing nephrotoxicity. However, nephrotoxicity seems to be more related to CIS peak concentrations than to the area under the curve (AUC) (Nagai et al., 1996), and should be higher when CIS is administered by IV. Last but not least, the administration of a nebulized CIS solution, during a phase I study, showed that the targeted dose was not reached following >6 h of nebulization (Wittgen et al., 2007). Besides, this involved heavy equipment (closed cabin, negative pressure rooms, high efficiency particulate air- HEPA filters) to limit the environmental contamination and exposure of the medical staff (Wauthoz et al., 2020).

Therefore, one strategy to overcome these limitations was to develop a CIS dry powder for inhalation (DPI) formulation (CIS-DPI) with controlled cisplatin release and with sustained lung retention to increase the exposure of the lung tissue and therefore of the lung tumour to the drug (Wauthoz et al., 2020). CIS-DPI has been previously developed using solid lipid microparticles embedding CIS into a lipid matrix (Amighi et al., 2020; Levet et al., 2016b). Delivering CIS-DPI during the resting period at a high frequency could be a promising strategy to expose the lung tumours and the locoregional invasion more often to CIS and therefore increase the opportunity to cure or to prolong the survival of the patients (Rosière et al., 2018).

In a phase II study, a significant increase in survival was reported for NSCLC patients treated with a combined group of 1/3 of the carboplatin dose by the pulmonary route and 2/3 by the IV route on day 1, when compared to the control group (3/3 of the carboplatin dose by the IV route) (Zarogoulidis et al., 2012). This can be related to a longer exposition of the tumour site, lymph nodes and systemic circulation to a therapeutic carboplatin concentration when compared to the control group (Zarogoulidis et al., 2012).

Therefore, combining both routes to deliver CIS can be promising as long as pulmonary and renal toxicities remain acceptable. Indeed, the dissolved part from CIS-DPI could further increase the maximum concentration peak related to the CIS-IV administration in the lungs and kidneys and be responsible for a higher pulmonary and renal toxicity. The aim of this study is to investigate lung and renal tolerance of: (i) CIS monotherapies administered three times a cycle using the pulmonary route for CIS-DPI and once a cycle for IV CIS (CIS-IV); and (ii) their combinations at different doses. Combinations were optimized in terms of doses (total and fractionated dose) and days of administration to find the best balance between the highest dosage and most frequent regimen (i.e. potentially related to efficacy) and their tolerance.

2. Materials and methods

2.1. Materials

Cisplatin was purchased from Umicore (Brussels, Belgium), hydrogenated castor oil from BASF (Ludwigshafen, Germany), TPGS from Biomadis (Paris, France) and formaldehyde (FMA) buffered pH 6.9 for histology from Sigma-Aldrich (St. Louis, USA). Phosphate buffer saline (PBS) was purchased from Life-Technologies (Merelbeke, Belgium) and isopropanol from Merck Millipore (Darmstadt, Germany). L-leucine and ethanol were obtained from Merck Millipore (Darmstadt, Germany). Primary antibodies used for immunostaining were rabbit anti-NGAL purchased from Invitrogen (Carlsbad, USA). The secondary antibody, the 3,3′- diaminobenzidine (DAB), the blocking kit and the avidin–biotin complex (ABC) kit were all obtained from Vector Labs (Peterborough, UK). Ultrapure water was obtained from a Pure-Lab Ultra® purification system (Elgan Lane End, UK). All solvents and chemicals were analytical grade.

2.2. CIS-DPI formulation production and characterization (DPI-0.5 and DPI-1)

CIS-DPI was produced and characterized as described in the patent (Amighi et al., 2020). Briefly, a suspension of CIS was prepared in ethanol (Merck, Darmstadt, Germany) at 5% (w/v) and was submitted to

size reduction (High speed and high-pressure homogenization). Excipients (hydrogenated castor oil, COH and d-α-Tocopherol polyethylene glycol 1000 succinate, TPGS) were added to the pre-heated (55 ± 5 °C) size-reduced suspension to target 50.0% (w/w) of cisplatin, 49.5% (w/w) of COH and 0.5% (w/w) of TPGS. The mixture was then spray-dried (Mini-Spray Dryer B-290, Büchi Labortechnik AG, Flawil, Switzerland), following the parameters previously optimized (Levet et al., 2016a). CIS-DPI was characterized *in vitro* in terms of cisplatin content (electrothermal atomic absorption spectrometry, (Levet et al., 2016a)), release ("paddle over disc" method, (Amighi et al., 2020)), geometric particle size distribution (laser diffraction (Levet et al., 2016a)), and aerodynamic particle size distribution (next generation impactor, NGI at 100 L/min during 2.4 s, with a pressure drop of 4 kPa, and a low resistance RS.01 Mod. 7 dry powder inhaler, following European Pharmacopeia 10 guidelines (Amighi et al., 2020)). The fine particle fraction (FPF) was calculated over the nominal dose, the mass median aerodynamic diameter (MMAD) and the geometric standard deviation (GSD) were determined using Copley Inhaler Testing Data Analysis Software 1 (Copley Scientific, Nottingham, UK). All these characteristics were analysed three times and expressed as mean ± standard deviation (SD).

CIS-DPI was diluted and blended with the diluent (mannitol leucine spray-dried powder), as described by Levet et al. (Levet et al., 2017a), to deliver ~ 1.0 mg of powder to mice for both doses to avoid any bias related to the delivered mass. Briefly, the diluent was added to CIS-DPI to target 1% and 2% CIS for DPI-0.5 (CIS-DPI at 0.5 mg/kg) and DPI-1 (CIS-DPI at 1.0 mg/kg), respectively, in a total mass of 250 mg using a 2 mL glass vial following the so-called sandwich method. Practically, to target 1% of CIS in DPI-0.5, an amount of 5 mg of CIS-DPI was added to 245 mg of diluent (mannitol leucine), and proportionally, to target 2% of CIS in DPI-1, an amount of 10 mg of CIS-DPI was added to 240 mg of diluent. The powders were blended using a Turbula 2C 3D motion mixer (Bachofen AG, Uster, Switzerland) at 46.2 rpm for 4 h. At the end of this process, the blend was sieved twice using a 355 μm stainless steel mesh to deagglomerate the particles. The blends were characterized in terms of cisplatin content and uniformity (expressed as a percentage of difference from the mean), as recommended by the European pharmacopeia v.10 (European pharmacopoeia 10, 2018).

2.3. *In vivo toxicity studies*

Female 6-week-old BALB/cAnNRj mice (16–18 g) (Janvier Labs, France) were kept under conventional housing conditions (12 h/12 h night and day cycles, 22 ± 2 °C, 55 ± 10% RH) and were given dry food and water *ad libitum*. All experiments and manipulations were performed in accordance with the ARRIVE guidelines and EU Directive 2010/63/EU for animal experiments, and were approved by the CEBEA (Comité d'Ethique et du Bien-Être Animal) of the faculty of medicine (ULB) under approval number 585 N. Mice were weighed three times per week and were euthanized if their weight loss exceeded 20% when compared to the first weighing day or 15% when compared to the last weighing.

2.3.1. *Cisplatin-based regimen administration*

2.3.1.1. Maximum tolerated dose evaluation. The CIS-DPI maximum tolerated dose (MTD) was defined as "the highest dose for which mean body weight (bw) loss did not exceed 5% w/w during the follow-up after the first dosing". A similar formulation developed in our laboratory has shown an MTD at 1 mg/kg (Levet et al., 2017b). Therefore, we tested this new formulation at the same dosage (DPI-1) and at a reduced dose of 50% (i.e. 0.5 mg/kg, DPI-0.5). Briefly, mice were anesthetized after being placed in a chamber with 3.5% of isoflurane for 5 min. Immediately after, mice were taken out of the chamber and put in an angled board to deliver the formulations directly into the mice trachea using an endotracheal device for dry powders (Dry Powder Insufflator™ model DP4-M®, Penn-Century) (Bivas-Benita et al., 2005).

CIS-IV MTD was defined as the "the lowest dose that increased the nephrotoxicity biomarkers and for which mean bw loss did not exceed 5% w/w". Five groups of six mice were administered CIS-IV one time per week for 3 weeks at 1.5, 1.75, 2.0, 2.25 and 2.5 mg/kg. They were compared to a negative control group (n = 6) that received only a saline solution. Briefly, mice were immobilized using a restraint device from which the tail protruded. Immediately after, the tail was vasodilated by applying a wet heated paper to facilitate the injection in the caudal vein. Blood was collected using *retro*-orbital terminal sampling and centrifuged to collect plasma, as discussed below. Plasma neutrophil-gelatinase associated lipocalin (NGAL), cystatin C and creatinine were quantified to evaluate the lowest dose at which the selected biomarkers increased. CIS solutions were prepared at 0.15, 0.175, 0.20, 0.225 and 0.25 mg/mL in 0.9% NaCl at pH 4 and were kept protected from light and used within 24 h. All the mice were weighed three times a week, i.e. on Mondays, Wednesdays and Fridays.

2.3.1.2. Monotherapies. Once the MTD was identified for CIS-IV and CIS-DPI, these formulations were tested first as monotherapies using either the endotracheal (ET) or IV (CIS-IV) route. One week was designated a cycle of treatment.

Four mouse groups were administered CIS using the ET route three times per cycle for two cycles (Fig. 1A), following the procedure described in the Section 2.3.1. The first treated group received the vehicle (DPI placebo without CIS, DPI-V (n = 14)), the second group CIS-DPI at 0.5 mg/kg (DPI-0.5) (n = 10) and the third group CIS-DPI at 1.0 mg/kg (DPI-1) (n = 9). Negative control groups received a saline solution (NaCl, 0.9%) (ET-saline) (n = 12) and the positive control group received ~1 μg of lipopolysaccharide (LPS) (ET-LPS) from *E. coli* (n = 6). The ET-saline group followed the same regimen as the treated groups (Fig. 1A). The ET-LPS group was administered the treatment 18 h before each sampling according to the kinetic of the inflammatory biomarkers, following the procedure described in the Section 2.3.1.1, and using an endotracheal device for solutions (Microsprayer™ model IA-1C® equipped with an FMJ-250 high-pressure syringe, Penn-Century). These biomarkers have been characterized by a peak between 12 and 24 h following exposure to LPS (Bondue et al., 2012). The control groups were re-evaluated each sampling day. All the ET-LPS groups increased all the biomarkers when compared to ET-saline. Biomarkers for the ET-LPS groups ranged between 74.7 and 209.6 pg/mL for TNF-α, 72.7 and 207.58 pg/mL for IL-6, 35.7 and 140.3 pg/mL for IL-1-ß, 290.5 and 433.28 pg/mL for CXCL1, 37.7 and 64.9 pg/mL for CXCL2 and 251.9 and 350.4 μg/mL for protein content. To simplify the figures, only figures for one ET-LPS group were reported to appreciate the difference between the tested groups and the LPS level.

ET groups were compared to IV groups, which were administered CIS the first day of each cycle for a total of three administrations (Fig. 1A). CIS-IV was administered at 2.0 mg/kg (IV-2) (n = 20) and 1.5 mg/kg (IV-1.5) (n = 12), following the procedure described in the Section 2.3.1.1. These groups were compared to saline (IV-saline) (n = 12). The last administration was performed 24 h before the first sampling according to the kinetics of nephrotoxicity biomarkers.

2.3.1.3. Combination therapies. Once the MTD determined for CIS-IV and CIS-DPI were tested as monotherapies, different regimens were made by combining CIS-IV and CIS-DPI at their MTD or by decreasing the IV MTD by 25% while maintaining the MTD for CIS-DPI or by delaying the administration between CIS-DPI from CIS-IV by 24 h. These adaptations (dose reduction and/or delayed days of administration) were integrated in this study to evaluate the pulmonary and renal tolerance of these adapted regimens in the case of cumulative toxicity that may result following the administration of CIS-IV and CIS-DPI at their MTD, and the same day.

To evaluate the tolerance of the combinations, CIS-IV and CIS-DPI

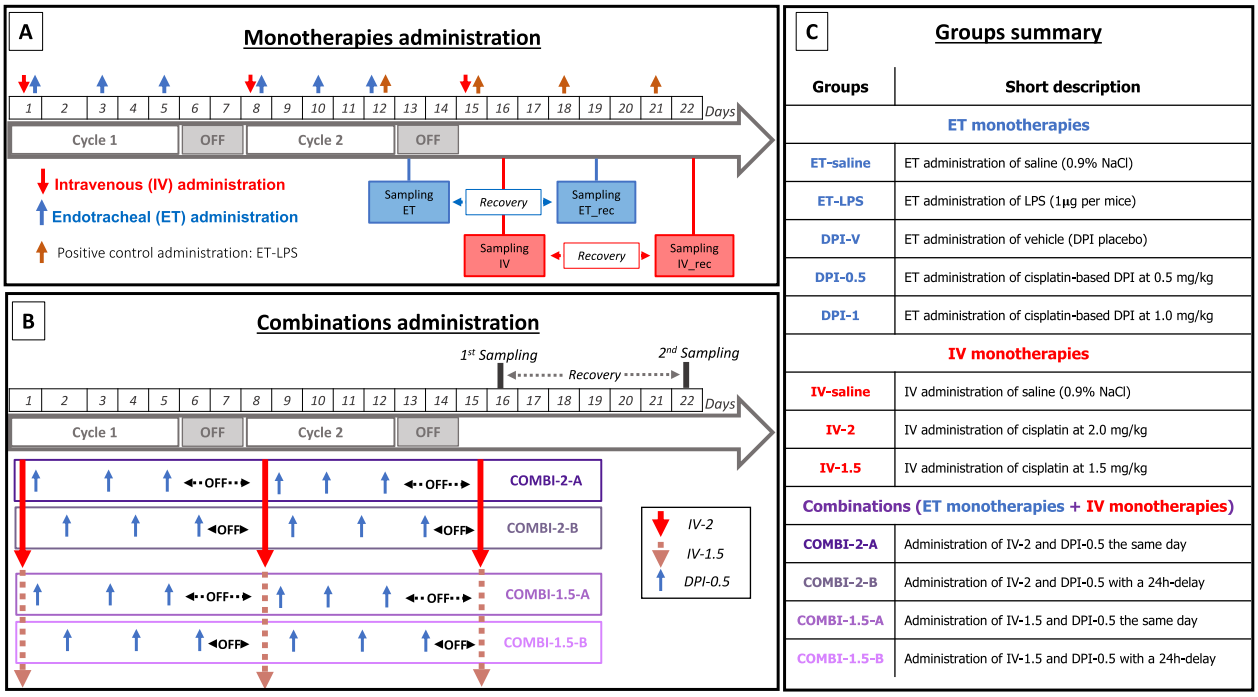


Fig. 1. Time course of CIS administrations (monotherapies (A) and combinations (B), respectively) and sampling procedures 24 h after the treatment administration and 1-week later recovery (rec) of all the groups (C).

were administered following four different regimens (Fig. 1B). COMBI-2 combined the IV and ET MTD (i.e. IV-2 and DPI-0.5). These formulations were either administered the same day within 1 h (COMBI-2-A) (n = 31) or delayed by 24 h (COMBI-2-B) (n = 36). COMBI-1.5 combined a decreased IV dose of 25% with the ET MTD (i.e. IV-1.5 and DPI-0.5) and were administered the same day (COMBI-1.5-A) (n = 9) or delayed by 24 h (COMBI-1.5-B) (n = 10). These groups were compared to the negative control group (IV-saline) (n = 20).

For both the monotherapy and the combination groups, the sampling was performed immediately or after a recovery period (rec groups) following the 2-week treatment, i.e. 24 h or 1 week after the last administration. For clarity, Fig. 1C summarizes the groups of mice exposed to the monotherapies or combinations described above.

2.3.2. *Pulmonary tolerance evaluation*

To evaluate the local tolerance, bronchoalveolar lavage fluid (BALF) and lungs were collected as described previously (Rosière et al., 2016). Briefly, the neck region was opened and a 20-gauge canula (Surflo® catheter, Terumo, Leuven, Belgium) was introduced in the trachea and fixed with a silk thread. The lungs were directly flushed three times using 0.7 mL PBS at 4 °C and BALF was collected in centrifuge tubes (VWR Chemicals, Leuven, Belgium). Lungs were harvested and immersed in FMA for 24 h, then cleaned in water for 15 min before being put in isopropanol for 24 h. Then, they were embedded in paraffin for histopathological analyses using haematoxylin-eosin staining. Analyses were performed in a randomized, blinded study by an independent pathologist following the process described by Jones et al. (Jones et al., 2014). This was performed for all mice lungs, using two impaired sections per lung. The severity of each observation was scored from 0 to 5, and the mean score was determined by calculating the average among each group. In addition, the frequency was evaluated and was expressed as the number of animals in which the observation was encountered.

BALF was kept on ice, vortexed and the total cell count was performed using an automated cell counter (Countess II FL, Life Technologies, Zellik, Belgium). BALF were immediately centrifuged at 160 g at 4 °C for 5 min. The supernatant was collected, aliquoted and stored at −80 °C. The packed cells were resuspended in 200 µL of cold PBS and then cytospinned to set them on slides. Slides were stained using May-Grünwald Giemsa stain to investigate the differential cell count. The alveolar macrophages (AM), polymorphonuclear neutrophils (PMN), lymphocytes (L) and eosinophils were counted manually on a total of 200 cells to determine the proportion of each cell type.

To evaluate the local tolerance, both inflammation and cytotoxicity were investigated. The pro-inflammatory cytokines (TNF-α, IL-6 and IL-1-ß), early neutrophil recruitment of specific mouse chemokines (CXCL1 and CXCL2) were selected. All these biomarkers were quantified using an ELISA method as described by the manufacturer (Duoset, RnD Systems, Abingon, UK).

To evaluate cytotoxicity, the lactate deshydrogenase (LDH) activity was quantified following the protocol described in the Cayman Chemical LDH Cytotoxicity Assay Kit (Ann Arbor, MI, USA). Results were expressed as $LDH/LDH_{Negative\ control}$. The total protein content was also quantified using a Pierce bicinchoninic acid (BCA) Protein Assay Kit (Thermo Fisher Scientific, Zellik, Belgium).

All analyses were performed in duplicate and each group was compared to positive (LPS) and negative (saline) controls, as described previously.

2.3.3. *Renal tolerance evaluation*

2.3.3.1. Acute kidney injury plasma biomarkers. In a preliminary study aiming to evaluate early acute kidney injury (AKI), the time course of several plasma and urine biomarkers of AKI was investigated after the administration of a single dose of CIS at 15 mg/kg using the intraperitoneal (IP) route (Holditch et al., 2019). Mice were euthanized 6, 12, 24, 48 and 72 h after CIS administration using an IP administration of sodium pentobarbital at 12 mg/kg. Urine was removed by a puncture directly in the bladder, aliquoted and stored at −80 °C. Blood and kidneys were collected and stored as described above. To evaluate early AKI, urinary and plasma NGAL, kidney injury molecule 1 (KIM-1) and plasma cystatin C were quantified in duplicate using a mouse-specific enzyme linked immunosorbent assay (ELISA) following the manufacturer's protocol (Duoset and Quantikine, RnD Systems, Abingdon, UK). Plasma creatinine was evaluated using a high-performance liquid

chromatography (HPLC) method and urinary creatinine was quantified using the Jaffé method as described previously (Debelle et al., 2003). Urinary NGAL and urinary KIM-1 were normalized using urinary creatinine.

Considering these preliminary results and the erratic availability of urine samples in mice, the following experimental procedures focussed on plasma biomarkers of AKI (NGAL, cystatin C and creatinine).

2.3.3.2. Sample collection, histology and immunohistochemistry. During sampling, blood was directly collected by *retro*-orbital puncture in lithium heparin tubes (Sarstedt, Cologne, Germany). Blood was centrifuged for 10 min at 2 000 g and 20 °C. Then, plasma was aliquoted and stored at −80 °C for further analysis. Kidneys were harvested from mice and put in a buffered 4% formalin solution for 24 h. They were then rinsed in water for 15 min, conserved in isopropanol for 24 h and then embedded in paraffin for histopathological analyses using haematoxylin-eosin (HE) and periodic acid schiff (PAS) staining. This was performed in a randomized, blinded study as described previously (Debelle et al., 2003). The severity of each observation was scored from 0 to 5, and the mean score was determined by calculating the average among each group.

NGAL immunostaining was also performed and was adapted from Luo et al. (2014). Briefly, once deparaffinized, kidney sections (5 µm) were rehydrated and washed in PBS. The heat antigen retrieval was performed by putting the sections in 10 mM of citrate buffer in a microwave for 15 min. The endogenous peroxidase activity was eliminated following incubation for 20 min in 3% H_20_2-CH_30H. After several washes in PBS-Tween 1% (v/v), the sections were blocked using a blocking solution (Vector Labs, Peterborough, UK) for 20 min in a humidity chamber. Sections were rinsed in the same buffer and incubated in a humidity chamber overnight with the primary antibody (Thermo Fisher Scientific, Zellik, Belgium) at 10 µg/mL. After the washing steps, sections were incubated with mouse anti-rabbit secondary antibody (Vector Labs, Peterborough, UK) for 30 min, before being washed again and incubated with the ABC solution (Vector Labs, Peterborough, UK). Sections were rinsed once again and DAB substrate (Vector Labs, Peterborough, UK) was added for 5 min. A haematoxylin counterstain was performed to identify the cell nuclei. Each time that the assay was conducted, a kidney section without primary antibody was assessed as a control to verify the specificity of the experiment.

2.4. Statistical analyses

All statistical tests were conducted using GraphPad PRISM® (7.0a) software. One-way ANOVA and the Bonferroni post-test were used to compare toxicity biomarkers *vs* their respective control groups (NGAL, creatinine, cystatin C, IL-6, TNF-α, IL-1β, CXCL1, CXL2, protein content and LDH ratio). Results were considered as statistically significant (*) for $p < 0.05$, very significant (**) for $p < 0.01$ and extremely significant for $p < 0.001$ (***) and for $p < 0.0001$ (****).

3. Results and discussion

3.1. Toxicity studies

CIS-DPI with appropriate particle size and drug release characteristics was developed to release CIS gradually from the particles (50.6 ± 0.3% after 6 h determined *in vitro* (n = 3)) in the aim to expose the lung and therefore the tumour to CIS while avoiding high maximum concentration (C_{max}) of dissolved CIS in the lungs and plasma. The CIS content was 49.5 ± 0.6% (n = 3), was adequate with the theoretical composition. CIS-DPI has demonstrated good geometric particle size distribution with a fraction of particles under 5 µm up to 91.4 ± 0.02% and Dv(50) of 2.240 ± 0.001 µm (n = 3). This was correlated to a good aerodynamic performance with a FPF of 52 ± 2% (n = 3), a MMAD of 2.01 ± 0.05 µm (n = 3), and a GSD of 1.81 ± 0.03 µm (n = 3) suitable for pulmonary drug delivery in humans. Indeed, it is commonly accepted that the particles with an aerodynamic diameter between 1 and 5 µm deposit optimally in the lower respiratory tract (Pilcer and Amighi, 2010). However, in the aim to deliver CIS-DPI to mice in the context of toxicity studies, it was required to dilute the spray-dried powder using the blend preparation procedure previously validated by our research group (Levet et al., 2017a). The CIS content in the blends were 1.03 ± 0.3% (n = 10) for DPI-0.5 and 1.95 ± 0.5% (n = 10) for DPI-1 with acceptable uniformity (100 ± 5%, n = 10) according to the European Pharmacopeia.

Toxicity was evaluated for both mono and combined IV and ET therapies to select the highest dose and frequency of administration that were well-tolerated.

The MTD was first determined for the ET or IV route. The CIS-IV MTD was observed at 2.0 mg/kg and CIS-DPI MTD at 0.5 mg/kg, as 1.0 mg/kg by ET route induces a bw decrease of −7 ± 3% (Fig. S1, supplementary material). Therefore, a combination was made of CIS-IV at 2.0 mg/kg and CIS-DPI at 0.5 mg/kg (COMBI-2) but also at a lower dose for CIS-IV (i.e. 1.5 mg/kg and CIS-DPI at 0.5 mg/kg, for COMBI-1.5). This second dosage was evaluated because combined administrations usually increase toxicity as CIS nephrotoxicity is dose-duration-frequency-dependent (Manohar and Leung, 2018). As anticipated, combining CIS using both routes increased the bw losses but these did not exceed 10% (Fig. S1, supplementary material). Moreover, during the recovery week, the bw increased for all groups except for COMBI-2 (A and B), under which some mice died (5% *vs* 2.5%, respectively). This showed that they were not able to recover from the toxicity generated by the cycles of administration of high doses of CIS administered using both routes under COMBI-2. This toxicity was also related to the invasive technique of administration (use of anaesthesia, endotracheal intubation and the delivery of several puffs per administration) as shown by the weight stabilisation for the negative control group during the treatment administration period.

To investigate the possible pulmonary and renal toxicities associated with monotherapies and combinations, biomarkers were quantified and histopathological analyses were assessed to identify the tissue damage and the inflammatory mediators involved.

In the case of monotherapies, the pulmonary tolerance following CIS-IV administration and the renal tolerance following CIS-DPI administration should be preserved, as the pharmacokinetic results demonstrated a seven-fold increase and decrease in C_{max} in lungs and plasma respectively for CIS-DPI in comparison to CIS-IV (C_{max} in the lungs for CIS-DPI: 19 ± 2 ng/mg *vs* 2.6 ± 0.8 ng/mg for CIS-IV, and C_{max} in the plasma for CIS-DPI: 0.7 ± 0.6 ng/µL *vs* 5 ± 1 ng/µL for CIS-IV. These results were generated following the methods described by (Levet et al., 2017a)).

As described previously, samples of all the groups were collected 24 h after the two cycles and one week later (rec groups) (Fig. 1) to evaluate potential pulmonary and/or renal toxicity found and their reversibility.

3.2. Pulmonary tolerance assessment of CIS-DPI, CIS-IV and their combinations

To investigate pulmonary tolerance, it was mandatory to evaluate inflammation as well as cytotoxicity related to CIS mode of action by both quantifying specific biomarkers and identifying lung damage. This was performed 24 h following the last treatment administration as these biomarkers are all involved in the early phase of inflammation. Inflammation, in its exudative phase, was evaluated in BALF to quantify the biomarkers in their site of origin (Bhargava et al., 2015). As TNF-α, IL-6 and IL-1-β have been widely investigated in animal models and remain the most frequently involved in the generation of animal toxicity, they were selected in this study (Abdulkhaleq et al., 2018; Bhargava et al., 2015). During a tolerance study of a similar formulation as that developed in our lab, an increased proportion of PMN was

observed (Levet et al., 2017b). Consequently, to estimate the PMN recruitment, mouse chemokines (CXCL1, CXCL2) were selected (De Filippo et al., 2013;). Cytotoxicity was evaluated by assessing LDH activity and the total protein content (Drent et al., 1996).

3.2.1. Pulmonary inflammation evaluation

As anticipated, all the positive controls (i.e. ET-LPS) led to higher values in terms of pro-inflammatory cytokine contents in BALF than their respective baseline (i.e. negative control groups: ET-saline and IV-saline) (Fig. 2). Indeed, these biomarkers are released in the acute-phase as a response to injury (Abdulkhaleq et al., 2018).

No significant TNF-α, IL-6 or IL-1β increase was observed following the administration of DPI-V, DPI-0.5, DPI-1 or COMBI-1.5-A (24 h and rec). TNF-α results showed a higher expression after the administration of IV when compared to ET (Fig. 2A). IV-1.5 and IV-2 were significantly higher when compared to their negative controls. Both COMBI-2-A and COMBI-2-B tended to increase the TNF-α levels, but this increase was only significant for COMBI-2-A, the highest drug and frequent dosage regimen ($p < 0.01$, Fig. 2A). One week later, these increases were maintained and COMBI-2-B_rec became significant (Fig. 2A), showing the expansion of the inflammation. However, TNF-α levels did not seem to increase following COMBI-1.5-A administration. This may be related to cytokine fluctuations as high variations are predicted during *in vivo* experiments. It was demonstrated that stress was able to increase pro-inflammatory cytokines levels and change their kinetics (Cheng et al., 2015). In our case, invasive techniques of administration (repetitive IV and ET administrations) could generate stress leading to unexpected fluctuations from the baseline. Biomarker levels must be interpreted with caution and should be combined with additional investigations such as cell counts and histology. A negative control group was sampled under the same conditions as each of the tested groups due to these day-to-day fluctuations.

IL-6 was significantly higher only for COMBI-2-A (Fig. 2B). IL-1β showed no significant increase in any groups when compared to the negative controls (Fig. 2C). CIS-IV seemed to increase TNF-α and IL-6 levels more than CIS-DPI. This may be explained by the fact that a higher solubilized CIS fraction was delivered to the lungs after the IV administration and may be responsible for a higher toxicity when compared to a controlled-release DPI formulation, for which CIS is gradually released (50.6 ± 0.3% after 6 h (n = 3), *in vitro* dissolution test for inhaled product). As described above, cytokine levels must be interpreted with caution as they might be influenced by mouse stress levels, and should be combined with other investigations.

To complete the pro-inflammatory cytokine evaluation, cells in the BALF (AM, PMN, L and eosinophils) were counted (Fig. S2, supplementary material). The total count showed a dose-dependent increase for DPI-0.5 and DPI-1 ($4 \pm 2 \times 10^6$ and $6 \pm 3 \times 10^6$ cells/mL, respectively *vs* $1.0 \pm 0.1 \times 10^6$ cells/mL for the negative control group). An increase was also observed for the combination groups when compared to their respective controls (COMBI-2-A, COMBI-2-B and COMBI-1.5-A, with $1.0 \pm 0.8 \times 10^6$ cells/mL *vs* $2.0 \pm 0.7 \times 10^5$ cells/mL, $7 \pm 5 \times 10^5$ cells/mL *vs* $2 \pm 0.7 \times 10^5$ cells/mL and $7 \pm 3 \times 10^6$ *vs* $9.0 \pm 0.2 \times 10^5$ cells/mL, respectively). This increase was reversible for all groups except DPI-0.5_rec. As PMN were the only cell type observed during the differential cell count analysis, other than AM and L, this increase was certainly related to both AM and PMN recruitment. AM in normal conditions are not adequate to assess phagocytosis (Moldoveanu et al., 2009). Thus their number is increased and their function increased by the dendritic cells in the case of inflammation and/or infection (Aggarwal et al., 2014; Moldoveanu et al., 2009). Moreover, PMN are the first inflammatory cells recruited directly at the acute inflammation site (Abdulkhaleq et al., 2018). It was therefore interesting to evaluate their proportion among the BALF cells.

As expected, all the positive control groups showed a significant PMN increase (Fig. 3A). No increase was observed when the vehicle (DPI-V) was administered, showing that the excipients were well-tolerated and did not induce inflammation. Therefore, all the following increases were attributable to the sustained release of CIS from the DPI formulation, and not to the presence of the vehicle. It was also noticed that all the groups that were administered CIS-DPI using the

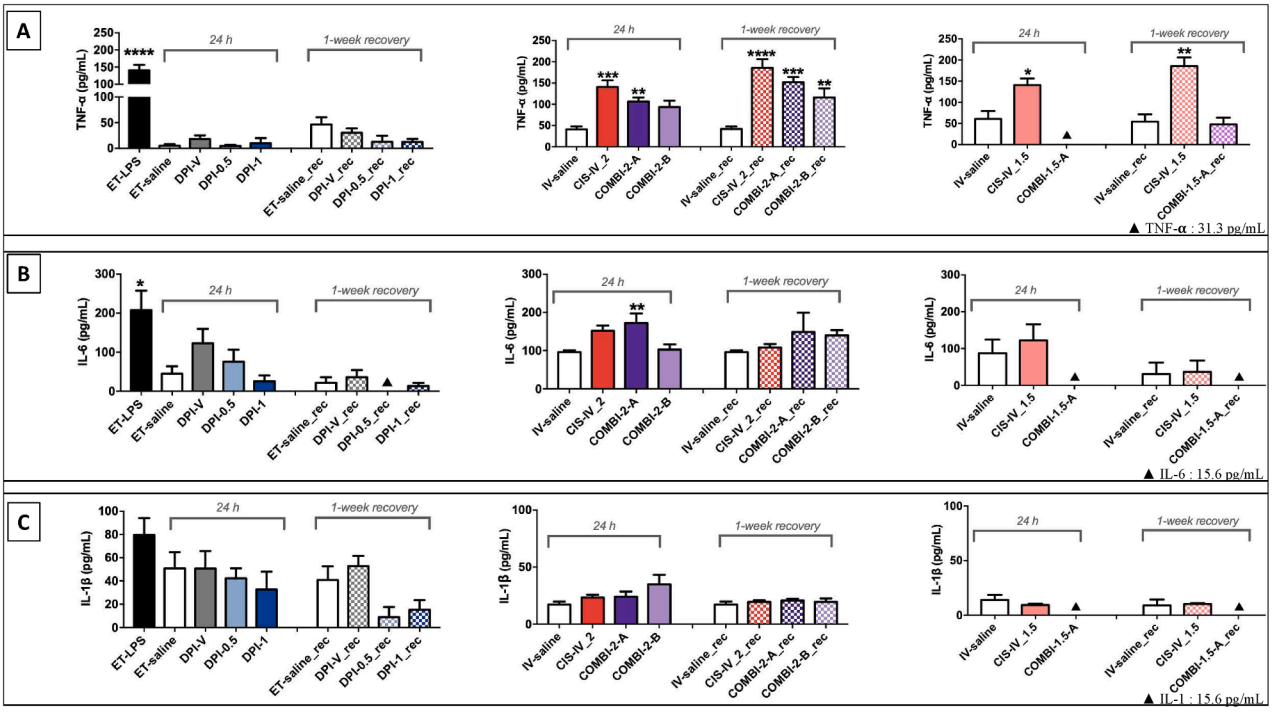


Fig. 2. Evaluation of TNF-α (A) IL-6 (B) and IL1-β (C) in BALF 24 h after the treatment administration and 1-week later recovery (rec). All the results are expressed as means ± SEM (N = 4–17). The statistical analyses were performed *vs* their corresponding negative control group using one-way ANOVA and Bonferroni's post-test (*** for $p < 0.001$,** for $p < 0.01$ and * for $p < 0.05$).

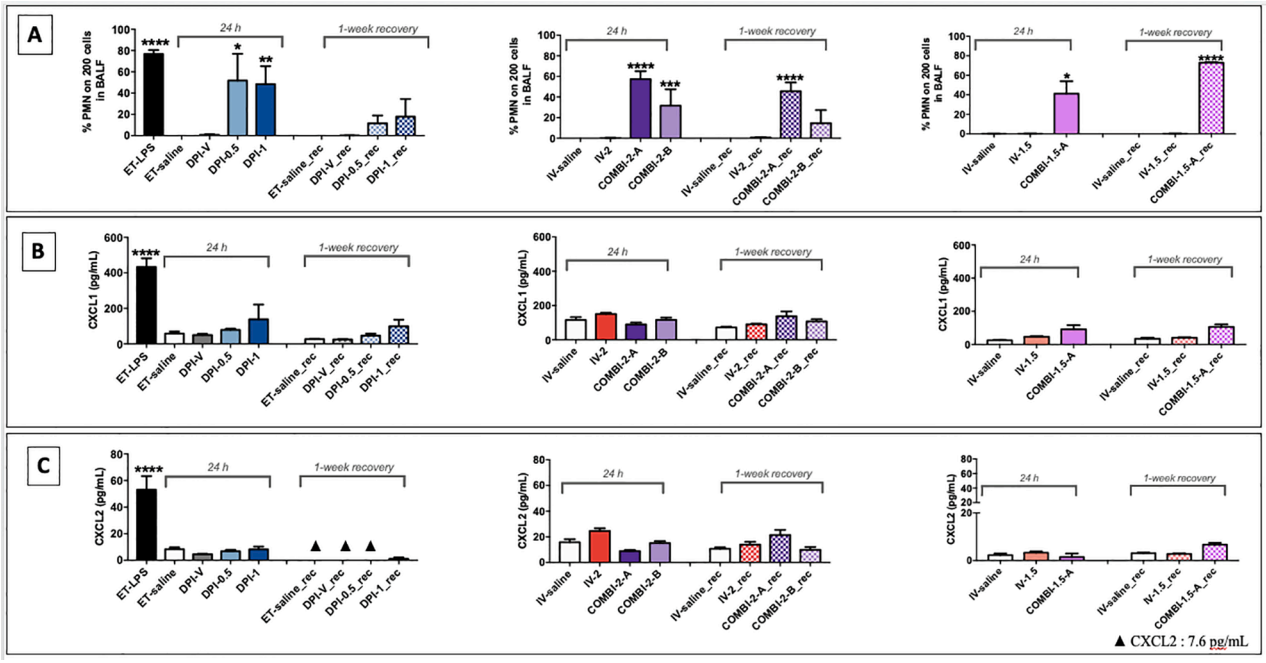


Fig. 3. Evaluation of polymorphonuclears neutrophils (PMN) as a proportion of 200 BALF cells (A), CXCL1 (B) and CXCL2 (C) in BALF 24 h after the treatment administration and 1-week later recovery (rec). All the results are expressed as means ± SEM (n = 4–17). The statistical analyses were performed *vs* their corresponding negative control group using one-way ANOVA and Bonferroni's post-test (*** for $p < 0.001$,** for $p < 0.01$ and * for $p < 0.05$).

ET route (as monotherapy or combination) increased the PMN proportion significantly in comparison with the IV route as monotherapy. This may be explained by the fact that, as demonstrated by pharmacokinetic studies, CIS-DPI led to a longer lung exposure to CIS than CIS-IV, during which CIS was slowly released from CIS-DPI particles ($AUC_{0-\infty}$ in the lungs for CIS-IV: 558 ng·min·mg^{-1} *vs* 4611 ± 932 ng·min·mg^{-1} for CIS-DPI) (Levet et al., 2017a). The most significant exposures were for COMBI-2-A and COMBI-1.5-A. This is understandable as in these combinations, CIS-DPI and CIS-IV were administered the same day, increasing local reaction induced by ET treatment when compared to IV administration alone. The evaluation of PMN levels one week later showed that the inflammation seemed to be reversible for DPI-0.5, DPI-1 and COMBI-2-B_rec, but seemed maintained or even increased for the combination groups that were administered treatment the same day (COMBI-2-A_rec and COMBI-1.5-A_rec, respectively) (Fig. 3A).

As PMN recruitment is partially controlled by CXCL1 and CXCL2 chemokines (De Filippo et al., 2013), these chemokines were therefore quantified in BALF. CXCL1 and CXCL2 were significantly higher for ET-LPS groups, when compared to their negative controls. As with PMN recruitment, CXCL1 and CXCL2 increased dose-dependently for DPI-0.5 and DPI-1 (Fig. 3B and 3C). The evaluation of CXCL1 and CXCL2 levels for COMBI-2-A and COMBI-1.5-A did not seem to increase following the 2-week administration period when compared to their negative controls, but were higher 1 week later. In contrast, CXCL1 and CXCL2 levels for DPI-0.5 and DPI-1 seemed to be reversible within 1 week. Therefore, administering CIS using both routes on the same day, regardless of their doses, could compromise the reversibility of the PMN recruitment as the delay of 24 h between the administrations seemed favourable for COMBI-2-B in comparison with COMBI-2-A. This irreversibility indicated a more intense local reaction for the combination groups administered the same day. These results are in line with the pro-inflammatory cytokines levels observed for COMBI-2-A and B, but not with the observations for COMBI-1.5-A (Fig. 2). As described above, pro-inflammatory cytokines levels may fluctuate depending on several factors (sampling day, mice stress) leading to unexpected results (Nassimi et al., 2010). Apart from inflammation, pulmonary tolerance should therefore be interpreted by considering cytotoxicity as well as lung damage.

3.2.2. *Cytotoxicity evaluation*

The protein content was higher for all groups administered treatment using the ET route (i.e. monotherapies and combinations) when compared to the IV groups. This may be correlated to a more prolonged exposure to CIS when administered in the lungs (controlled-release form) as no higher protein content was observed following the administration of CIS-IV. This increase was even significant for DPI-0.5_rec ($p < 0.05$, Fig. 4A). Indeed, in the case of cell lysis, the protein content is released from cytosol, which makes it an interesting cytotoxicity biomarker. The protein content for COMBI-2-A was significantly higher after the 2-week administration ($p < 0.01$, Fig. 4A) and was maintained 1 week later ($p < 0.05$, Fig. 4A). COMBI-1.5-A protein levels were higher but not significant because of the high variation observed within this group.

$LDH/LDH_{Negative\ control}$ showed no significant increase following the administration of monotherapies (Fig. 4B). Nevertheless, DPI-1 and IV-2_rec were nearly two-fold higher when compared to their negative controls. This was not observed for DPI-0.5 or IV-1.5. LDH is found in the cytoplasm of the cell of various tissue types (brain, lung, lymph nodes, etc.) and increases in the case of cell damage or death (Drent et al., 1996). Consequently, LDH seems to be also an interesting biomarker for cytotoxicity. Moreover, the LDH ratio was more than three-fold higher following the 2-week regimen of COMBI-2-A, and was even higher 1 week later ($p < 0.01$, Fig. 4B). LDH was significantly higher for COMBI-1.5-A ($p < 0.05$, Fig. 4B) but was reversible within 1 week. This difference can be explained by the fact that a higher IV dose was administered for COMBI-2-A in comparison with COMBI-1.5-A (2.0 mg/kg *vs* 1.5 mg/kg).

Despite the fact that the protein content evaluation included all the proteins that were retrieved in the cytosol following cell lysis and that the LDH activity was specific to this unique protein, these cytotoxicity biomarkers tended to demonstrate similar trends. Indeed, no higher cytotoxicity was observed following the administration of monotherapies. However, once combined, a higher cytotoxicity was found in the groups that were administered the same day (COMBI-2-A and

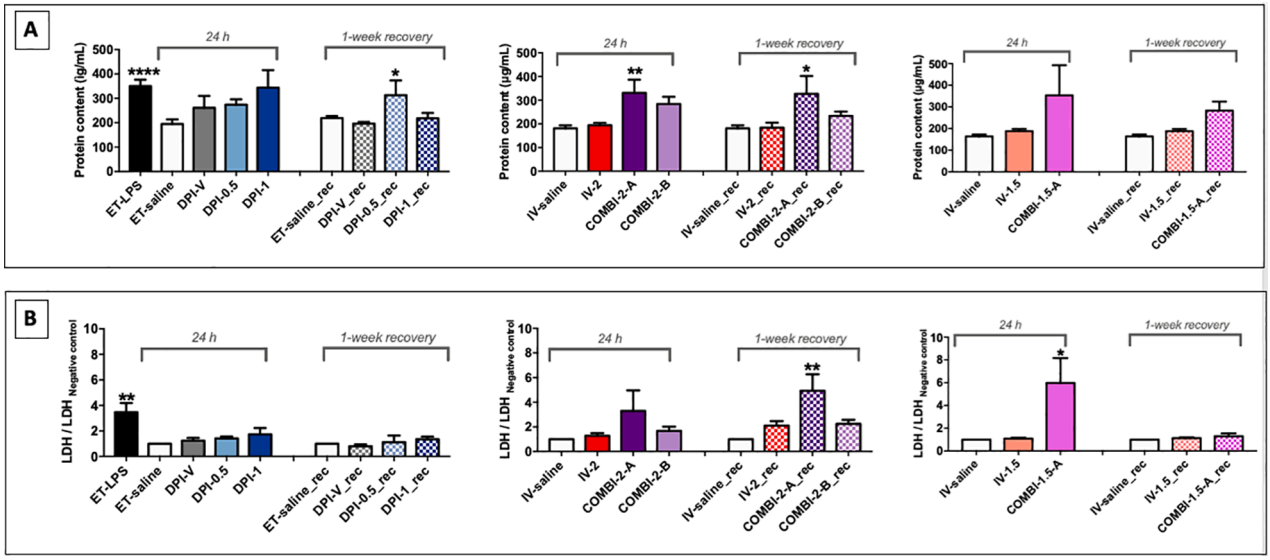


Fig. 4. Evaluation of the total protein content (A), the LDH/LDH $_{(Negative\ control)}$ (B) in BALF 24 h after the treatment administration and 1-week later recovery (rec). All the results are expressed as means ± SEM (n = 4–17). The statistical analyses were performed *vs* the corresponded negative control group using one-way ANOVA and Bonferroni's post-test (*** for $p < 0.001$, ** for $p < 0.01$ and * for $p < 0.05$).

COMBI-1.5-A), 24 h after the last administration (Fig. 4). This was also observed one-week later for the group administered with the highest dose, (COMBI-2-A_rec, Fig. 4).

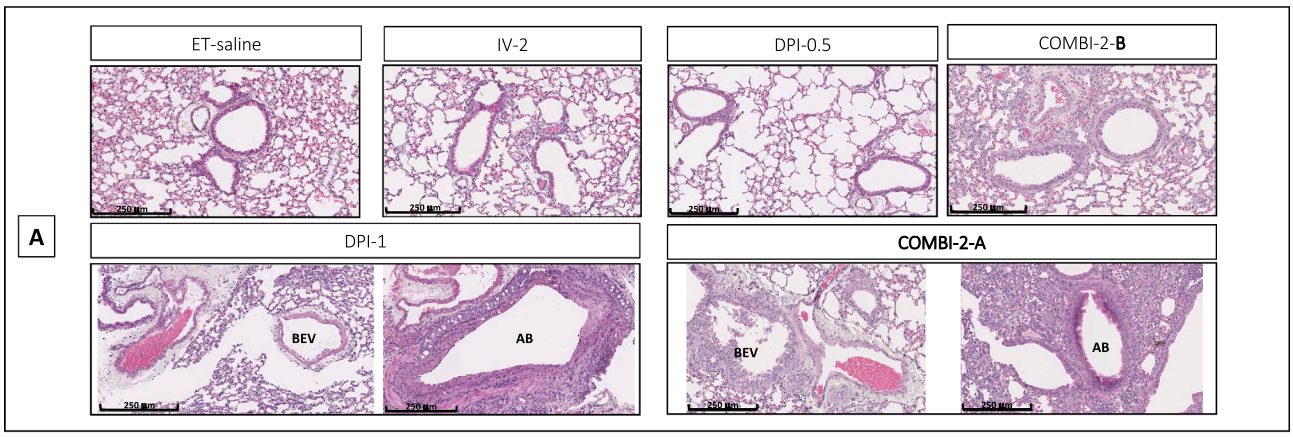
ET and IV treatments seem to activate two different inflammation processes. Indeed, CIS-IV was characterized by increased TNF-α, IL-6 levels when compared to CIS-DPI. In contrast, CIS-IV showed no increase in terms of AM and PMN recruitments (total and differential cell counts) nor did PMN-related chemokines (CXCL1 and CXCL2). As the pulmonary tolerance impairment seemed to be related to higher concentration peaks in the lungs (whole CIS dose solubilized for CIS-IV *vs* a part of CIS released from CIS-DPI particles), CIS-IV may have induced epithelial cell damage, and injured the type II alveolar cells, which are involved in the airways' innate immunity (Hussell and Bell, 2014). This is the most well-described mechanism for cytotoxic injury (Adamson, 1984; Darwiche et al., 2013). As a consequence of this lung damage, pro-inflammatory cytokines (mainly TNF-α, IL-6 and IL-1- β) were secreted to initiate local inflammation (Hussell and Bell, 2014). Other mediators may have been secreted but were not evaluated in this study, such as reactive oxygen species and platelet-activating factors leading to the release of arachidonic acid from membrane lipids. This fatty acid is involved in the production of eicosanoids, which stimulates tissue inflammation (Moldoveanu et al., 2009).

As CIS-DPI was delivered repetitively in the form of solid-lipid microparticles from which CIS had to be released slowly, local damage was therefore prevented. It should be noted that following the administration of DPI-1 using the Dry Powder Insufflator^{TM} model DP4-M®, the CIS mass recovered in the lungs was 5.4 ± 0.7 µg (n = 12), which corresponded to 26 ± 3% of the delivered dose. This was in the same range as previously published by our research group (Levet et al., 2017a). As these microparticles were PEGylated, they were able to escape to AM recognition and uptake since AM identify hydrophobic surfaces more easily (Amighi et al., 2020; Levet et al., 2017a). AM may have started to identify these particles in a time-delayed manner and secreted pro-inflammatory cytokines (mainly TNF-α, IL-6 and IL-1β) and chemokines, leading to AM and PMN recruitments (Bhargava et al., 2015; Darwiche et al., 2013). Moreover, as CIS was entrapped into lipid microparticles, its release from these particles took several hours as was shown from pharmacokinetic studies obtained previously. Following this release, the excipient was entrapped by the AM and, its digestion started with its incorporation into the lysosomes. Hydrogenated castor oil is a triglyceride that can be potentially hydrolysed into glycerol and free fatty acids by the action of the lysosomal acid lipase A (Li and Zhang, 2019). Low PEGylated and amphiphilic excipients such as TPGS can be solubilized, absorbed in the systemic circulation before being eliminated mostly by urinary excretion (Baumann et al., 2014). Consequently, for a controlled-release formulation, AM seemed to control the phagocytosis more efficiently by secreting pro-inflammatory cytokines smoothly, preventing any major peak in their levels in comparison with CIS-IV. Moreover, except for TNF-α and IL-6 fluctuations and considering the PMN proportion, the combination groups seemed to induce higher toxicity effects than CIS-IV.

3.2.3. *Histopathological analyses*

Histopathological analyses showed no major damage for the monotherapy groups (DPI-V, DPI-0.5, IV-1.5 and IV-2) (Fig. 5). Nevertheless, some rare interstitial PMN were observed for the ET-LPS groups. However, bronchiolar epithelial vacuolation and acute bronchopneumonia were scored 1 for 40% of the mice among the DPI-1 group. Both kinds of damage were reversible 1 week later. COMBI-2-A was characterized by an increased bronchiolar epithelial vacuolation and acute bronchopneumonia (score 1).

The bronchiolar epithelial vacuolation and acute bronchopneumonia observed for DPI-1 and COMBI-2-A were scored 0.4 and 0.2, respectively. Cytoplasmic vacuolation is the creation of vacuoles in animal cells exposed to stressful stimuli, including chemotherapeutic drugs, affecting the cell cycle and migration (Shubin et al., 2016; Zong et al., 2011). This phenomenon has been observed for weak basic lipophilic compounds that contain amine groups such as CIS (Shubin et al., 2016). Indeed, in the extracellular medium, CIS can easily cross the membranes through passive diffusion. Once in the cytoplasm, the pH is higher and the chloride concentration is lower, CIS loses its chloride ligands to hydroxyl groups, and is transformed into positively-charged metabolites (monoaquacisplatin: $[Pt(NH_3)_2Cl(OH_2)]^+$, and diaquacisplatin: $[Pt(NH_3)_2(OH_2)_2]^{2+}$), which are no longer able to diffuse through the plasma membrane (Corinti et al., 2017; Shubin et al., 2016). The accumulation of these charged metabolites increases the intraorganellar osmotic pressure. The equilibration of the osmotic pressure is assured by water diffusion, leading to the formation of vacuoles (Shubin et al., 2016). Irreversible vacuolization can affect the endoplasmic reticulum as well as the endosomal-lysosomal system and Golgi apparatus, leading to cell death (Shubin et al., 2016). Histopathological analyses have already reported a vacuolization in the lungs related to amine



B Observations

Observations	Controls		ET - Monotherapies						IV - Monotherapies				Combinations					
	ET-saline	ET-LPS	DPI-V	DPI-V_rec	DPI-0.5	DPI-0.5_rec	DPI-1	DPI-1_rec	IV-1.5	IV-1.5_rec	IV-2	IV-2_rec	COMBI-2-A	COMBI-2-A_rec	COMBI-2-B	COMBI-2-B_rec	COMBI-1.5-A	COMBI-1.5-A_rec
Bronchiolar epithelial vacuolation (BEV)	0	0	0	0	0	0	0.4	0	0	0	0	0	0,2	0	0	0	0	0
Acute bronchopneumonia (AB)	0	0	0	0	0	0	0,4	0	0	0	0	0	0,2	0	0	0	0	0
Perivascular oedema	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Alveolar luminal macrophages	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0
Intra alveolar fibrin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Intra alveolar haemorrhage	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Scale: 20% animals; 40% animals; 60% animals; 80% animals; 100% animals

Fig. 5. Histopathology of lung tissue exposed to monotherapies and combinations. Representative images of treated groups (A) and heat map of adverse observations and severity scores depending on lung tissue histopathology (B).

containing chemotherapeutics such as bleomycin (Zong et al., 2011). Also, CIS has already been associated with vacuolation in liver injury (Huang et al., 2015).

The light and transient acute bronchopneumonia observed with DPI-1 and COMBI-2-A_rec has already been described for inhaled CIS delivered by nebulization (Selting et al., 2008).

An increased number of alveolar luminal macrophages was also observed and was scored from 1 to 4. Moreover, some perivascular oedema and PMN were found for these groups. This was certainly related to a higher phagocytosis activity by the AM and an increased ingestion of the debris by the PMN (Moldoveanu et al., 2009). These observations seemed to be transient and more related to the ET treatment than IV, as no tissue damage was observed following CIS-IV administration, even at the highest dosage, CIS-IV MTD. Staggering these administrations for 24 h (COMBI-2-B) prevented the development of all these observations, even at the highest dose (Fig. 5). Moreover, combination groups at a lower dose (COMBI-1.5-A) did not show any tissue damage (Fig. 5).

As observed for biomarker quantification, and cell-count analysis, all these observations tended to describe an increased local inflammation for DPI-1 and for the highest and more frequent combination group (COMBI-2-A). Nevertheless, the quantitative techniques seemed to be more sensitive to dose and frequency of administration changes than histology, as no histological damage was observed for COMBI-2-B and COMBI-1.5-A.

It is also important to note that since its first use and unlike other cytotoxic drugs (bleomycin, gemcitabine and mitomycin), CIS has not shown any major pulmonary toxicity when administered using the IV route, except some hypersensitivity reactions and in some rare cases, bronchospasm (Adamson, 1984; Collis, 1980; Sanctis et al., 2011). In one case report, CIS was reported to induce eosinophilic pneumonia but this seems to be very rare as it was only described for one patient (De Giacomi et al., 2018; Ideguchi et al., 2014). These observations are therefore in line with what was observed for CIS-IV, as no major tissue damage was noticed during this study. These results confirmed that the increased TNF-α and IL-6 cytokines for IV groups were not related to any major lung damage. However, when administered by nebulization in the lungs, CIS induced bronchitis, dyspnoea, severe pneumonitis and mild to moderate fibrosis (Selting et al., 2008; Wittgen et al., 2007). Delivering CIS as a controlled-release DPI could have prevented the development of these side-effects, thus improving its tolerance.

Overall, cytokine evaluation showed a good tolerance for DPI-V, DPI-0.5 and DPI-1. TNF-α and IL-6 increased for the IV monotherapies (IV-1.5 and IV-2), COMBI-2-A and COMBI-2-B. These increases seemed to be maintained after the 1 week of recovery. Moreover, PMN increased dose-dependently for all groups that were administered DPI (i.e. monotherapies and combinations) and were only reversible 1 week later for the DPI monotherapies (DPI-0.5, DPI-1) and the combination group, for which CIS-IV and CIS-DPI administration were staggered over 24 h (COMBI-2-B). These results are in line with the cytotoxicity evaluation in terms of evolution and reversibility. The histological analyses showed a transient increase in bronchiolar epithelial vacuolation and a reversible acute bronchopneumonia for DPI-1 and COMBI-2-A. An increased number of luminal AM as well as PMN were observed for the combination group (COMBI-2-A), showing a more intense phagocytic activity when compared to other groups. Adding DPI-0.5 to IV-2 seems to have a higher impact than if DPI-0.5 was added to IV-1.5 in terms of inflammation, cytotoxicity and their reversibility.

3.3. Renal tolerance assessment of CIS-DPI, CIS-IV and their combinations

3.3.1. Preliminary study for the selection of AKI biomarkers

To assess early AKI, novel biomarkers have been intensively investigated, and have proven their sensitivity in both human and animal studies (Charlton et al., 2014; Ostermann et al., 2020). They are capable of detecting minor tubules and glomeruli injuries, even when creatinine levels remain in normal ranges (Schley et al., 2015). It is important to select optimal biomarkers not only on their specificity and selectivity but also according to their kinetics. It was therefore mandatory to assess a preliminary study to detect the most pertinent timing to collect the NGAL, KIM-1, cystatin C and creatinine.

During this preliminary study, plasma creatinine, plasma cystatin C and both urine and plasma NGAL and KIM-1 were quantified. Plasma

NGAL increased significantly 24 h following the AKI-induced model (Fig. 6). Both plasma cystatin C and creatinine also increased 24 h following the induction and were significant only 48 h and 72 h later. Plasma KIM-1 levels remained low and no significant difference was observed when compared to the negative control (Fig. 6). KIM-1 is a transmembrane glycoprotein expressed at the apical membrane of proximal tubule cells that increases in the case of ischemic or toxic injury (Luo et al., 2014; Wasung et al., 2015). In the literature, similar studies have shown that plasma KIM-1 increased up to 5 days after AKI, and was not as predictive as it was expected to be (Togashi et al., 2012). Urinary NGAL and KIM-1 showed a high variation between the time points (Fig. 6). Therefore, plasma NGAL, cystatin C and creatinine were selected for the renal tolerance evaluation. These biomarkers were collected 24 h following the last administration as they were all higher than the negative control. It seems important to note that this study aimed to evaluate the earliest phase in AKI for repeated (i.e. not single) administrations. Moreover, this timing also had to match the optimal kinetics timing (i.e. 24 h following the last dose) of the pulmonary biomarkers as both pulmonary and renal tolerance were evaluated at the same time for the same mice. As described above, these AKI biomarkers were also evaluated 1 week later to assess the reversibility of CIS induced nephrotoxicity.

3.3.2. *MTD evaluation*

For this evaluation, it was mandatory to include nephrotoxicity for the determination of CIS-IV MTD as it is a CIS dose-limiting toxicity (DLT) that imposes hours of hydration, massive side effects and separated cycles of administration. This was observed for CIS-IV at 2 mg/kg, as plasma NGAL, cystatin C and creatinine were higher when compared to their negative control ($p < 0.05$, $p < 0.01$, $p > 0.05$, respectively, data not shown).

3.3.3. *Monotherapies evaluation*

IV-CIS administration increased the biomarkers slightly higher than for CIS-DPI (Fig. 7). Indeed, NGAL, cystatin C and creatinine levels for IV-2 were higher than those for DPI-1 when compared to their respective negative controls. It is important to mention that high variability is predicted for *in vivo* experiments and that it is crucial to compare each group to its relative negative control. Considering this purpose, the negative control group followed the same scheme of administration and sampling. NGAL concentration was 366 ± 40 pg/mL for IV-2 and 396 ± 61 pg/mL for DPI-1 *vs* their respective baselines, 233 ± 20 pg/mL for IV-saline and 335 ± 56 pg/mL for ET-saline. Moreover, cystatin C concentration was 1024 ± 48 pg/mL for IV-2 and 812 ± 56 pg/mL for DPI-1 *vs* their respective baselines, 798 ± 26 pg/mL for IV-saline and 648 ± 79 pg/mL for ET-saline. Finally, creatinine concentration was 0.107 ± 0.005 mg/dL for IV-2 and 0.097 ± 0.005 mg/dL for DPI-1 *vs* their respective baselines, 0.075 ± 0.005 pg/mL for IV-saline and 0.081 ± 0.004 pg/mL for ET-saline (Fig. 7). This was related to a higher systemic exposure following IV administration when compared to DPI. As plasma concentration peaks are related to renal injury (Nagai et al., 1996), IV-CIS rapidly reached the kidneys. It showed a higher C_{max} than CIS-DPI, resulting in AKI. Interestingly, our PK results showed that the C_{max} in plasma for DPI-1 was 0.7 ± 0.6 pg/mL *vs* 5 ± 2 pg/mL for CIS-IV, at 1.5 mg/kg (data not shown). Administering a lower dose of a prolonged-release CIS formulation using the ET route three times per cycle limited CIS peak concentration in the proximal tubule and improved its tolerance by the kidneys. This was observed for a lower cumulative dose and also for a higher dose of CIS-DPI (i.e. DPI-1, three times per cycle for two cycles) when compared to CIS-IV (i.e. IV-1.5, one time per cycle for a total of three administrations).

NGAL, cystatin C and creatinine showed no major increase following the administration of DPI-V, DPI-0.5 and DPI-1, whether this was done after the 2-week treatment or after the 1-week recovery ($p > 0.05$, Fig. 7). All these biomarkers tended to increase following the administration of IV-2 (Fig. 7). This increase was significantly different for cystatin C ($p < 0.01$) and creatinine ($p < 0.001$) and was maintained at significant levels during the recovery week for cystatin C. The 25%-fold reduction of the IV dose (i.e. IV-1.5) showed lower cystatin C and creatinine levels. IV-1.5 showed a slightly but non-significant increase in NGAL and cystatin C ($p > 0.05$, Fig. 7A and 7B) that was also maintained after 1 week of recovery. Plasma NGAL showed no significant difference when compared to the control groups. Therefore, IV_1.5 seemed to be better tolerated by the kidneys than IV-2 and seemed to be preferable to combine with CIS-DPI at 0.5 mg/kg.

3.3.4. *Combination evaluation*

Levet et al. showed that the plasma C_{max} was reached immediately (~5 min) after the administration of CIS using the IV route at 1.25 mg/kg and was seven-fold lower 24 h later. This result was correlated to the platinum concentration in the kidneys, which was five-fold lower 24 h following the administration of CIS-IV (Levet et al., 2017a). Therefore, it was considered that 24 h were enough to consider CIS as being majorly eliminated. Consequently, mice received DPI-0.5 either the same day

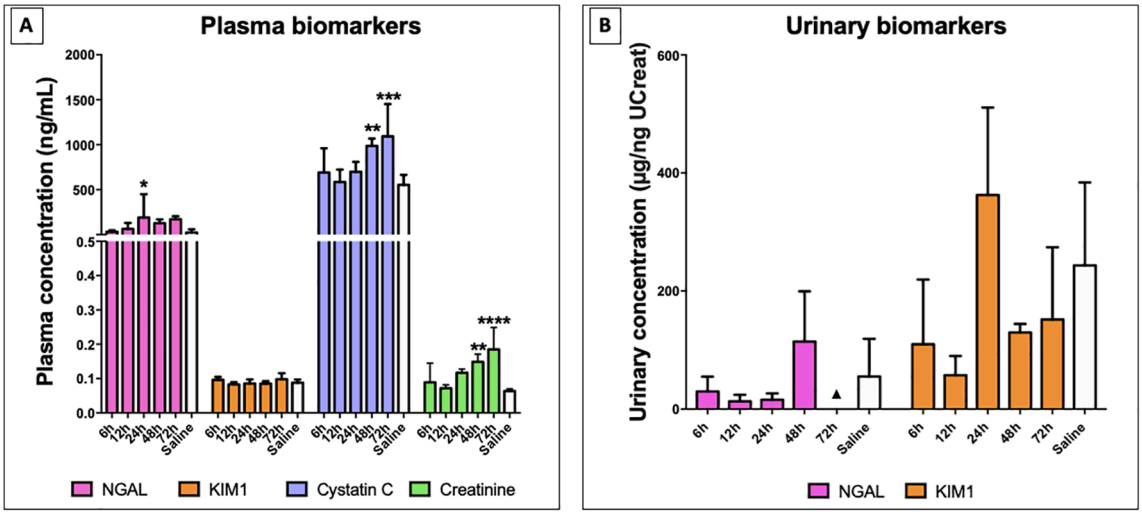


Fig. 6. Evaluation of plasma (A) and urinary (B) NGAL, KIM-1, cystatin c and creatinine 6 h, 12 h, 24, 48 and 72 h following the induction of an acute kidney injury model. All the results are expressed as means ± SEM (N = 5–6). The statistical analyses were performed *vs* the control group using one-way ANOVA and Bonferroni's post-test (*** for $p < 0.001$,** for $p < 0.01$ and * for $p < 0.05$).

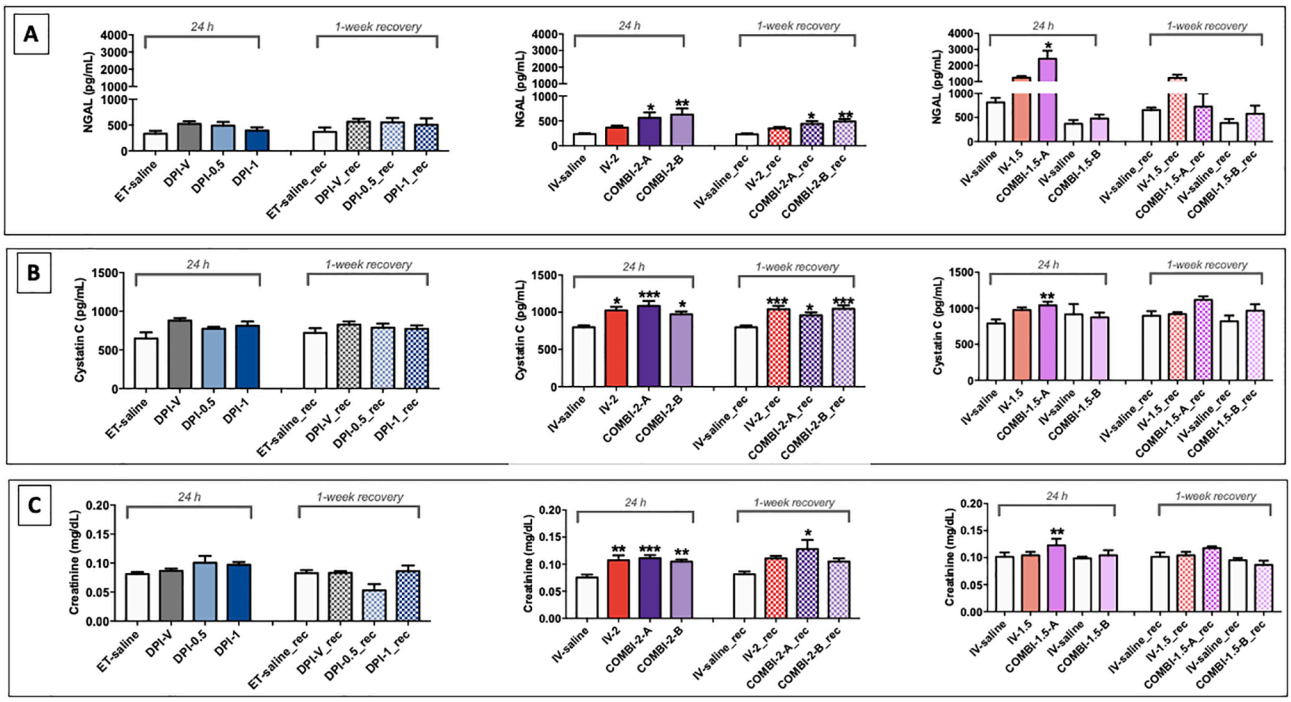


Fig. 7. Evaluation of plasma NGAL (A), cystatin c (B) and creatinine (C) 24 h after the treatment administration and 1-week later recovery (rec). All the results are expressed as means ± SEM (n = 4–17). The statistical analyses were performed *vs* the corresponding negative control group using one-way ANOVA and Bonferroni's post-test (*** for $p < 0.001$,** for $p < 0.01$ and * for $p < 0.05$).

(within 1 h) or 24 h following CIS-IV (IV-2 or IV-1.5) administration.

Administering DPI-0.5 and IV-2 (COMBI-2) increased significantly all the biomarkers when administered the same day (COMBI-2-A; NGAL: $p < 0.05$, cystatin C: $p < 0.001$, creatinine: $p < 0.001$, Fig. 7) or 24 h later (COMBI-2-B ; NGAL: $p < 0.01$, cystatin C: $p < 0.05$, creatinine: $p < 0.01$, Fig. 7). These increases were not reversible even after 1 week of recovery. DPI-0.5 was administered when IV-2 had shown signs of AKI and its addition seemed to increase AKI.

Administering DPI-0.5 and IV-1.5 the same day (COMBI-1.5-A) increased significantly all the biomarkers (NGAL: $p < 0.05$, cystatin C and creatinine: $p < 0.01$, Fig. 7). This increase seemed reversible within 1 week (COMBI-1.5-A_rec). When DPI-0.5 was delivered 24 h after IV-1.5 (COMBI-1.5-B and rec), no significant difference was observed between all the biomarker levels and their negative controls, whether immediately after the treatment or after the 1-week recovery ($p > 0.05$, Fig. 7). In contrast to observations from the combination of the IV and ET respective MTDs (COMBI-2), the administration of DPI-0.5 the same day as IV-1.5 increased the C_{max}, resulting in reversible AKI. Therefore, decreasing the IV dose by 25% in the combination (COMBI-1.5) is preferred as the generated AKI seemed reversible after 1-week of recovery. Moreover, delaying the DPI-0.5 administration to CIS-IV (COMBI-1.5-B) by 24 h was preferred to prevent any AKI complication.

AKI biomarkers are divided into two categories, whether they report on kidney function, tubule function or damage (Charlton et al., 2014). Creatinine and cystatin C are considered as functional biomarkers of AKI whereas NGAL is an upregulated protein that occurs during AKI (Charlton et al., 2014). Cystatin C is a small protein that is freely filtered by the glomerulus, and is almost completely reabsorbed and degraded in the proximal tubule (Charlton et al., 2014; Vaidya et al., 2009). Its increase in plasma reveals a filtration defect (Charlton et al., 2014) as its filtration is performed inefficiently by the kidneys, as a consequence of AKI and so it accumulates in plasma. NGAL, as its name indicates, is involved in neutrophil maturation and renal tubular damage after its release by activated PMN, epithelial cells and kidney tubular cells following cell damage or inflammation (Ning et al., 2018). Nevertheless, during PMN activation, its dimeric form prevails whereas in tubular cells the monomeric and heterodimeric form are produced (Charlton et al., 2014; Kaucsár et al., 2016). Many clinical studies have revealed that NGAL remains very low in biological fluids in the steady-state levels and is up-regulated in the case of AKI at an early stage (Charlton et al., 2014; Vaidya et al., 2009; Wasung et al., 2015). In any case, urinary and plasma NGAL has showed similar sensitivity in many studies, which indicates NGAL specificity to kidneys (Kaucsár et al., 2016).

Nevertheless, as PMN were retrieved in BALF, it was mandatory to verify whether NGAL increases in plasma were attributed to pulmonary PMN increase or to AKI. However, this investigation was a verification because following IV-2 and IV-1.5 administration, no significant PMN increase in BALF was observed but NGAL in plasma was higher when compared to the negative groups.

To investigate NGAL increases, NGAL was quantified in BALF. It showed no significant increase between all the groups (Fig. S3, supplementary material), except for ET_LPS ($p < 0.001$). However, NGAL in BALF increased slightly and dose-dependently for ET groups following PMN increase. Moreover, NGAL also increased in some combination groups when compared to their respective IV monotherapies (Fig. S3, supplementary material). The differences observed in terms of NGAL levels between COMBI-2 and IV-2 and between COMBI-1.5-A and IV-1.5 are attributed to CIS-DPI administration. Indeed, as PMN also increased for the same combination groups, NGAL was therefore released in the lungs (Fig. 3.A and Fig. S3, supplementary material).

Nevertheless, NGAL levels were>40-fold higher in plasma than in BALF. Even if this necessitates further investigation, NGAL in BALF seemed to be correlated to PMN recruitment and may represent an interesting novel marker to detect pulmonary inflammation.

To complete the investigation of the specificity of NGAL, a qualitative immunohistochemistry study was assessed to identify NGAL in the kidneys (Fig. 8, Fig. S4). The negative control groups (ET-saline and IV-saline) did not show any labelling, showing no NGAL in these sections. Following the administration of the IV monotherapies (IV-2 and IV-1.5), NGAL was retrieved in the renal cortex and more precisely in the proximal tubules, as described in the literature (Charlton et al., 2014; Kaucsár et al., 2016; Luo et al., 2014). Brown staining was only detected

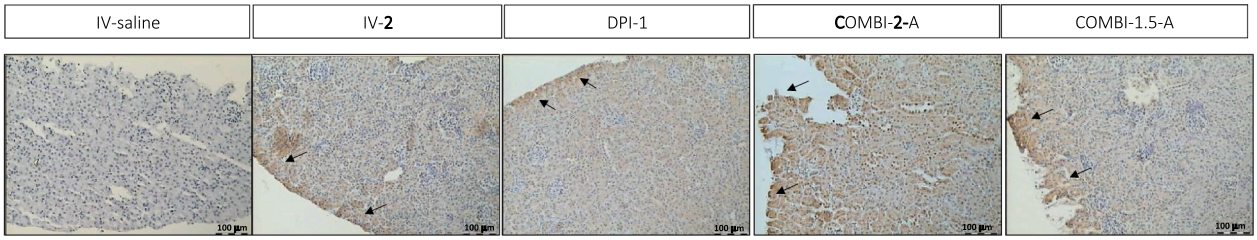


Fig. 8. Representative results of NGAL immunostaining (magnitude × 100) from mice exposed to the most drastic conditions in terms of CIS nephrotoxicity: IV-2, DPI-1, COMBI-2-A, COMBI-1.5-A and the corresponding negative control group IV-saline.

in the area of tubule proximal area, and not in the glomeruli (Fig. 8).

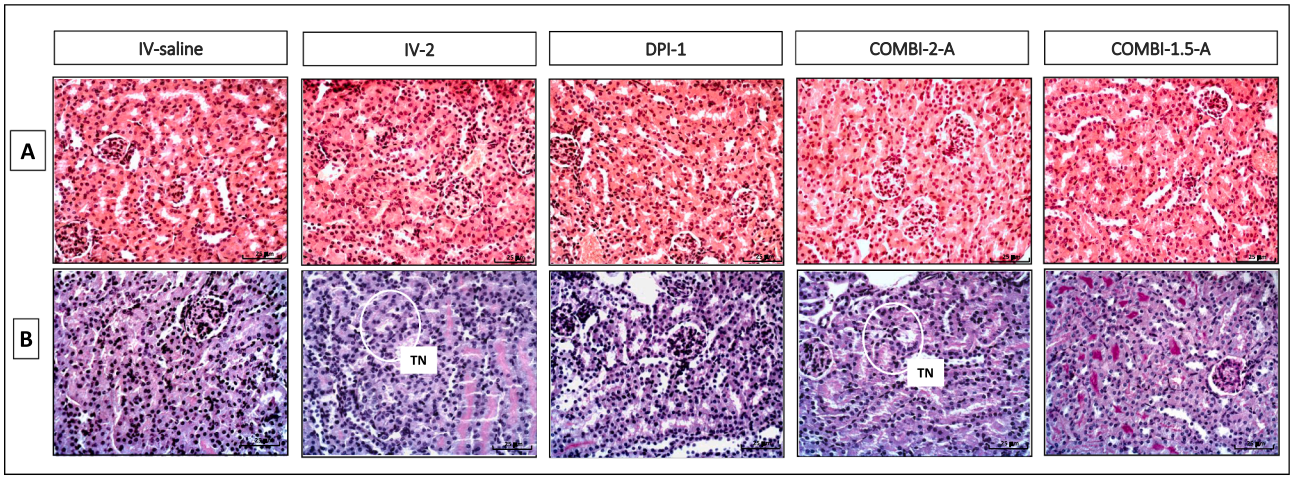
All combination groups showed that NGAL was concentrated in proximal tubules and seemed to be more intense for the groups that were exposed to CIS-DPI and CIS-IV on the same day (COMBI-2-A and COMBI-1.5-A). These results supported what was observed for NGAL quantification in plasma (Fig. 7). Consequently, plasma NGAL was a good biomarker of AKI, as widely described in the literature (Charlton et al., 2014; Luo et al., 2014; Ning et al., 2018; Ostermann et al., 2020; Togashi et al., 2012).

Moreover, it is important to mention that the selected biomarkers tended to describe the same trends for all the groups, as no major difference between NGAL, cystatin C and creatinine was noticed. This increased the reliability of our mouse model in terms of selected biomarkers and adequate timing for sampling. Plasma NGAL and cystatin C seemed to be therefore as reliable as plasma creatinine could be. Nevertheless, the novel biomarkers seem to be more sensitive to primitive injuries, as demonstrated for IV-2_rec and COMBI-2-B_rec.

In these groups (IV-2, COMBI-2-A, COMBI-2-B, COMBI-1.5-A), NGAL, cystatin C and creatinine levels significantly increased, suggesting a severe AKI complication compared to the other groups (ET groups, IV-1.5). This result could be related to high concentration of CIS and its metabolites (monoaquacisplatin: $[Pt(NH_3)_2Cl(OH_2)]^+$, and diaquacisplatin: $[Pt(NH_3)_2(OH_2)_2]^{2+}$) in the proximal tubular epithelium. They are able to interact with glutathione leading to the formation of a complex that can be metabolized by specific enzymes resulting in the formation of a reactive thiol, reported to be a more potent nephrotoxin (Karasawa and Steyger, 2015; Manohar and Leung, 2018) These metabolites were able to induce several chemokines and cytokines to promote: (i) inflammation driven by leukocytes leading to NGAL increase; and/or (ii) alteration of specific cytoplasmic structures (Bunel et al., 2017; Karasawa and Steyger, 2015; Manohar and Leung, 2018). As an overall consequence of these observations, a dramatic filtration decrease, reflected by an increased plasma cystatin C level, confirmed the occurrence of AKI (Bunel et al., 2017).

3.3.5. *Histopathological analyses*

Following the analyses of HE and PAS staining kidney sections, no major kidney injury was observed following the administration of the ET and IV monotherapies or the combination groups (Fig. 9, Figs. S5 and S6, supplementary material). A light tubular necrosis and lymphocytic infiltration scored 1 for IV-2. Moreover, the administration of CIS-DPI and CIS-IV at their MTD (COMBI-2) seemed to induce a transient tubular necrosis and lymphocytic infiltration, that were greater for COMBI-2-A than COMBI-2-B. These observations seemed to identify slight and transient tubular necrosis and inflammation for the highly dosed IV monotherapy as well as its combination with CIS-DPI. However, the tubular necrosis damage that was identified was described as “a rare single necrotic tubule”, and the lymphocytic infiltration as “a few



C Observations

Observations	Control	ET Monotherapies				IV Monotherapies				Combinations							
	IV-saline	DPI-0.5	DPI-0.5_rec	DPI-1	DPI-1_rec	IV-1.5	IV-1.5_rec	IV-2	IV-2_rec	COMBI-2-A	COMBI-2-A_rec	COMBI-2-B	COMBI-2-B_rec	COMBI-1.5-A	COMBI-1.5-A_rec	COMBI-1.5-B	COMBI-1.5-B_rec
Tubular atrophy	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tubular necrosis (TN)	0	0	0	0	0	0	0	**0.3**	0	**1**	0	**0.4**	0	0	0	0	0
Lymphocytic infiltration	0	0	0	0	0	0	0	**0.3**	0	**0.7**	0	**0.4**	0	0	0	0	0
Interstitial fibrosis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Fig. 9. Representative renal tissue histological analyses (HE (A), PAS (B), magnitude x400) from mice exposed to the most drastic conditions in terms of CIS nephrotoxicity: IV-2, DPI-1, COMBI-2-A, COMBI-1.5-A and the corresponding negative control group IV-saline and summary of semi-quantitative score of tubulo-interstitial lesions (C).

scattered cells" and did not seem significant. As already explained above for pulmonary histology, biomarker quantification seems to be more sensitive in detecting injury than histology can be.

In conclusion, DPI-V, DPI-0.5 and DPI-1 showed a good pulmonary and renal tolerance as they did not increase significantly any local or systemic biomarker. Nevertheless, DPI-0.5 and DPI-1 increased the PMN proportion transiently, showing that a local inflammation was being initiated. However, DPI-0.5 remains the MTD to use in combination as the bw does not decrease below 5%. IV-1.5 and IV-2 were less well-tolerated than DPI as they increased dose-dependently both pulmonary and systemic biomarkers. Adding DPI-0.5 to IV-2 decreased the pulmonary tolerance (i.e. cytokine levels, PMN proportion and cytotoxicity) and increased AKI induced by IV-2 alone. Therefore, this combination cannot be used for further efficacy study. When the total IV dose was decreased to 25% in the combination therapy, pulmonary tolerance remained acceptable as no additional pulmonary damage was observed when compared to IV-1.5, except for a transient PMN increase, as observed following DPI-0.5. However, the kidneys were still damaged under this regimen. Staggering the DPI administration to IV administration by 24 h seemed to be enough to prevent renal damage. Therefore, COMBI-1.5-B seems to be the best regimen to be evaluated during efficacy studies.

4. Conclusion

Administering a CIS-based controlled-release DPI formulation following a 2-week regimen was overall well-tolerated by the lungs except for a transitory PMN increase. None of the renal biomarkers increased significantly. Administering CIS using IV at its MTD increased both the pulmonary pro-inflammatory cytokines and the renal biomarkers, resulting in both renal and pulmonary damage. Therefore, flattening the pulmonary and plasmatic peaks by developing a controlled-release DPI to deliver cisplatin enhanced both pulmonary and renal tolerance compared to IV.

Regimens were optimized to find the highest and most frequent drug dosage while maintaining pulmonary and renal tolerance. The pulmonary and renal tolerance were impaired as soon as CIS-DPI and CIS-IV were administered at their respective MTDs. Fractionating the total dose between IV and DPI (i.e. decreasing the IV dose by 25%) was a good strategy to improve lung tolerance and to hinder any AKI complication. Staggering the DPI administration from IV administration by 24 h was enough to prevent any signs of AKI. This regimen was therefore selected to assess efficacy in different mouse lung carcinoma models. Future studies should evaluate the efficacy of the selected regimen in murine lung carcinoma models.

CRediT authorship contribution statement

S. Chraibi: Conceptualization, Methodology, Validation, Investigation, Writing - original draft, Project administration. **R. Rosière:** Conceptualization, Methodology, Validation, Investigation, Writing - review & editing, Supervision, Project administration. **E. De Prez:** Investigation, Resources. **M.H. Antoine:** Investigation, Resources, Writing - review & editing. **M. Remmelink:** Methodology, Investigation, Resources, Writing - review & editing. **I. Langer:** Methodology, Resources, Writing - review & editing. **J. Nortier:** Methodology, Resources, Writing - review & editing. **K. Amighi:** Conceptualization, Methodology, Resources, Writing - review & editing, Supervision. **N. Wauthoz:** Conceptualization, Methodology, Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: 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 material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpharm.2021.120425.

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