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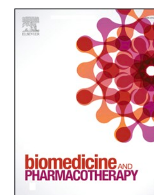

Preclinical tolerance evaluation of the addition of a cisplatin-based dry powder for inhalation to the conventional carboplatin-paclitaxel doublet for treatment of non-small cell lung cancer

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ABSTRACT

Despite the advances in targeted therapies and immunotherapy for non-small cell lung cancer (NSCLC) patients, the intravenous administration of carboplatin (CARB) and paclitaxel (PTX) in well-spaced cycles is widely indicated for the treatment of NSCLC from stage II to stage IV. Our strategy was to add a controlled-release cisplatin-based dry-powder for inhalation (CIS-DPI-ET) to the conventional CARB-PTX-IV doublet, administered during the treatment off-cycles to intensify the therapeutic response while avoiding the impairment of pulmonary, renal and haematological tolerance of these combinations. The co-administration of CIS-DPI-ET (0.5 mg/kg) and CARB-PTX-IV (17–10 mg/kg) the same day showed a higher proportion of neutrophils in BALF (35 ± 7% *vs* 1.3 ± 0.8%), with earlier regenerative anaemia than with CARB-PTX-IV alone. A first strategy of CARB-PTX-IV dose reduction by 25% also induced neutrophil recruitment, but in a lower proportion than with the first combination (20 ± 6% *vs* 0.3 ± 0.3%) and avoiding regenerative anaemia. A second strategy of delaying CIS-DPI-ET and CARB-PTX-IV administrations by 24 h avoided both the recruitment of neutrophils in BALF and regenerative anaemia. Moreover, all these groups showed higher cytotoxicity (LDH activity, protein content) with no higher renal toxicities. These two strategies seem interesting to be assessed in terms of antitumor efficacy in mice.

1. Introduction

The improvement in terms of screening as well as the development of new personalized medicines (targeted therapies, immunotherapy), along with a reduction in incidence (USA, 2013–2016), has led to a reduced death rate related to lung cancer [1,2]. Worldwide, lung cancer remains a major public health problem. It is still the most frequent and deadliest among all cancers, for both sexes combined, with 11.6% of total cases and 18.4% of total cancer deaths in 2018 [3]. Therefore there is still an urgent need to increase treatment effectiveness by developing new treatment strategies and drug combinations or by repurposing current therapies.

Conventional platinum-based chemotherapy combining a platinum compound (*i.e.* cisplatin (CIS) or carboplatin (CARB)) to another antineoplastic agent such as paclitaxel (PTX) or pemetrexed (recommended for non-squamous histology) is still widely recommended for the

Abbreviations: AKI, Acute Kidney Injury; AM, Alveolar macrophages; AUC, Area under the curve; BALF, Bronchoalveolar lavage fluid; BCA, Bicinchoninic acid; Bw, Body weight; CARB, Carboplatin; CIS, Cisplatin; DPI, Dry powder for inhalation; EOS, Eosinophils; ET, Endotracheal; FMA, Formaldehyde; NT-GRA, Granulocytes-neutrophils; GRA, Granulocytes; Hb, Haemoglobin; HE, Haematoxylin-Eosin; Ht, haematocrit; IV, Intravenous; KIM-1, Kidney Injury Molecule 1; LDH, Lactate Dehydrogenase; LPS, Lipopolysaccharide; LYM, Lymphocytes; MCH, Mean cell haemoglobin; MCHC, Mean corpuscular haemoglobin concentration; MCV, Mean corpuscular volume; MON, Monocytes; MPV, Mean platelet volume; MTD, Maximum Tolerated Dose; NGAL, Neutrophil-Gelatinase Associated Lipocalin; NSCLC, Non-Small-Cell Lung Cancer; PAS, Periodic Acid-Schiff; PBS, Phosphate Buffer Saline; PLT, Platelets; PTX, Paclitaxel; RBC, Red blood cells; RDW, Red cells diameter width; TPGS, Tocopherol poly(ethylene glycol) 1000 succinate; WBC, White blood cells.

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treatment of non-small cell lung cancer (NSCLC, 85% of total lung cancer cases) [4]. Among all these platinum-based chemotherapies, the combination of CARB-PTX is widely used. It is recommended as a neo-adjuvant and adjuvant therapy to surgery (for patients with comorbidities or not able to tolerate cisplatin due to its dose-depended nephrotoxicity) and to radiotherapy in early stages. CARB-PTX is a first-line therapy for advanced or metastatic disease, with no genomic alterations (EGFR, ALK, ROS1, BRAF), and is used in combination with immunotherapy (in the case of programmed death ligand-1 (PDL-1), tumour proportion score < 50%) [4].

CARB and PTX co-administration seems to be synergistic as they both induce cell apoptosis [5]. This is mainly due to their different mechanisms of action: CARB mainly acts as a non-cell cycle specific-agent and crosslinks DNA, whereas PTX binds to the subunits of microtubules specifically leading to cell-cycle blockade at the G2/M phase [6]. As cytotoxic drugs target highly proliferative cells, including healthy and cancerous cells, this synergistic effect also results in dose-limiting myelosuppression (*i.e.* mainly leucopoenia and thrombocytopenia), leading to severe outcomes such as infections and bleeding disorders [7–10]. Therefore it is recommended to monitor regularly the haematological parameters (white blood cell and platelet counts) and to initiate the next treatment cycle only when these parameters are normalized. Moreover, myelosuppression, and more specifically anaemia, can also occur indirectly following renal damage, leading to a decreased production of erythropoietin by the kidneys [11,12]. Among the various types of anaemias, acquired haemolytic anaemias are often associated with cytotoxic drugs such as CARB [13–15]. Following red blood-cell destruction, the reticulocyte count increases, which indicates a regenerative anaemia [13,15,16].

To minimize these side effects, the doses and regimen have had to be optimized to find the best balance between clinical efficacy and tolerance [17]. Therefore the CARB dose is calculated using the Calvert formula. This is because myelosuppression seems to be more precisely forecast using the patient's renal function, age and area under the curve (AUC) than body surface area [18]. Consequently CARB (AUC 6 mg/mL.min) and PTX (200 mg/m^2) are recommended to be administered over 5 h of infusion in spaced-out cycles (the first day of each cycle of 21 days for 4 cycles) [4].

During the intervals between treatment cycles, all the body tissues recover as they are no longer exposed to any cytotoxic drug. This leads to tumour repopulation and to treatment failure [19]. On the other hand, recent studies have shown that the combination of different drugs seems to be an interesting strategy for cancer therapy [20]. In this case, toxicity will therefore be the limiting factor and must be managed as a priority [20].

The administration of a sustained-release CIS-based dry powder for inhalation directly into the lungs during the off-cycles is a promising strategy to intensify the treatment effectiveness. This is because the frequency of administration as well as the exposure of the tumour and its locoregional environment to CIS will be increased [21,22]. This is even more important as pulmonary-tumour platinum concentration is correlated with a reduction in the tumour size and with an improvement in terms of survival and recurrence of the disease [23].

In our previous study, a cisplatin-based DPI (CIS-DPI) was formulated to deliver CIS sustainably and to expose the lungs (and therefore the tumour) to cytotoxic drug for several hours [24]. As described in our previous preclinical work, the main limitation related to the combination of both these routes of administration (endotracheal (ET) and intravenous (IV)) was lung and renal tolerance [25]. The results showed that the administration of the CIS-DPI three times a week for 2 consecutive weeks at its maximum tolerated dose (MTD) repeatedly as a monotherapy did not induce any pulmonary or renal toxicity. In contrast, the combination of CIS-DPI and CIS-IV administered once a week for 3 consecutive weeks at their respective MTD, with the first doses administered at the same day, induced non-reversible pulmonary and renal toxicities even after one week of recovery. It was mandatory to decrease the CIS-IV MTD by 25% and stagger ET from IV administrations by 24 h to avoid additional pulmonary and renal injury [25]. However, even considering these adaptations (decreased dose and delay), the selected regimen demonstrated a therapeutic intensification in the M109 lung carcinoma orthotopic model in mice. However, the regimen could have decreased the full therapeutic potential of this strategy of combination [24].

As it is widely known that CARB has a better toxicological profile than CIS [10], it was therefore interesting to evaluate the possibility to combine CIS-DPI (ET route) with CARB-PTX (IV route), the same day at their MTD. However, clinical studies have demonstrated increased leucopoenia and thrombocytopenia when CIS and CARB were administered together using the IV route [26,27]. To the best of our knowledge, the combination of CIS using the ET route and CARB using the IV route has not yet been reported.

Therefore the aim of this study was to (i) evaluate pulmonary, renal and haematological tolerance of the combination of CIS-DPI and CARB-PTX and (ii) to optimize their combinations in terms of doses and regimen.

2. Materials and methods

2.1. Materials

Cisplatin and carboplatin were purchased from Umicore (Pilar, Argentina) and paclitaxel from Carbosynth (Berkshire, UK). Hydrogenated castor oil (Kolliwax®, HCO) and polyoxyl 35 castor oil (Cremophor®, EL) were supplied by BASF (Ludwigshafen, Germany). D-α-Tocopherol poly(ethylene glycol) 1000 succinate (TPGS) was purchased from Biomadis (Paris, France) and 4% formaldehyde solution (FMA) for histology was purchased from Sigma-Aldrich (St. Louis, USA). Phosphate buffer saline (PBS) was provided by Life Technologies (Merelbeke, Belgium) and isopropanol and ethanol absolute (≥ 99.8%) and L-leucine by Merck Millipore (Darmstadt, Germany). UltraPure Milli-Q water was purified using a Pure-Lab Ultra® purification system (Elgan, Lane End, UK). All solvents and chemicals used were of analytical grade.

2.2. In vivo toxicity studies

All experiments and manipulations 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 faculty of medicine (ULB) under approval number 585 N. Female 6- to 8-week old BALB/cAnNRj mice (16–22 g) (Janvier Labs, France) were given dry food and water *ad libitum* and were maintained in conventional housing conditions (12 h/12 h night and day cycles, 22 ± 2 °C, 55 ± 10% RH). Mice were weighed three times per week and were euthanized if one of the endpoints described in the protocol was reached. During the MTD evaluation, mice were weighed up to five times per week.

2.3. Formulations and administration for in vivo experiments

2.3.1. CIS-DPI blend

The CIS-DPI was produced as described by Chraibi et al. [24]. Blends with an appropriate diluent for *in vivo* administration were prepared to deliver ~1.0 mg of powder to mice, as described previously [25]. For this purpose, CIS-DPI was weighed and diluted at 1% (w/w) in the spray-dried mannitol/leucine 10:1 diluent. The uniformity of CIS content in the blend was determined as recommended by the European Pharmacopeia, v.10. It was satisfactory, with a coefficient of variation below 5% (*i.e.* 1.12 ± 0.03% (CV% 3%), n = 10).

The *in vivo* administration of the CIS-DPI blend by the endotracheal route (CIS-DPI-ET) consisted of the anaesthesia of mice using 3.5% isoflurane at 0.8–1.0 L/min for 5 min. After this, mice were positioned

on an angled board to deliver CIS-DPI blend directly into the mice trachea using a DP4-M dry powder insufflator (Penn-Century, Wyndmoor, PA, USA).

2.3.2. CARB-PTX solution

CARB solution was prepared at 5 mg/mL in saline (0.9% NaCl) and diluted using the same vehicle at the appropriate concentration. This solution was kept protected from light, at 4 °C, for maximum 3 days. PTX solution was prepared at 6 mg/mL using a mixture of Cremophor El® and absolute ethanol (50:50 v/v) and diluted in saline at the appropriate concentration. CARB-PTX solution was prepared immediately before use and renewed each day. The vehicle-IV group received the same proportion of each excipient as in the MTD dose, which corresponded to 83.3% (v/v) saline, 8.3% (v/v) Cremophor EL® and 8.3% (v/v) ethanol absolute. The *in vivo* administration of CARB-PTX solution or vehicle was made by the IV route (tail vein).

2.4. Regimen administration

2.4.1. CARB/PTX ratio

To be closest to clinical practice, doses were based on the ratio of CARB and PTX human doses recommended for lung cancer patients with no-comorbidities (PTX: 200 mg/m^2, CARB: AUC 6 mg/mL x min) [4]. For this purpose, the CARB dose was calculated using the Calvert formula, which involves different factors such as the patient sex, age, serum creatinine and weight [28]. In this study, it was interesting to determine this dose for a normal patient with no co-morbidities as the study was conducted on healthy mice. Thus this corresponded to the maximum CARB dose that can be administered.

The patient's age was fixed at 71 years old as it is the median age for lung cancer patients [29]. The serum creatinine levels were considered in the normal ranges, 0.72–1.18 for males and 0.55–1.02 for females, as described by Ceriotti et al. [30]. The corresponding doses were calculated based on a body weight of 60 kg. The CARB doses were expressed as mg/kg and their mean was calculated (9 mg/kg). The PTX dose expressed in mg/m^2 (200 mg/m^2) was converted to mg/kg as described by Reagan et al. [31], and was fixed at 5.4 mg/kg. The related CARB/PTX ratio was therefore calculated (9.1/5.4) and set at about 1.7. This ratio was maintained between the CARB and PTX doses during the MTD investigation study on mice.

2.4.2. Maximum tolerated dose determination

The MTD of CARB-PTX IV was defined in this study as the highest dose at which mean body weight (bw) loss did not exceed 5% w/w for any of the 3 tested animals for the whole of the applied regimen and for 28 days following the first dosing. This limit of 5% is a good threshold as *(i)* this treatment is intended to be combined to another treatment (*i.e.* CIS-DPI-ET) and as, *(ii)* a bw decrease of less than 10% w/w was observed for combinations (*i.e.* CIS-DPI-ET and CIS IV) in which each monotherapy dose was selected with a threshold of 5% w/w [25]. Six groups of three female 6-week old BALB/cAnNRj mice were administered CARB-PTX solution using the IV route the first day of each week for 3 consecutive weeks (days 1, 8 and 15) at 8-5 mg/kg (dose 1), 17-10 mg/kg (dose 2), 25-15 mg/kg (dose 3), 34-20 mg/kg (dose 4), 42-25 mg/kg (dose 5) and 51-30 mg/kg (dose 6) for CARB and PTX respectively. (Fig. S1, supplementary data). These groups were compared to the negative control group (vehicle-IV).

2.4.3. Administration of IV doublets, CIS-DPI and control groups

CARB-PTX was administered as an IV doublet at its MTD (i.e. CARB-PTX-IV; 1.7 mg/mL for CARB and 1.0 mg/mL for PTX) and at 75% of the MTD dose (i.e. CARB-PTX- 0.75: 1.3 mg/mL for carboplatin and 0.75 mg/mL for paclitaxel) to healthy female 6-week old BALB/cAnNRj mice and was compared to the vehicle (*i.e.* vehicle-IV). CARB-PTX-IV (n = 16), CARB-PTX-0.75 (n = 16) and vehicle-IV (n = 24) were administered by IV following the regimen described for MTD determination (day 1, 8 and 15) (Fig. 1A).

CIS-DPI-ET at its MTD (*i.e.* 0.5 mg/kg [25]) (n = 30) was administered using the ET route three times per week for 2 consecutive weeks (days 1, 3, 5, 8, 10, 12) (Fig. 1A). The positive control group was administered with ~1 µg of lipopolysaccharide (LPS) from E.coli in the mice trachea (n = 21) using a MicroSprayer® Aerosolizer model IA-1C (1.5” tip) with an FMJ-250 high-pressure syringe (Penn-Century, Wyndmoor, PA, USA), as described previously [25]. The LPS-ET group was administered with LPS 18 h before each sampling, followed the kinetics of the inflammatory biomarkers (peak between 12 and 24 h following the LPS administration [32]). Samples from both the positive (LPS-ET) and the negative control (vehicle-IV) groups were collected each sampling day (n = 3). Samples from each type of control group were then pooled together and annotated with their group name, if sampled 24 h after the last administration, or annotated with the suffix '_rec' to their group name if sampled after one week of recovery, to evaluate myelotoxicity and pulmonary and renal toxicities.

2.4.4. Combinations administration

Once CARB-PTX-IV, CARB-PTX-0.75 and CIS-DPI-ET were evaluated separately, they were combined following three different regimens (Fig. 1B). This was done to evaluate the input of a reduced IV dose or delayed days of administration, in case of cumulative toxicities. The first regimen (COMBI-1) (n = 28) combined CARB-PTX-IV (*i.e.* administered on days 1, 8, 15) and CIS-DPI-ET administered three times per a weeek, starting at day 1 (*i.e.* days 1, 3, 5, 8, 10, 12). The second regimen (COMBI-1–24 h) (n = 29) combined CARB-PTX-IV (days 1, 8, 15) and CIS-DPI-ET, administered 24 h later than in the first regimen (*i.e.* on days 2, 4, 6, 9, 11, 13). The third regimen (COMBI-0.75) (n = 26) combined the administration of CARB-PTX- 0.75 (*i.e.* on days 1, 8, 15) and CIS-DPI-ET, three times a week starting the same day (*i.e.* days 1, 3, 5, 8, 10, 12). In COMBI-1 and COMBI-0.75, ET administrations on day 1 and day 8 were performed maximum one-hour after the IV administrations on day 1 and 8. The myelotoxicity and pulmonary and renal toxicities were assessed 24 h after the last administration (group name annotation) and after one week of recovery (group name with '_rec' suffix annotation).

2.5. Pulmonary tolerance evaluation

To evaluate the pulmonary tolerance, both inflammation and cytotoxicity were investigated. To do so, bronchoalveolar lavage fluid (BALF) as well as lung tissue were collected [25]. Briefly, following blood sampling, BALF was sampled by means of 3 × 0.7 mL of PBS at 4 °C through a 20-gauge canula (Surflo® catheter, Terumo, Leuven, Belgium) inserted in the trachea. BALF samples were kept on ice during the sampling sessions and then vortexed. The total cell count was measured using an automated cell counter (Countess II FL, Life Technologies, Merelbeke, Belgium). Supernatants were extracted following a centrifugation step (160 g, 4 °C, for 5 min), and immediately aliquoted and stored at − 80 °C. The packed cells were re-suspended in 200 µL of cold PBS and then centrifuged using a cytospin to set them on slides. To investigate the differential cell count, alveolar macrophages (AM), neutrophil granulocytes (NT-GRA), lymphocytes (LYM) and eosinophils (EOS) were counted manually on a total of 200 cells to calculate the proportion of each cell type after slide staining using May-Grünwald Giemsa stain.

To evaluate pulmonary inflammation, pro-inflammatory cytokines (TNF- α, IL-6 and IL-1-ß) and chemokines (CXCL1 and CXCL2) were quantified following an ELISA method as explained by the manufacturer (Duoset and Quantikine, RnD Systems, Abingdon, UK).

To evaluate cytotoxicity, the total protein content was quantified using a Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Fischer Scientific, Zellik, Belgium). The lactate dehydrogenase (LDH) activity was also investigated using a Cayman Chemical LDH Cytotoxicity Assay Kit (Ann Arbor, MI, USA) following the protocol specified by

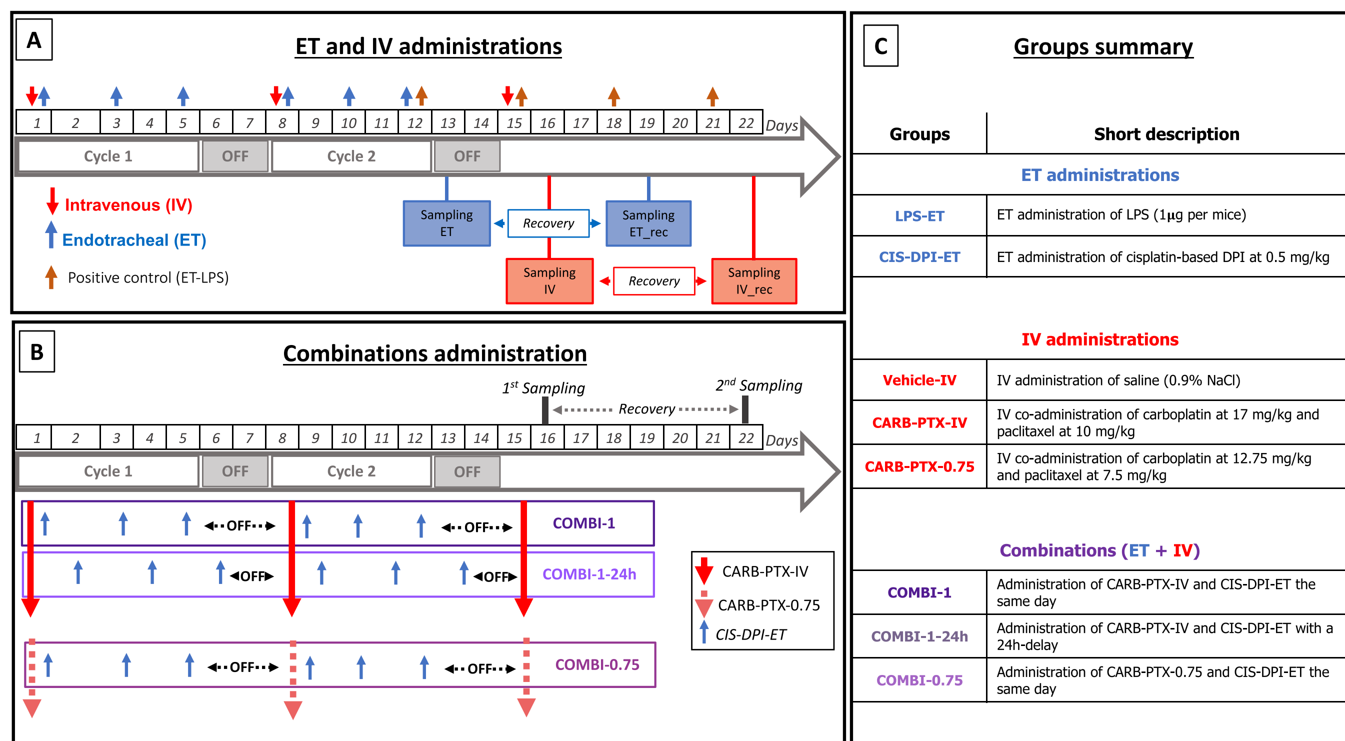


Fig. 1. Scheme of administration of ET and IV doublets treatments (A) and their combinations (B) and sampling procedures 24 h after the treatment administration and one week later (_rec) for all groups (C)

the manufacturer. Results were expressed as LDH/LDH $_{\text{Vehicle-IV}}$. All analyses were assessed twice, and each group was compared to the related positive (LPS-ET) and negative control (vehicle-IV) groups.

To evaluate lung histopathology, lungs were harvested following BALF collection and were immediately put in FMA for 24 h. Lungs were washed in water for 15 min and put in a PBS solution until paraffin embedding and haematoxylin-eosin (HE) staining of two impaired sections per lung. Lung histopathology analysis was performed by an independent pathologist following the description given by Jones et al. [33]. The severity of each observation (bronchiolar epithelial vacuolation, acute bronchopneumonia, perivascular oedema, luminal AM, intra-alveolar fibrin and intra-alveolar haemorrhage) was scored 0–5 and the mean score was calculated as the average of each group. The frequency was also assessed as the number of animals for whom the observation was detected.

2.6. Renal tolerance evaluation

Following the injection of an intraperitoneal lethal dose of sodium pentobarbital at 12 mg/kg, blood was directly collected in lithium-heparin tubes (Sarstedt, Cologne, Germany) and was centrifuged (2000 g, 20 °C, for 10 min) to collect plasma that was aliquoted and stored at − 80 °C for further analysis. The kidneys were collected and put in FMA for 24 h, rinsed in water for 15 min and preserved in isopropanol until their embedding in paraffin. The slides were prepared and stained using HE and periodic acid-Schiff (PAS). Renal histopathology was investigated following the protocol of Debelle et al. [34]. Tubular atrophy, tubular necrosis, inflammatory infiltrates and interstitial fibrosis were scored 0–5, and their mean score was calculated.

To evaluate acute kidney injury (AKI), three plasma biomarkers, neutrophil gelatinase-associated lipocalin (NGAL), cystatin-c and creatinine were selected, as described previously [25]. NGAL and cystatin-c were quantified following an ELISA protocol as specified by the manufacturer (Duoset and Quantikine, RnD Systems, Abingdon, UK). Plasma creatinine was evaluated using a high performance liquid chromatography (HPLC) method, as previously described by Debelle et al. [34].

2.7. Myelosuppression evaluation

Three to four drops of blood were collected by retro-orbital puncture in Minicollect® K3EDTA tubes (Greiner Bio-One, Vilvoorde, Belgium). Tubes were immediately gently inverted manually before being put in an automated rotary mixer to gently invert the tubes for 15 min. They were immediately placed at 4 °C until analysis, which was performed a maximum of 4 h following the sampling.

To evaluate myelotoxicity, the total count of red blood cells (RBC) and white blood cells (WBC), including basophils and NT-GRA, eosinophils (EOS), lymphocytes (LYM) and monocytes (MON), was evaluated, as well as each WBC type proportion among all WBC. Haemoglobin (Hb), haematocrit (Ht), mean corpuscular volume (MCV), mean cell haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), and red cell distribution width (RDW) were also determined. The total count of platelets (PLT) as well as the mean platelet volume (MPV) were investigated. All these parameters were determined using a haemocytometer (Scil Vet abc Plus+®, Altorf, France) [35].

2.8. Statistical analyses

All statistical tests were assessed using GraphPad PRISM® (7.0a) software. One-way ANOVA and the Bonferroni post-hoc test were selected to compare myelotoxicity parameters (total and differential WBC count, Ht, Hb, MCV, MCH, MCHC, RDW, PLT and MPV), the toxicity biomarkers (NGAL, creatinine, cystatin-c, IL-6, TNF- α, IL-1β, CXCL1, CXL2, protein content, and LDH ratio) and the differential BALF cell counts (PMN, AM and LYM) *vs* their respective control groups. Moreover, to evaluate the effect of the addition of CIS-DPI-ET to CARB-PTX-IV, COMBI-1 and COMBI-1–24 h were compared to CARB-PTX-IV and COMBI-0.75 was compared to CARB-PTX-0.75. To evaluate the staggering effect on the different parameters, COMBI-1 and COMBI-

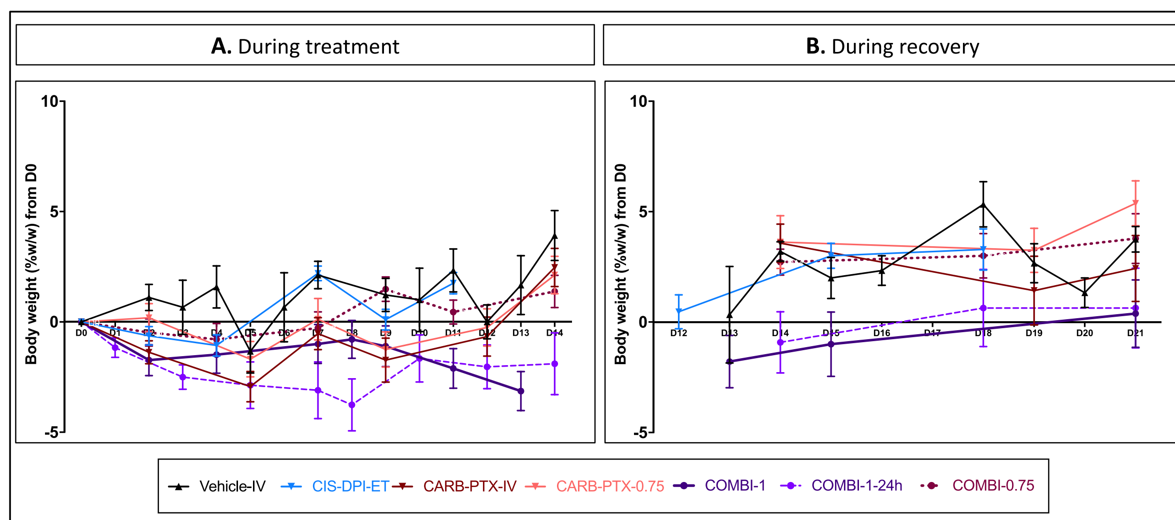


Fig. 2. Body weight profiles following carboplatin-paclitaxel administration using the IV route (IV doublets) at different doses, cisplatin-based dry powder formulation using the ET route (CIS-DPI-ET) and their combinations *vs* the vehicle-IV group. All results are expressed as means ± SEM (n = 8–15).

1–24 were also compared. 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 $p < 0.0001$ (****).

3. Results

3.1. Determination of MTD for CIS-DPI and CARB-PTX-IV

Pulmonary and renal toxicities as well as myelotoxicity (dose-limiting toxicity of CARB-PTX) were investigated to select the safest but also least-spaced and most highly dosed regimen that can be applied to treat lung cancer. To do so, it was mandatory to identify the MTD, as defined previously, for both routes, following the ET and IV regimens as described in Fig. 1A. CIS-DPI MTD was fixed at 0.5 mg/kg, as reported in our previous work [25]. Regarding the results (Fig. S1, supplementary data), CARB-PTX-IV MTD was assessed to be 17 and 10 mg/kg for CARB and PTX respectively using the CARB/PTX ratio of 1.7, as explained in Section 2.3.2. In fact, dose 3 (CARB/PTX 25–15 mg/kg) showed a limited bw loss during the treatment administrations. However, bw started decreasing on day 21 until reaching − 10.3 ± 0.5% on day 26, leading to mice euthanasia. The lower dose (dose 2: CARB/PTX 17–10 mg/kg) did not show any major bw loss during the treatment administration and bw increased during the 28 days follow-up. The lower dose was therefore chosen as the MTD.

3.2. Tolerance of CIS-DPI-ET, CARB-PTX-IV and their combinations

Once the MTD was determined, CIS-DPI-ET and CARB-PTX-IV were administered starting the same day (COMBI-1) at their respective MTD. However, as the co-administration of CIS-DPI-ET and CARB-PTX-IV may induce cumulative toxicities, two strategies were developed. The first was staggering CARB-PTX-IV administration from CIS-DPI-ET administration by 24 h (COMBI-1–24 h); the second was the reduction of the IV dose by 25% as is commonly carried out in clinical practice to limit the risk of cumulative toxicity (COMBI-0.75) [10]. To evaluate the pulmonary and renal tolerance due to the administration of CIS-DPI, it was mandatory to investigate histopathological damage (on lung and renal tissues) as well as specific biomarker levels following CIS-DPI-ET, CARB-PTX-IV and their combinations [25,36]. Moreover, myelotoxicity was assessed to evaluate the dose-limiting toxicity (DLT) of CARB-PTX by counting WBC, RBC and PLT, as well as their related parameters.

3.3. Bw profiles and general evaluation

The bw profiles of all the groups, illustrated in Fig. 2A, fluctuated during the treatment period but remained above − 5%. However, even if their evaluation is crucial during preclinical studies, the reason of bw fluctuations was difficult to identify. Indeed it can be attributed to both a limited physiological growth due to the stress generated by the techniques of administration (*i.e.* limited bw increases in vehicle-IV group), and to the toxicity of the treatments (*i.e.* higher bw losses for the treated groups than vehicle-IV group), that can counteract each other.

CARB-PTX-IV and CARB-PTX-0.75 bw profiles were similar and the bw losses decreased proportionally to the dose (Fig. 2A). The highest bw losses for these groups were observed on day 5 at − 4 ± 1% and − 2 ± 1%, respectively. Moreover, CIS-DPI-ET did not induce any major bw losses, with the highest bw loss observed on day 4 at − 1.0 ± 0.5% as already described [25]. Combining CIS-DPI-ET and CARB-PTX-IV the same day (COMBI-1) or 24 h later (COMBI-1–24 h) was similarly well-tolerated by the mice. Their highest bw losses were observed on day 13 at − 3 ± 1% and on day 8 at − 4 ± 1%, respectively. However, decreasing the dose by 25%, as done by combining CIS-DPI-ET and CARB-PTX-0.75 the same day (COMBI-0.75), seemed to be better tolerated than COMBI-1 and COMBI-1–24 h. The highest bw loss for this group was observed on day 8 at − 1 ± 1%.

During the one-week recovery period, all bw increased and were positive at the end as mice were no longer exposed to any cytotoxic drug. This showed that bw losses were reversible.

3.4. Evaluation of pulmonary toxicity

Pulmonary inflammation and cytotoxicity were evaluated following the ET administrations of CIS (CIS-DPI-ET), IV doublets and their combinations. As previously described, mouse groups were sampled 24 h following the last administration and after one week of recovery. This was done to evaluate toxicity in its early acute phase and its possible reversibility within one week, as described in our previous preclinical study [25].

3.4.1. Inflammation

3.4.1.1. Total BALF cells

3.4.1.1.1. IV doublets and CIS-DPI-ET. The repeated administration of CIS-DPI-ET following the selected regimen tended to increase total BALF cells, although this was non-significant in comparison with the

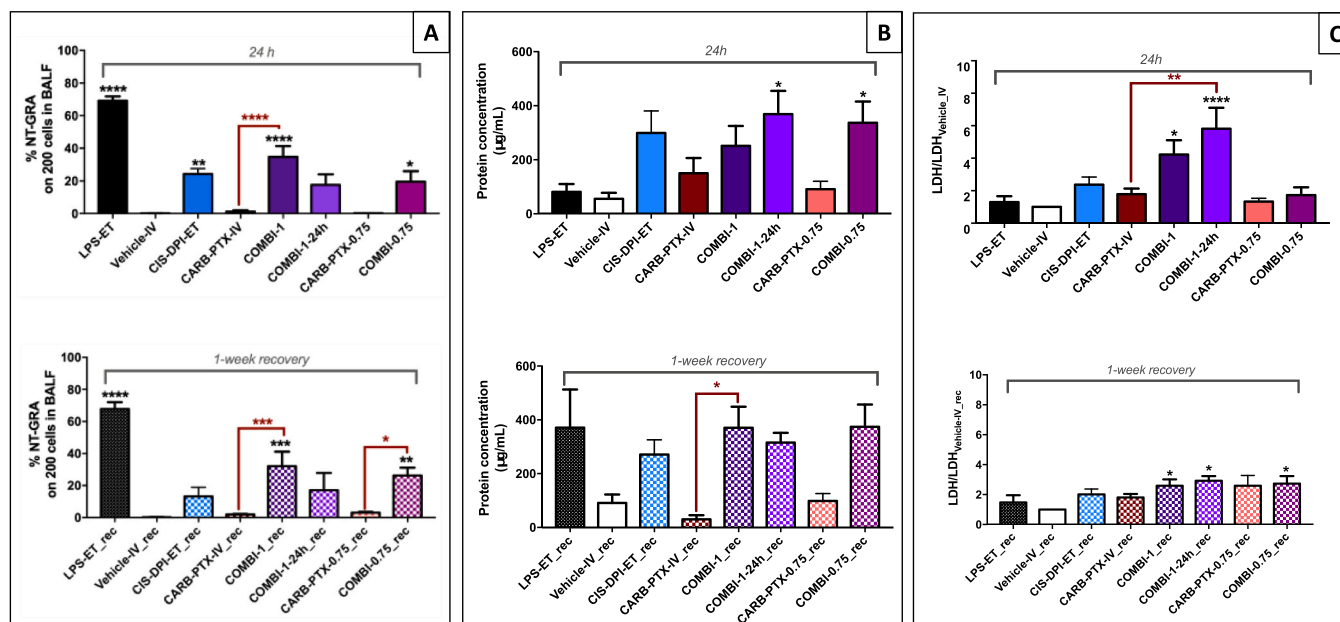


Fig. 3. Evaluation of neutrophil granulocytes (NT-GRA) as a percentage of 200 BALF cells (A), total protein concentration (B), and the ratio of LDH activity reported on the LDH activity of the vehicle-IV or vehicle-IV_rec (C) in BALF 24 h following the last treatment administration and after one week of recovery (_rec). All results are expressed as means ± SEM (n = 8–15). Statistical analyses were performed *vs* the vehicle-IV group (black) or *vs* the selected groups as illustrated (red), using one-way ANOVA and Bonferroni's post-hoc test (**** for $p < 0.0001$, *** for $p < 0.001$, ** for $p < 0.01$ and * for $p < 0.05$).

vehicle-IV group at both sampling times ($p > 0.05$, Fig. S2A, supplementary data), as previously observed [25]. However, this increase was fully reversible after one week of recovery ($p > 0.05$, Fig. S2B, supplementary data). In contrast, the administration of CARB-PTX-IV at both doses did not induce any BALF cell recruitment at either sampling time ($p > 0.05$, Fig. S2, supplementary data).

3.4.1.1.2. Combinations. To evaluate the effect of the addition of CIS-DPI-ET to the IV chemotherapy, the combination groups were compared first to the vehicle-IV group and then to their respective IV doublets.

The co-administration of CIS-DPI-ET and CARB-PTX-IV, irrespective of the IV-dose and the scheme of administration, activated BALF-cell recruitment in comparison with the vehicle-IV control group 24 h after the last treatment administration (COMBI-1: $1.9 \pm 0.7 \times 10^7$ cells/mL, COMBI-1–24 h: $2.1 \pm 0.5 \times 10^6$ cells/mL and COMBI-0.75: $1.3 \pm 0.6 \times 10^7$ cells/mL, *vs* $7 \pm 3 \times 10^5$ cells/mL, Fig. S2A, supplementary data) and were all reversible after one week of recovery ($p > 0.05$, Fig. S2B, supplementary data). This increase was only statistically higher for COMBI-1 in comparison with the vehicle-IV control group and in comparison with CARB-PTX-IV ($p < 0.05$ and $p < 0.01$, respectively, Fig. S2A, supplementary data). Moreover, this acute increase was not significant for COMBI-1–24 h and COMBI-0.75 in comparison with their respective IV doublets ($p > 0.05$, Fig. S2A, supplementary data). Therefore, the administration of CIS-DPI-ET and CARB-PTX-IV at their MTD on the same day induced higher inflammatory-cell recruitment to trigger inflammation when compared to CARB-PTX-IV alone. However, this reaction seemed to be lessened when CIS-DPI-ET administration was staggered from CARB-PTX-IV administration by 24 h, or when the CARB-PTX-IV dose was reduced by 25% (CARB-PTX-0.75).

3.4.1.2. Proportion of cells in BALF

3.4.1.2.1. IV doublets and CIS-DPI-ET. Among the total BALF cell increase, the proportions of AM, NT-GRA and LYM were assessed. All LPS-ET positive control groups induced a significant increase in NT-GRA compared to the vehicle-IV group ($p < 0.0001$, LPS-ET: 69 ± 2%, ET-LPS_rec: 68 ± 4% *vs* vehicle-IV: 0.1 ± 0.1%, vehicle-IV_rec: 0.2 ± 0.2%, Fig. 3A), as previously observed [25].

The BALF cell count increase observed for CIS-DPI-ET (Fig. S2, supplementary data) was related to higher NT-GRA in comparison with the vehicle-IV group ($p < 0.01$, 24 ± 3% *vs* 0.1 ± 0.1%, Fig. 3A) and was reversible within one week of recovery. However, the administration of IV doublets at both doses did not increase NT-GRA proportions at either sampling time (Fig. 3A).

3.4.1.2.2. Combinations. The combination of IV doublets and CIS-DPI-ET increased all NT-GRA proportions (COMBI-1: 35 ± 7%, COMBI-1–24 h: 18 ± 7%, COMBI-0.75: 20 ± 6% *vs* vehicle-IV: 0.1 ± 0.1%, Fig. 3A). This increase was only significant for the groups that were administered CIS-DPI-ET and CARB-PTX-IV the same day (COMBI-1 and COMBI-0.75), in comparison with the vehicle-IV group ($p < 0.0001$ and $p < 0.05$, Fig. 3A), and was not reversible after one week of recovery ($p < 0.001$ and $p < 0.01$, Fig. 3A).

COMBI-1 increased significantly the NT-GRA proportion when compared to CARB-PTX-IV-MTD ($p < 0.0001$, Fig. 3A), which was not reversible after one week of recovery ($p < 0.001$, Fig. 3A). Moreover, COMBI-1–24 h showed a lower increase in NT-GRA than COMBI-1 ($p < 0.05$, Fig. 3A).

COMBI-1 and COMBI-0.75 showed that the pulmonary inflammation initiated 24 h following the treatment administration was not reversible within one week. This demonstrated a more prolonged inflammation for these groups in comparison to COMBI-1–24 h.

3.4.1.3. Inflammation biomarkers in BALF

3.4.1.3.1. CXCL1 and CXCL2. The mouse chemokines (CXCL1, CXCL2) involved in NT-GRA recruitment, were quantified in BALF supernatant (Table 1). The LPS-ET positive control groups demonstrated significantly higher concentrations of both chemokines, when compared with the vehicle-IV group ($p < 0.0001$, Table 1). For IV doublets and CIS-DPI-ET, no major difference was observed when compared with the vehicle-IV group 24 h after the last treatment administration ($p > 0.05$, Table 1) or after one week of recovery ($p > 0.05$, Table). Similarly, for the combinations, no significant difference was observed between them and the vehicle-IV group or and their corresponding IV doublets, at both sampling times ($p > 0.05$, Table 1).

Table 1
Evaluation of chemokines (CXCL1 and CXCL2), pro-inflammatory cytokines (TNF-**α**, IL-6, and IL1-**β**) in BALF and biomarkers of acute kidney injury (cystatin-c and creatinine) in plasma 24 h following the last treatment administration and after one week of recovery (_rec). All the results are expressed as means ± SEM (n = 8–15). The statistical analyses were performed *vs* the vehicle-IV group using one-way ANOVA and Bonferroni's post-hoc test (**** for $p < 0.0001$).

Biomarkers	Chemokines in BALF		Pro-inflammatory cytokines in BALF			Acute kidney injury biomarkers in plasma	
	CXCL1 (pg/mL)	CXCL2 (pg/mL)	TNF- α (pg/mL)	IL-6 (pg/mL)	IL-1-β (pg/mL)	Cystatin-c (pg/mL)	Creatinine (pg/mL)
			24 h after treatment administration				
LPS-ET	330 ± 71****	49 ± 15****	150 ± 31****	54 ± 10****	87 ± 10****	–	–
Vehicle-IV	30 ± 6	2.6 ± 0.6	57 ± 11	11 ± 2	18 ± 3	943 ± 37	0.13 ± 0.01
CIS-DPI-ET	33 ± 6	5.9 ± 0.4	33 ± 4	16 ± 10	9 ± 3	989 ± 34	0.133 ± 0.006
CARB-PTX-IV	38 ± 13	1.8 ± 0.2	60 ± 12	12 ± 5	13 ± 4	974 ± 24	0.118 ± 0.006
COMBI-1	44 ± 6	3.1 ± 0.5	45 ± 8	9 ± 2	13 ± 4	949 ± 38	0.132 ± 0.008
COMBI-1–24 h	38 ± 8	2.5 ± 0.5	71 ± 29	11 ± 5	23 ± 8	961 ± 35	0.137 ± 0.008
CARB-PTX-0.75	22 ± 3	1.2 ± 0.5	47 ± 6	21 ± 7	8 ± 2	1020 ± 34	0.125 ± 0.003
COMBI-0.75	50 ± 5	1.2 ± 0.2	47 ± 6	8 ± 3	22 ± 5	911 ± 41	0.112 ± 0.006
			One week of recovery after the treatment administration				
LPS-ET_rec	405 ± 98****	37 ± 9****	261 ± 153****	70 ± 16****	58 ± 11****	–	–
Vehicle-IV_rec	42 ± 7	6.1 ± 0.7	51 ± 11	11 ± 3	20 ± 4	953 ± 31	0.119 ± 0.006
CIS-DPI-ET_rec	48 ± 8	8 ± 2	30 ± 5	15 ± 3	15 ± 5	1039 ± 37	0.131 ± 0.006
CARB-PTX-IV_rec	67 ± 33	6.1 ± 0.5	46 ± 12	9 ± 4	9 ± 1	884 ± 43	0.12 ± 0.01
COMBI-1_rec	49 ± 7	7 ± 1	16 ± 4	3 ± 1	18 ± 6	907 ± 39	0.126 ± 0.005
COMBI-1–24h_rec	67 ± 21	6.7 ± 0.9	35 ± 7	3 ± 1	12 ± 2	923 ± 31	0.124 ± 0.005
CARB-PTX-0.75_rec	22 ± 2	4.6 ± 0.2	26 ± 2	7 ± 4	7 ± 3	1013 ± 39	0.135 ± 0.007
COMBI-0.75_rec	53 ± 12	9 ± 2	26 ± 5	5 ± 2	21 ± 6	1005 ± 33	0.14 ± 0.01

3.4.1.3.2. TNF-α, IL-6 and IL-1-β. TNF-α, IL-6 and IL-1-β were quantified to evaluated pro-inflammatory responses following the treatment administration. As expected, all positive control groups (LPS-ET) were significantly higher than the vehicle-IV groups ($p < 0.0001$, Table 1). Following the administration of CIS-DPI-ET, IV doublets and their combinations, at all doses and regimens, no significant increase was observed at either sampling time. Moreover, TNF-α, IL-6 and IL-1-β levels were all lower one week later (Table 1).

3.4.2. Cytotoxicity

3.4.2.1. Protein content. Following the administration of the IV doublets or CIS-DPI-ET, protein concentration levels in BALF increased, dose-dependently. However, these were not significant in comparison with the vehicle-IV group 24 h after the last treatment administration ($p > 0.05$, CIS-DPI-ET: 300 ± 81 µg/mL, CARB-PTX-IV: 151 ± 56 µg/mL, CARB-PTX-0.75: 91 ± 29 µg/mL *vs* 56 ± 23 µg/mL, Fig. 3B), which remained high but still not significant after one week of recovery ($p > 0.05$, Fig. 3B). Although non-significant, CIS-DPI-ET protein concentration was more than 5-fold higher than in the vehicle-IV group and 2- and 3-fold higher than the CARB-PTX-IV and CARB-PTX-0.75 groups, respectively, 24 h after the last treatment administration.

The combination of both CIS-DPI-ET and CARB-PTX-IV irrespective of the dose or regimen showed higher protein concentrations than the vehicle-IV group (COMBI-1: 252 ± 74 µg/mL, COMBI-1–24: 369 ± 86 µg/mL, COMBI-0.75: 337 ± 78 µg/mL *vs* 56 ± 23 µg/mL, Fig. 3B). However, this was only significant for COMBI-1–24 h and COMBI-0.75 at 24 h after the last treatment administration ($p < 0.05$, Fig. 3B) but not after one week of recovery ($p > 0.05$, Fig. 3B).

None of the combination groups increased significantly the total protein concentration in comparison with their respective IV doublets, 24 h after the treatment. However, after one week of recovery, only the COMBI-1 presented a total protein concentration significantly higher than the CARB-PTX-IV. This was due to the addition of CIS-DPI-ET the same day ($p < 0.05$, Fig. 3B) as its co-administration with CARB-PTX-IV would have led to a cumulative cytotoxicity from both CARB-PTX-IV and CIS-DPI-ET leading to potentially higher protein content for this group.

3.4.2.2. LDH activity. As observed for the protein concentration evaluation, the LDH activity was higher following the administration of IV doublets and CIS-DPI-ET than for the vehicle-IV group but without significant difference ($p > 0.05$, Fig. 3C), at both sampling times. However, all combination groups increased significantly the LDH ratios 24 h after the last treatment administration (COMBI-1: 4.2 ± 0.9, COMBI-1–24 h: 6 ± 1, COMBI-0.75: 1.8 ± 0.5, *vs* vehicle-IV group: 1, Fig. 3C) except for COMBI-0.75 ($p > 0.05$). These groups were all significantly higher after one week of recovery ($p < 0.05$, Fig. 3C) when compared to the vehicle-IV group. Moreover, a significant increase in LDH activity was shown for COMBI-1–24 h when compared to CARB-PTX-IV 24 h after the last treatment administration ($p < 0.01$, Fig. 3C). However, this increase was not maintained after one week of recovery ($p > 0.05$, Fig. 3C).

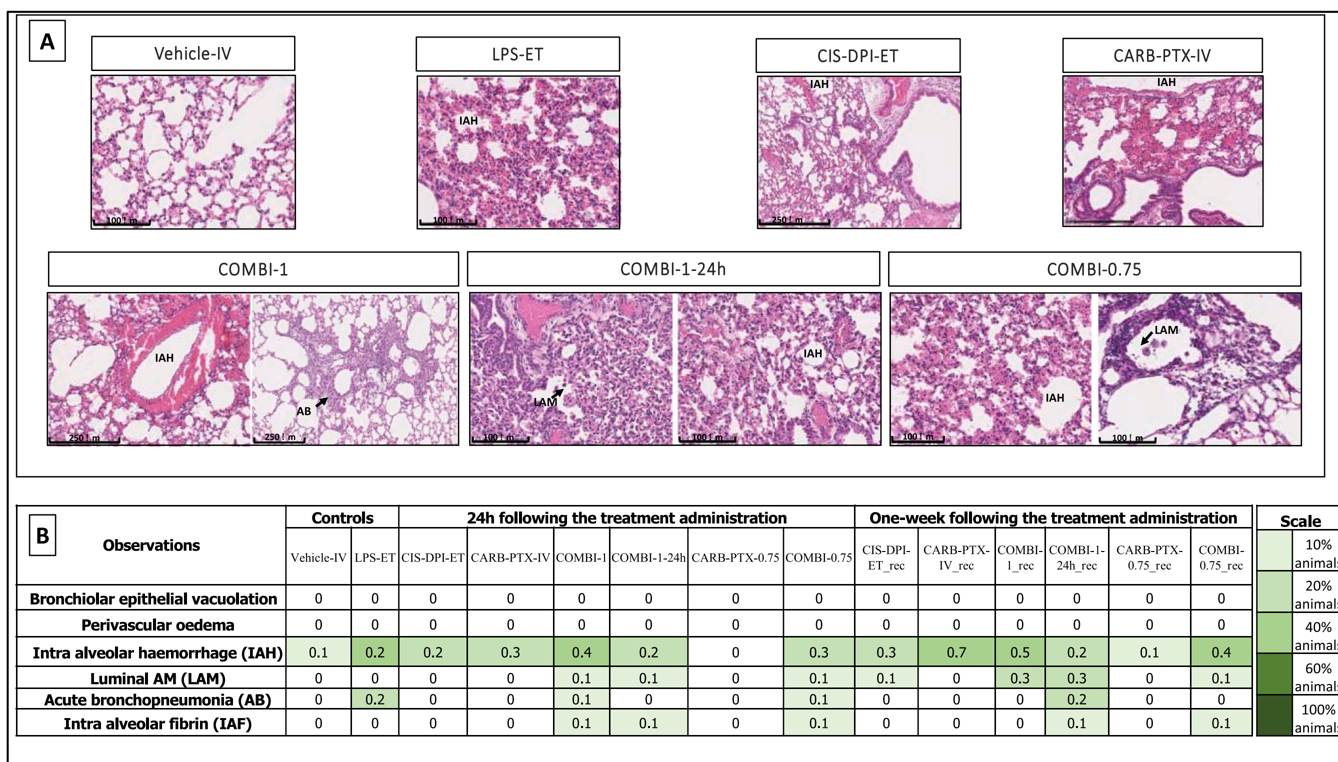
3.4.3. Histopathological analysis

As previously observed during the evaluation of the pro-inflammatory cytokines and chemokines as well as during the cytotoxicity evaluation (Table 1), no major lung damage was observed for IV doublets and CIS-DPI-ET administered alone or in combinations. The scores of all the adverse observations were less than 1 on a severity score scale of 5 and occurred in a maximum of 45% of the mice (Fig. 4B).

3.4.3.1. Vacuolation and perivascular oedema. None of the groups displayed bronchiolar epithelial vacuolation or perivascular oedema.

3.4.3.2. Intra-alveolar haemorrhage. All the groups except CARB-PTX-0.75, displayed intra-alveolar haemorrhage. This was certainly related to the technique of sampling as blood may have not been completely removed before the paraffin-embedding. This could be also attributed to the BALF collection procedure. However, intra-alveolar haemorrhage seemed to be more intensely and frequently encountered for some groups than for the vehicle-IV (Fig. 4B).

3.4.3.3. Luminal alveolar macrophages. In terms of luminal AM, which can be a sign of ongoing acute inflammation, CIS-DPI-ET and IV doublet groups showed a score of 0/5 24 h after the last administration (Fig. 4). They also showed this after one week of recovery except for CIS-DPI-ET_rec, which had a score of 0.1/5 (Fig. 4B). However, once results for each combination were combined, a score of 0.1/5 was observed for all combinations for 7% of mice (COMBI-1, COMBI-1–24 h and COMBI-1–0.75) 24 h after the last treatment administration (Fig. 4B). That increased to 0.3/5 for COMBI-1_rec and COMBI-1–24h_rec for 27% and 23% of mice, respectively. However, for COMBI-0.75, the initial score of 0.1/5 was maintained after one week of recovery for 7% of mice



B Observations	Controls		24h following the treatment administration						One-week following the treatment administration					
	Vehicle-IV	LPS-ET	CIS-DPI-ET	CARB-PTX-IV	COMBI-1	COMBI-1-24h	CARB-PTX-0.75	COMBI-0.75	CIS-DPI-ET_rec	CARB-PTX-IV_rec	COMBI-1_rec	COMBI-1-24h_rec	CARB-PTX-0.75_rec	COMBI-0.75_rec
Bronchiolar epithelial vacuolation	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Perivascular oedema	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Intra alveolar haemorrhage (IAH)	0.1	0.2	0.2	0.3	0.4	0.2	0	0.3	0.3	0.7	0.5	0.2	0.1	0.4
Luminal AM (LAM)	0	0	0	0	0.1	0.1	0	0.1	0.1	0	0.3	0.3	0	0.1
Acute bronchopneumonia (AB)	0	0.2	0	0	0.1	0	0	0.1	0	0	0	0.2	0	0
Intra alveolar fibrin (IAF)	0	0	0	0	0.1	0.1	0	0.1	0	0	0	0.1	0	0.1

Fig. 4. Histopathology of lung tissue exposed to IV doublets, ET monotherapy and their combinations. Representative images of treated groups (A) and heat map of adverse observations (in scale of concerned proportion of mice) and severity scores (from 0–5) depending on lung tissue histopathology (B) (Optical microscopy, x400).

(Fig. 4B).

3.4.3.4. Acute bronchopneumonia. Acute bronchopneumonia was scored at 0/5 for CIS-DPI-ET and IV doublets at both doses and sampling times. This was also scored at 0.1/5 for COMBI-1, 0/5 for COMBI-1–24 h and 0.1/5 for COMBI-0.75 24 h after the last treatment administration (Fig. 4B). This was observed for 13% of mice, for both groups and was already reported where there was CIS nebulization [37]. However, this observation was reversible within one week for the lower- dosed regimen of both groups (COMBI-1 and COMBI-0.75) and was not reversible for the highest dosed group (0.2/5 for COMBI-1–24 h) (Fig. 4B).

3.4.3.5. Intra-alveolar fibrin. Intra-alveolar fibrin was scored at 0/5 for CIS-DPI-ET IV doublets at both sampling times (Fig. 4B). For the combination groups (COMBI-1, COMBI-1–24 h and COMBI-0.75) this showed a low score of 0.1/5 and was observed for only for 7% of the mice (Fig. 4B) and was maintained after one week of recovery except for COMBI-1 (Fig. 4B).

3.5. Evaluation of nephrotoxicity

The DLT of CIS is cumulative nephrotoxicity. It is therefore important that CIS-DPI-ET does not induce nephrotoxicity as this treatment is designed to be administered during off-cycles as a complement to IV doublets [24]. Moreover, it is important to verify that there is no accumulation of toxicity on the kidneys using the combinations as CARB can also impact their function [38].

3.5.1. Plasma biomarkers

3.5.1.1. NGAL

3.5.1.1.1. IV doublets and CIS-DPI-ET. The repeated administration of CIS-DPI-ET did not induce any major significant NGAL difference in comparison with the vehicle-IV group ($p > 0.05$), both 24 h after the last administration (107 ± 32 pg/mL *vs* 300 ± 35 pg/mL, Fig. 5A) and after one week of recovery (Fig. 5A). IV doublets showed a dose-dependent but non-significant increase in NGAL when compared to the vehicle-IV group 24 h after the last treatment administration (CARB-PTX-IV: 602 ± 108 pg/mL, CARB-PTX-0.75: 539 ± 43 pg/mL *vs* 300 ± 35 pg/mL, Fig. 5A). The NGAL concentrations decreased for all groups and were all non-significant after one week of recovery ($p > 0.05$, Fig. 5A).

3.5.1.1.2. Combinations. The co-administration of CIS-DPI-ET and CARB-PTX-IV, at both doses and regimens, increased NGAL concentrations to higher than with the vehicle group-IV (COMBI-1: 939 ± 287 pg/mL, COMBI-1–24 h: 575 ± 45 pg/mL, COMBI-0.75: 715 ± 72 pg/mL *vs* 300 ± 35 pg/mL, Fig. 5A). The increase was significant only for COMBI-1 ($p < 0.01$, Fig. 5A), 24 h after the last treatment administration. These increases were all reversible after one week of recovery (Fig. 5A). This result was expected, as COMBI-1 was the most highly dosed regimen in which CIS-DPI-ET and IV doublets administrations were less spaced. In comparison with their relative IV groups, all the combinations showed a non-significant increase in NGAL concentration at both sampling times (Fig. 5A).

3.5.1.2. Cystatin-c and creatinine. The repeated administration of CIS-DPI-ET, IV doublets and their combinations did not induce higher cystatin-c or creatinine levels when compared to the vehicle-IV group ($p > 0.05$, Table 1), at either sampling time (Table 1).

3.5.2. Histopathological analysis

Following the analysis of PAS and HE staining, no AKI was noticed for all groups of all doses and regimens (Fig. 5B) at both sampling times. These results confirmed observations during the plasma biomarker evaluation.

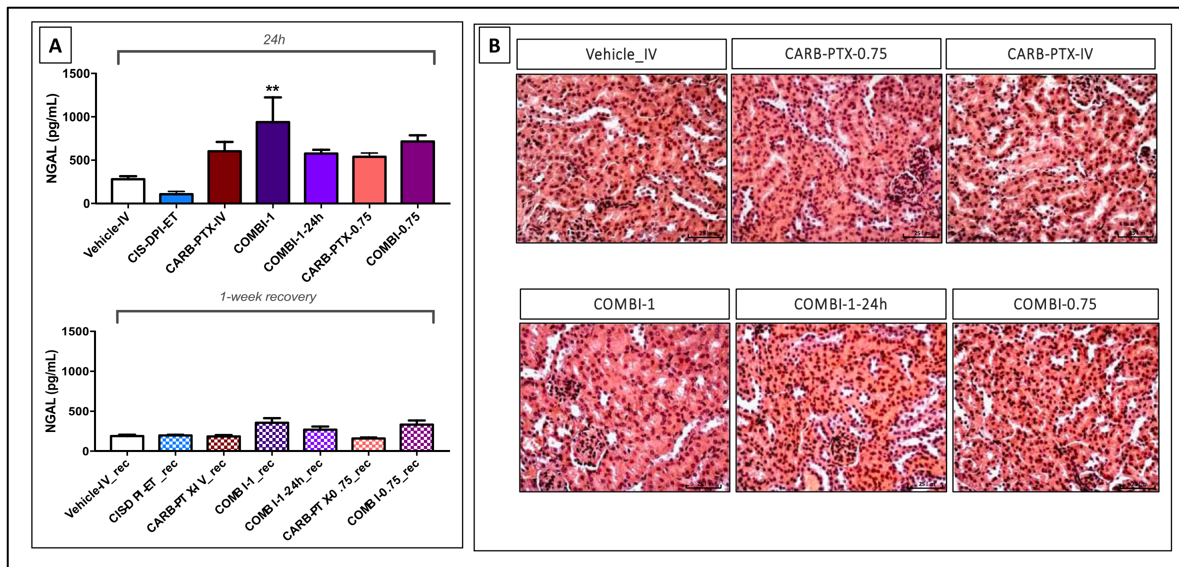


Fig. 5. Evaluation of plasma NGAL (A) 24 h following the last treatment administration and after one week of recovery (_rec). All results are expressed as means ± SEM (n = 8–15). The statistical analyses were performed *vs* the vehicle-IV group (black) or *vs* the selected groups as illustrated (red), using one-way ANOVA and Bonferroni's post-hoc test (** for $p < 0.01$). Representative renal tissue histological analyses (HE, magnification x400) in selected mice groups (B).

3.6. Evaluation of myelotoxicity

Myelosuppression is an adverse effect often responsible for the interruption of the treatment by 3 weeks, which it is the time necessary to renew bone-marrow stem cells [19]. Therefore, it is crucial that the CIS-DPI does not induce cumulative myelosuppression if it is administered during the off-cycles of IV chemotherapy. To verify this, the total numbers of WBC, RBC and PLT, and their related parameters, were determined 24 h after the treatment administration regimen and after one week of recovery.

To improve the clarity of the figures, WBC results are represented in Fig. 6A without the statistical analyses specified hereunder.

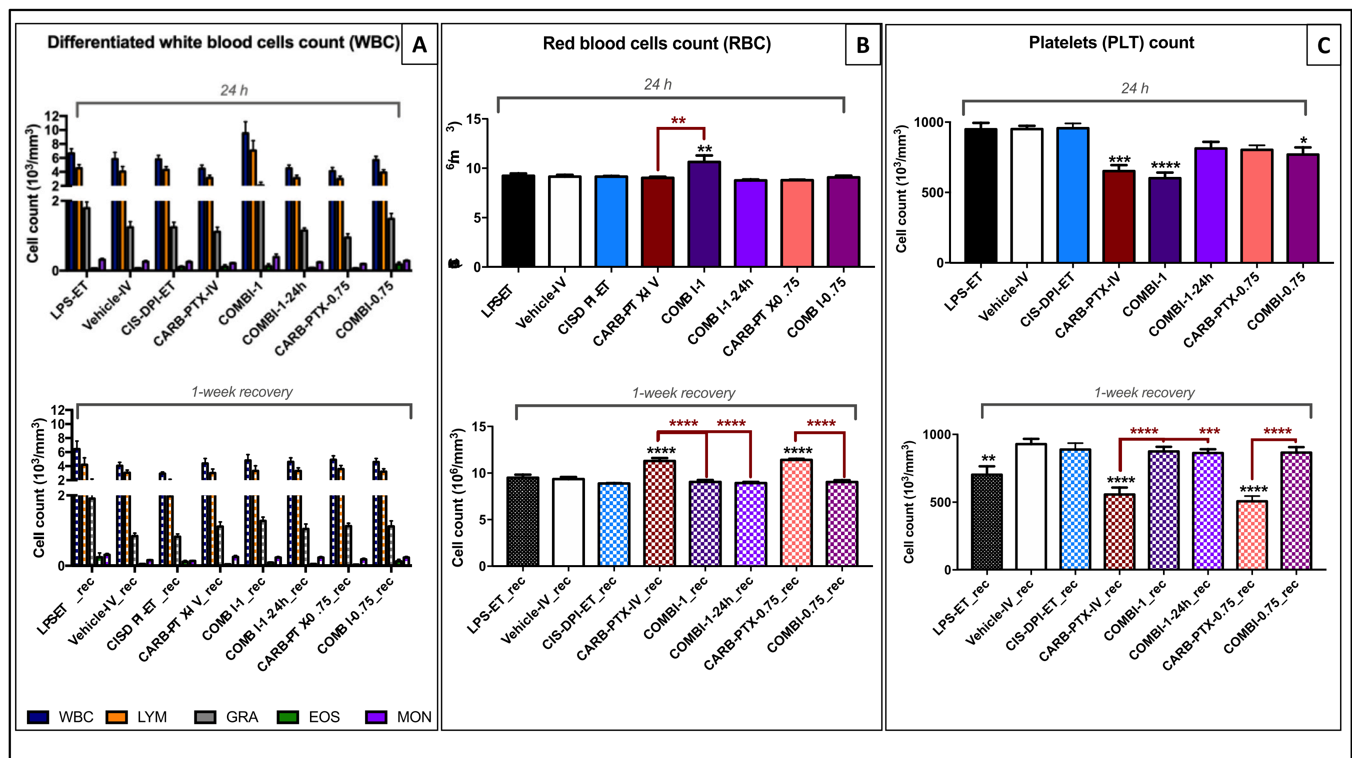


Fig. 6. Evaluation of the differentiated total cell count of white blood cells (WBC) (A), red blood cells (RBC) (B) and platelets (PLT) (C) in blood, 24 h following the last treatment administration (A) and after one week of recovery (_rec). All results are expressed as means ± SEM (n = 8–15). The statistical analyses were performed *vs* the vehicle-IV group (black) or *vs* the selected groups as illustrated (red), using one-way ANOVA and Bonferroni's post-hoc test (**** for $p < 0.0001$, *** for $p < 0.001$, ** for $p < 0.01$ and * for $p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Evaluation of mean corpuscular haemoglobin concentration (MCHC), haemoglobin (Hb), haematocrit (Ht), mean corpuscular volume (MCV), red cell distribution width (RDW), mean cell haemoglobin (MCH) and mean platelet volume (MPV) in blood, 24 h following the last treatment administration and after one week of recovery (_rec). All results are expressed as means ± SEM (n = 8–15). The statistical analyses were performed *vs* the vehicle-IV group (black) or *vs* the selected groups as illustrated (red), using one-way ANOVA and Bonferroni's post-hoc test (**** for $p < 0.0001$, *** for $p < 0.001$, ** for $p < 0.01$ and * for $p < 0.05$).

Parameters	MCHC (g/dL)	Hb (g/dL)	Ht (%)	MCV (%)	RDW (µm^3)	MCH (pg)	MPV (µm^3)
24 h after the treatment administration							
LPS-ET	34.8 ± 0.4	15.6 ± 0.2	44.9 ± 0.9	49.1 ± 0.2	12.7 ± 0.1	17.1 ± 0.2	6.1 ± 0.1
Vehicle-IV	34.3 ± 0.2	15.5 ± 0.3	45.2 ± 0.8	49.3 ± 0.2	12.8 ± 0.1	16.9 ± 0.1	6.0 ± 0.1
CIS-DPI-ET	34.4 ± 0.2	15.4 ± 0.2	44.8 ± 0.6	48.8 ± 0.1	12.8 ± 0.1	16.9 ± 0.1	6.2 ± 0.1
CARB-PTX-IV	34.5 ± 0.2	15.5 ± 0.2	44.9 ± 0.7	49.8 ± 0.3	13.0 ± 0.1	17.2 ± 0.1	6.1 ± 0.1
COMBI-1	34.3 ± 0.1	18.2 ± 1.0****	53.5 ± 11.2***	50.2 ± 0.2**	13.0 ± 0.1	17.2 ± 0.1	6.2 ± 0.1
COMBI-1–24 h	33.8 ± 0.2	14.8 ± 0.2	43.9 ± 0.5	50.2 ± 0.1**	13.0 ± 0.1	16.9 ± 0.1	6.3 ± 0.1
CARB-PTX-0.75	34.3 ± 0.2	15.1 ± 0.2	43.9 ± 1.5	50.0 ± 0.2	13.2 ± 0.1**	17.2 ± 0.1	6.0 ± 0.1
COMBI-0.75	34.1 ± 0.1	15.4 ± 0.3	45.2 ± 0.9	49.9 ± 0.2	12.9 ± 0.1	17.0 ± 0.1	6.4 ± 0.1
One-week recovery after the treatment administration							
LPS-ET_rec	34.5 ± 0.4	16.1 ± 0.5	46.6 ± 1.7	49.0 ± 0.2	12.9 ± 0.1	17.0 ± 0.2	6.5 ± 0.2**
Vehicle-IV_rec	34.4 ± 0.2	15.8 ± 0.3	46.0 ± 1.0	49.3 ± 0.2	13.0 ± 0.1	16.9 ± 0.1	6.0 ± 0.1
CIS-DPI-ET_rec	34.7 ± 0.2	15.3 ± 0.1	44.0 ± 0.5	49.5 ± 0.1	12.9 ± 0.1	17.2 ± 0.1	5.9 ± 0.1
CARB-PTX-IV_rec	33.8 ± 0.2	19.4 ± 0.6****	57.4 ± 1.7****	50.8 ± 0.3***	14.1 ± 0.2****	17.2 ± 0.1	6.3 ± 0.2
COMBI-1_rec	34.6 ± 0.3	15.7 ± 0.3	45.5 ± 1.0	50.2 ± 0.2	13.6 ± 0.1*	17.4 ± 0.2	6.0 ± 0.1
COMBI-1–24h_rec	33.8 ± 0.3	15.2 ± 0.2	45.1 ± 0.6	50.5 ± 0.2**	13.5 ± 0.1	17.1 ± 0.1	6.1 ± 0.1
CARB-PTX-0.75_rec	33.8 ± 0.2	19.5 ± 0.3****	57.9 ± 1.0****	50.6 ± 0.2**	13.3 ± 0.1	17.1 ± 0.1	6.0 ± 0.1
COMBI-0.75_rec	34.2 ± 0.2	15.4 ± 0.3	44.9 ± 0.9	49.7 ± 0.4	13.3 ± 0.1	17.0 ± 0.2	6.1 ± 0.1

3.6.1. *White blood cells*

3.6.1.1. IV doublets and CIS-DPI-ET. The administration of CIS-DPI-ET did not induce any leukopenia or WBC increase in comparison with the vehicle-IV group ($p > 0.05$, Fig. 6A). The administration of the IV doublets induced a non-significant slight decrease in the total WBC *vs* the vehicle-IV group (CARB-PTX-IV: $4.5 \pm 0.5 \times 10^3$ cells/mm^3 and CARB-PTX-0.75: $4.1 \pm 0.5 \times 10^3$ cells/mm^3, respectively *vs* $5.8 \pm 0.9 \times 10^3$ cells/mm^3, Fig. 6A). These differences were all reversible after the recovery week ($p > 0.05$, Fig. 6A). However, it should be noted that for the positive LPS-ET_rec groups, the proportions of GRA and LYM were significantly higher in comparison to the vehicle-IV group ($p < 0.001$, Fig. 6A).

3.6.1.2. Combinations. The administration of COMBI-1 increased significantly the total WBC count *vs* the vehicle-IV group: $10 \pm 2 \times 10^3$ cells/mm^3 *vs* $5.8 \pm 0.9 \times 10^3$ cells/mm^3 ($p < 0.05$, Fig. 6A) 24 h after the last treatment administration. This was observed for all the different WBC but was only significant for the total number of LYM ($p < 0.05$, Fig. 6A) 24 h after the last treatment administration. This increase was reversible after one week of recovery as no significant difference was observed between this group and the vehicle-IV group ($4.8 \pm 0.8 \times 10^3$ cells/mm^3 *vs* $4.0 \pm 0.5 \times 10^3$ cells/mm^3) ($p > 0.05$, Fig. 6A). Moreover, after one week of recovery, the proportion of LYM remained significant and the proportion of GRA became significant for COMBI-1 in comparison to the vehicle-IV group ($p < 0.05$ and $p < 0.01$, respectively). The total WBC count of COMBI-1 was very significant when compared to CARB-PTX-IV ($p < 0.01$, Fig. 6A) 24 h after the last treatment administration. This was related to higher LYM ($p < 0.01$), GRA ($p < 0.05$) and MON ($p < 0.05$) for COMBI-1 in comparison with CARB-PTX-IV. In contrast, this was not the case for COMBI-1–24 h or COMBI-0.75 24 h after the last treatment administration as no significant difference was observed *vs* the vehicle-IV group or their respective IV doublets ($p > 0.05$, Fig. 6A).

Moreover, the difference in terms of WBC counts 24 h after the last treatment administration between COMBI-1–24 h and COMBI-1 or between COMBI-0.75 and COMBI-1 was significant, in both cases ($p < 0.001$, $p < 0.01$, respectively, Fig. 6A). This was related to the increase in LYM ($p < 0.001$) and GRA ($p < 0.05$) for COMBI-1 and to the increase in LYM ($p < 0.01$) for COMBI-0.75 when compared to COMBI-1–24 h. All differences observed between COMBI-1–24 h and COMBI-1 or between COMBI-0.75 and COMBI-1 were reversible one week later ($p > 0.05$, Fig. 6A). The 24 h delay and the dose reduction therefore both seemed useful to limit the inflammation.

3.6.2. *Red blood cells*

3.6.2.1. IV doublets and CIS-DPI-ET. The administration of CIS-DPI-ET or IV doublets did not show any significant decrease in terms of total RBC count, Hb and Ht 24 h after the last treatment administration when compared to the vehicle-IV group (Fig. 6B and Table 2). However, after one week of recovery, total RBC count, Hb, and Ht were significantly higher for the IV doublets at both doses (CARB-PTX-IV and CARB-PTX-0.75) ($p < 0.0001$, Fig. 6B and Table 2).

Moreover, both IV doublets were also characterized by significantly higher MCV (macrocytosis) after one week of recovery in comparison with the vehicle-IV group ($p < 0.001$ for CARB-PTX-IV and $p < 0.01$ for CARB-PTX-0.75) (Table 2). In addition, the RDW of IV doublets was significantly different ($p < 0.01$, Table 2).

3.6.2.2. Combinations. COMBI-1 induced significantly higher numbers of RBC ($10.6 \pm 0.7 \times 10^6$ cells/mm^3 *vs* $9.1 \pm 0.2 \times 10^6$ cells/mm^3, $p < 0.01$, Fig. 6B), Hb (18.2 ± 1.0 g/dL *vs* 15.5 ± 0.8 g/dL, $p < 0.0001$, Table 2) and Ht (54 ± 11% *vs* 45.2 ± 0.8%, $p < 0.001$, Table 2) than vehicle-IV group, 24 h after the last treatment administration. This increase was reversible after one week of recovery as no significance difference was observed with the vehicle-IV group (Table 2). However, the RDW was significantly higher for COMBI-1 in comparison with the vehicle-IV group (Table 2).

Total RBC count, Hb and Ht of COMBI-1 were also significantly higher than for CARB-PTX-IV ($p < 0.01$, $p < 0.0001$ and $p < 0.001$, respectively) and COMBI-1–24 h ($p < 0.0001$, for all), 24 h after the last treatment administration (Fig. 6B and Table 2).

Consequently, the administration of COMBI-1 seemed to induce an increased number of RBC (polycythaemia) 24 h after the last treatment administration. This was much earlier than with both IV doublets (CARB-PTX-IV and CARB-PTX-0.75).

COMBI-1–24 h did not increase significantly RBC, MCHC, Hb, Ht or RDW in comparison with the vehicle-IV group or CARB-PTX-IV at either sampling time ($p > 0.05$, Fig. 6B, Table 2). However, both COMBI-1 and COMBI-1–24 h were characterized by a significant increase in the MCV, in comparison with the vehicle-IV group ($p < 0.01$) although not with the CARB-PTX-IV group ($p > 0.05$), 24 h after the last treatment administration (Table 2). On the other hand, the MCV increase was maintained for COMBI-1–24 h after one week of recovery in comparison to the vehicle-IV group ($p < 0.01$) (Table 2). COMB-0.75 did not induce

any increase in terms of RBC count or their related parameters (Fig. 6B, Table 2), for either sampling time. It should be noted that for all groups, MCH and MHCH were similar to those for the vehicle-IV group.

3.6.3. Platelets

Thrombocytopenia is one of the most common cumulative side effects of CARB-PTX doublet chemotherapy [7–10]. It was therefore mandatory to investigate total PLT count and MPV for all the tested groups.

3.6.3.1. CIS-DPI-ET and IV doublets. CIS-DPI-ET did not decrease the total PLT count as no significant difference with the vehicle-IV group was observed at either sampling time ($p > 0.05$, Fig. 6C). On the other hand, the administration of CARB-PTX-IV induced a significant decrease in total PLT count, in comparison with the vehicle-IV group ($p < 0.001$, Fig. 6C), which remained one week after administration ($p < 0.0001$, Fig. 6C). In contrast, this was not the case for CARB-PTX-0.75, as no significant difference was observed with the vehicle-IV group ($p > 0.05$, Fig. 6C) 24 h after the last treatment administration, although this was the case after one week of recovery ($p < 0.0001$, Fig. 6C).

None of the CIS-DPI-ET or IV doublets increased MPV in comparison with the vehicle-IV group at either sampling time ($p > 0.05$, Fig. 6C).

3.6.3.2. Combinations. The administration of COMBI-1 or COMBI-0.75 led to a significantly lower PLT count than for the vehicle-IV group 24 h after the last administration and was proportional to the administered dose ($p < 0.00001$ and $p < 0.05$, respectively, Fig. 6C). However, these lower PLT counts were reversible after one week of recovery with no significant difference with the vehicle-IV group ($p > 0.05$, Fig. 6C).

COMBI-1–24 h showed no significant difference in total PLT count compared with the vehicle-IV group at either sampling time ($p > 0.05$, Fig. 6C). Moreover, COMBI-1–24 h had a significantly higher total PLT count than COMBI-1, 24 h after the last administration ($p < 0.001$, Fig. 6C).

No significant difference was observed between the combination groups and their respective IV doublets 24 h after the treatment. However, a significant difference was observed for all the combination groups one week later, as the decreases observed with IV doublets were significantly higher than with their respective combination groups (COMBI-1 ($p < 0.0001$), COMBI-1–24 h ($p < 0.001$) *vs* CARB-PTX-IV, Fig. 6C, and COMBI-0.75 *vs* CARB-PTX-0.75 ($p < 0.0001$), Fig. 6C).

None of the combination groups increased MPV in comparison with the vehicle-IV group at either sampling time ($p > 0.05$, Fig. 6C).

4. Discussion

It was fundamental to verify that the addition of CIS-DPI-ET during IV doublet off-cycles did not impair general tolerance (*i.e.* bw losses) or pulmonary, renal and haematological tolerance. Indeed, these resting cycles are crucial for recovery before the administration of the following treatment cycle. The bw profiles demonstrated limited bw losses during the treatment administration and increased bw during recovery. This matched the general tolerance cited above.

The investigation of the pulmonary tolerance of CIS-DPI-ET and IV doublets separately and in combination was necessary as platinum-based agents and taxanes have been reported to induce hypersensitive reactions in lungs [37]. This occurred with acute onset of bronchospasm, dyspnoea, cough and hypotension, leading to treatment discontinuation [37]. Indeed, 30% of patients treated with paclitaxel developed, within the first 10 min of infusion, acute pneumonia and hypersensitivity pneumonitis in addition to the reactions described above [37].

In our study, pulmonary inflammation and cytotoxicity were evaluated directly in BALF to increase the specificity of the lung-related tolerance. As inflammatory cells are involved in the earliest phase of inflammation, BALF-packed cells were evaluated in terms of differential and total cell counts [16,39]. A higher total cell recruitment (total BALF cell analysis) was detected for the groups exposed to CIS-DPI-ET locally (CIS-DPI-ET, COMBI-1, COMBI-1–24 h and COMBI-0.75). This recruitment was related to higher NT-GRA cell proportions in comparison to their respective IV doublets (Fig. 3, Figure S.2, supplementary data). This demonstrated a more intense inflammation for these groups related to the prolonged exposure to CIS following CIS-DPI-ET administration. Indeed, NT-GRA are the most common cells among GRA and are recruited at the site of inflammation to help remove phagocytosis debris by releasing reactive oxygen species, antimicrobial proteins and degradative enzymes [40].

It should be noted that in our previous preclinical study, we demonstrated that the administration of the diluent (used for the preparation of CIS-DPI blend) following the scheme described in Fig. 1A did not induce any inflammation in mice. Indeed, the administration of the diluent did not lead to any bw loss, lung inflammatory reaction (*i.e.* no increase in pro-inflammatory cytokines, chemokines, or proportion of GRA-NT in BALF), lung cytotoxicity (*i.e.* no increase in protein content) or AKI (*i.e.* no increase in NGAL, cystatin-c, creatinine) [25]. Consequently, the adverse effects observed in lung and kidneys following CIS-DPI-ET administration were only attributed to CIS, not to the diluent or to the technique of administration [25].

As cell recruitment occurs using specific mouse chemokines, CXCL1 and CXCL2 were also quantified in BALF [41]. However, the observed lung inflammatory reactions did not seem to be related to higher CXCL1 and/or CXCL2 levels (Table 1). This might have been related to the fact that NT-GRA had already been retrieved in BALF and needed no further recruitment. Moreover, these chemokines may have increased gradually during the treatment administration and may have normalized once NT-GRA were recruited in BALF. Indeed, these chemokines increase in the early phase of inflammation and also seem to have an active role in the regulation and homeostasis of NT-GRA recruitment [42,43]. As pro-inflammatory cytokines are well-known to take an active part in the generation of acute inflammation, TNF-α, IL-6 and IL-1-β were quantified in BALF supernatant. Indeed, these cytokines are the most involved in inflammation in both human and mouse models [39,44]. In our study, except for the positive control (LPS-ET), none of the treated groups demonstrated a higher lung inflammation (Table 1). However, in our previous preclinical study, in which CIS-DPI-ET and CIS IV were combined, a higher pulmonary inflammation was reported following their administration on the same day at their MTD (*i.e.* 0.5 mg/kg for CIS-DPI-ET and 2 mg/kg for CIS IV) in terms of NT-GRA recruitment, TNF- α and IL-6 levels [25]. It has been reported that the administration of CIS using nebulization through the ET route was responsible for severe pneumonitis and mild to moderate fibrosis during a preclinical study on dogs [45]. Moreover, its administration in a phase I study was correlated to dyspnoea, bronchitis and hoarseness, which were scored 1–2 above 4 [46]. This may have been avoided in our study by developing CIS as a controlled-release formulation. Indeed, CIS-DPI-ET was formulated using 50% w/w of pharmaceutical grade lipids excipients (*i.e.* 49.5% w/w of HCO and 0.5% w/w of TPGS) and was able to release CIS *in vitro* gradually from solid-lipid microparticles (~50% after 24 h *vs* 80% from uncoated-CIS DPI powder) [24]. This was correlated to good lung targeting and retention in mouse lungs following a pharmacokinetic study conducted on healthy mice [24]. This controlled-release strategy was selected to avoid a high CIS dose to be directly available in the lungs and inducing a local inflammation, as observed by Levet et al., for an immediate release of CIS-based DPI [47].

Lung cytotoxicity was evaluated by quantifying the LDH activity and total protein content. Cytotoxicity leads to cell rupture, leading to the release of their contents in the extracellular compartment. The determination of the total protein content is therefore correlated to cell lysis and to cytotoxicity. By the same logic, LDH is an enzyme that is retrieved in the cytoplasm of different tissues and increases in the case of cell rupture or death. As its concentration is up to 500-fold higher in lung tissue than in plasma, it was therefore interesting to quantify LDH in BALF [48].

Results tended to show that CIS-DPI-ET administered using the ET route seemed to induce higher cytotoxicity in BALF than the IV doublets (Fig. 3). These results were similar to those obtained for CIS-DPI-ET and CIS IV during our previous preclinical study [25]. This can be explained by a more prolonged and more frequent exposure to the cytotoxic drug following CIS-DPI-ET in comparison with IV doublets [25]. Considering both these biomarkers, the combination groups seemed characterized by higher protein content when compared to their relative IV doublets. This was predictable as cytotoxicity is related to the total cytotoxic drug dose (higher dose for combinations *vs* IV doublets or CIS-DPI-ET).

The histopathological analyses did not demonstrate any major lung damage for any of the groups and confirmed the results obtained with the pulmonary biomarkers. Indeed, vacuolation (score 0/5), perivascular oedema (score 0/5), major intra-alveolar haemorrhage (score 0.7/5), acute bronchopneumonia (score 0.2/5) and intra-alveolar fibrin (0.1/5) were all scored less than 1 (Fig. 4). Luminal AM investigation in lung sections demonstrated a slight transient increase for the combination groups. The recruitment of AM related to the administration of CIS-DPI-ET, as observed during BALF cell counts, was to ensure phagocytosis as this is the main innate defence mechanism (Fig. S2, supplementary data) [39]. Moreover, an increased blood congestion and a higher blood flow are observed in inflammation to ensure inflammatory cell recruitment and extravasation [49], which may explain the intensified intra-alveolar haemorrhage and luminal AM observations in lung sections. Furthermore, “tissue factor” is an integral plasma membrane protein expressed by AM, lung epithelium and fibroblasts, which increases in lung or pleural injuries to initiate the extrinsic pathway of coagulation [50]. In the sites of injury, this pro-coagulation factor activates thrombin leading to localized fibrin sections [50]. This is a well-known reaction to inflammation [50] and may explain the slight intra-alveolar fibrin observations.

These results seemed to demonstrate a good pulmonary tolerance of all combinations. Indeed, except for total BALF cell and GR-NT proportion increases, CIS-DPI-ET, IV doublets and their combinations at their MTD, administered on the same day, did not seem to induce acute pulmonary inflammation. However, these combination groups seemed to induce a higher cytotoxicity than their respective IV doublets (COMBI-1–24 h *vs* CARB-PTX-IV), which would be favourable for treatment efficacy. This tended to be associated to higher but still light lung damage for these groups in comparison to IV doublets. It is important to report that higher lung inflammation was observed following the administration of CIS IV at its MTD (*i.e.* 2.0 mg/kg) when combined with CIS-DPI-ET, the same day or 24 h later in a previous preclinical study [25]. All lung-related inflammatory reactions were reversible within one week of recovery, except for NT-GRA recruitment [25]. The reported inflammation was avoided only when the CIS IV dose was reduced by 25%. The combination of CARB-PTX IV doublets and CIS-DPI-ET seemed to be overall better tolerated by the lungs than the previous CIS IV and CIS-DPI-ET combinations [25]. Indeed, while the combination of CARB-PTX-IV and CIS-DPI-ET did not induce higher pro-inflammatory cytokines, the combination of CIS IV and CIS-DPI-ET induced an irreversible increase in TNF-a levels and a reversible increase in IL-6 levels [25]. Moreover, luminal AM observation was scored higher for the combination of CIS IV and CIS-DPI-ET than CARB-PTX-IV and CIS-DPI-ET (2.0 *vs* 0.1, respectively), demonstrating a more intense phagocytotic reaction for this combination [25]. These differences may be related to the fact that carboplatin has a slower rate of aquation and therefore a lower cytotoxic activity than cisplatin, leading to a better overall toxicity profile [51,52]. Therefore considering only lung-related inflammatory reactions, the co-administration of CARB-PTX-IV and CIS-DPI-ET the same day should be considered while the combination of CIS IV at its MTD and CIS-DPI-ET on the same day should be avoided.

As described previously, the investigation of AKI is mandatory to assess the safety of the combination groups as platinum compounds are reported to be highly nephrotoxic [38]. To evaluate early AKI, novel biomarkers that have already proven their specificity and sensitivity in both preclinical and clinical studies were selected [53]. In our previous preclinical study, AKI was detected 24 h after its induction using NGAL, cystatin-c and creatinine in plasma [25]. AKI biomarkers are divided into tubule-function biomarkers (cystatin-c, creatinine) and kidney-damage biomarkers (NGAL) [25,53] and should be both considered for AKI assessment [54]. On the one hand, the functional biomarker cystatin-c, is freely filtered and almost completely reabsorbed and degraded in the proximal tubule. Therefore, it increases if there is a filtration defect [53,55]. On the other hand, NGAL is secreted by NT-GRA and kidney tubular cells in the early stages of renal damage or inflammation [53,55,56].

The administration of CIS-DPI-ET following three administrations a week for 2 consecutive weeks was well-tolerated by the kidneys as no early or delayed AKI was notified, as already demonstrated in our previous preclinical study (Fig. 5, Table 2) [25]. Indeed, no significant increase in NGAL, cystatin-c, or creatinine was identified following the administration of CIS-DPI-ET at 0.5 mg/kg or even at 1 mg/kg [25]. Moreover the administration of CARB-PTX-IV at its MTD did not seem to induce AKI, whereas this was observed following the administration of CIS IV at its MTD (*i.e.* 2.0 mg/kg) [25]. It is widely known that, unlike CIS, CARB does not interact with the organic copper transporter 2 (hOCT2). This results in a lower concentration of CARB in the proximal tubule cells than CIS leading to less renal damage [51].

The combination of CIS-DPI-ET and CARX-PTX IV induced higher NGAL levels, but with similar cystatin-c and creatinine levels, resulting in significantly higher damage and/or inflammation than with the vehicle-IV group. However, this increase was reversible within one week, and was not significantly different from CARB-PTX-IV. In addition, no renal injury or higher plasma AKI biomarkers were observed for the other groups (Fig. 5, Table 2). This showed that the strategies of dose reduction by 25% or a delay in administrations were promising to avoid any major biomarker increase when compared to groups treated with IV doublets. In contrast, the combination of CIS-DPI-ET and CIS IV at their respective MTD, the same day or 24 h later, induced non-reversible AKI, with increases in NGAL, cystatin-c and creatinine levels [23]. It was mandatory to reduce the IV dose by 25% and stagger the administrations by 24 h to avoid AKI [23]. As expected, the combinations with CIS IV seemed to induce higher AKI than those with IV CARB-PTX as no dose or regimen adaptations were required. These last results validated our preclinical AKI model and confirmed its appropriateness, as CARB is widely described to be less nephrotoxic than CIS [10].

Last but not least, haematological tolerance was assessed to ensure that the addition of CIS-DPI-ET did not impair the regeneration of bonemarrow stem cells that normally occurs during the off-cycles [19]. To evaluate myelotoxicity, which is the CARB-PTX DLT, total cell counts of WBC, RBC and PLT as well as their related parameters were investigated.

Except for ET-LPS and COMBI-1, no significant differences in terms of WBC counts in any group with vehicle-IV was reported (Fig. 6A) 24 h after the last administration or after one week of recovery. The significantly higher MON, GRA and LYM retrieved in the blood for COMBI-1 in comparison with CARB-PTX-IV seemed to be related to the AM and NT-GRA recruitments detected in BALF following CIS-DPI-ET administrations (Figs. 3A, 6A, S2, supplementary data), 24 h after the last administration. This increase was intensified when combined on the same day with CARB-PTX-IV, as discussed previously and in regard to the result obtained with LPS-ET groups (higher MON, GRA and LYM than in the vehicle-IV group). Indeed, MON, the principal immune effector cells, are retrieved in circulating blood (40%) and in all tissues in a steady state (60%) [16]. They are capable of differentiating into macrophages or dendritic cells as soon as they cross the endothelium [57]. In the case of inflammation, their number is increased to ensure phagocytosis [39]. Following this innate response, dendritic cells can migrate to lymph nodes and induce an immune reaction by stimulating LYM. In parallel, GRA basophils release histamine and serotonin to maintain the acute-phase response [39]. This explains how MON, GRA (mainly

NT-GRA and basophils) and LYM were recruited in the early phase of pulmonary inflammation following CIS-DPI-ET administration and how they were retrieved in both blood and BALF (Fig. 3A, 6A, S2, supplementary data).

One week later, both total BALF cells and WBC counts for COMBI-1 were reversible and similar to the vehicle-IV group. However, the proportion of GRA and LYM in blood remained significantly higher than the baseline and for IV doublets, as observed for the NT-GRA proportion in BALF (Fig. 3A, 6A). The similarity between these trends confirmed that the WBC count increases seemed related to pulmonary inflammation.

The evaluation of anaemia demonstrated a higher RBC count (polycythaemia), MCHC, Hb, Ht and RDW for COMBI-1 one week earlier than for the IV doublets, while no significant difference was reported with the other combination groups (Fig. 6B, Table 2). In addition, as described above, COMBI-1 also induced an increased plasma GRA proportion in the blood (Fig. 6A). It has been reported that both of these observations are increased as a response to anaemia, as erythropoietin production is raised and may be detected from a higher RBC count, Hb and Ht, describing polycythaemia [16]. Indeed, it has been described that an increase in RBC count (polycythaemia) is observed 7–14 days after an acute drop due to bone-marrow erythrocyte production, depending on the species [15]. This may explain why the RBC counts of IV doublets were higher after one week of recovery. The last IV administration could have led to a drop in RBC and to their generation 7 days later, which corresponded to the results from the second sampling time (Fig. 6B).

The observed anaemia might be attributed to CARB as it is was reported that this drug was able to destroy mature RBC (haemolysis) through three different mechanisms [15]. Moreover, higher MCV (macrocytosis) was observed in the mice treated with COMBI-1 and COMBI-1–24 (Table 2). Besides, macrocytosis is described to be often associated with polycythaemia in mice undergoing exuberant regenerative responses to anaemia [16]. Macrocytosis is rarely observed in patients undergoing chemotherapy; however, it was reported in patients undergoing antimetabolite-based chemotherapy [58]. Besides, a recent study reported that macrocytosis was also associated with CARB-PTX doublet in ovarian cancer patients [59].

Thrombocytopenia was investigated by means of PLT count. The results from CARB-PTX-IV and CARB-PTX-0.75 24 h after the last treatment demonstrated that the strategy of dose reduction of 25%, as in clinical practice, was successful to prevent a PLT count drop (Fig. 6C). Moreover, the strategy of delaying the administrations by 24 h was also promising as no significant difference in PLT counts was observed for COMBI-1–24 h in comparison with the vehicle-IV group, at either sampling time. However, PLT count decrease was observed and/or worsened one week later for the IV doublet groups. This has already been observed 24 h after the last administration for CARB-PTX-IV, COMBI-1 and COMBI-0.75. For the RBC count, our hypothesis is that the addition of CIS-DPI-ET may have led to earlier thrombocytopenia for the combination groups, which was reversible within one week. Moreover, no significance difference in MPV was observed for any group at either sampling time. This showed that no higher PLT production due to hypoxia-induced thrombocytopenia was observed [16], which was consistent with our previous observations.

Furthermore, it was interesting to observe that total PLT counts for LPS-ET_rec were significantly lower than for the vehicle-IV group ($p < 0.01$, $703 \pm 62 \times 10^3$ cells/mm^3 *vs* $930 \pm 39 \times 10^3$ cells/mm^3, Fig. 6C). PLT are known to be recruited at the site of inflammation as they have important roles in the inflammation initiation by (i) secreting adhesion molecules to aggregate with WBC and (ii) stimulating NT-GRA, MON and LYM using chemotactic chemokines for WBC to form aggregates and launch inflammation [60]. Consequently, a reduced number of circulating PLT could be available for cell count if there is local inflammation [60]. This is also a hypothesis for the reduction in PLT for COMBI-1 and COMBI-0.75 24 h after the last administration (Fig. 5C).

The comparison between COMBI-1 and COMBI-1–24 h in terms of pulmonary and haematological tolerance was interesting for the evaluation of the added value of the 24 h delay between IV doublets and CIS-DPI-ET administration in the design of the regimens. A significantly lower proportion of NT-GRA in BALF ($p < 0.05$) together with lower WBC ($p < 0.001$) and PLT counts ($p < 0.01$) in blood were detected for COMBI-1–24 h in comparison with COMBI-1. This demonstrated that staggering the administrations by 24 h seemed to avoid (i) the recruitment of pro-inflammatory cells in BALF (especially NT-GRA) and consequently in blood (WBC count) as well as (ii) the sequestration of PLT in the lungs leading to higher PLT in blood available for count.

Considering all these results, even if the combination groups (COMBI-1, COMBI-0.75) induced thrombocytopenia, the addition of CIS-DPI-ET to IV chemotherapy did not induce higher thrombocytopenia than was observed with IV doublets. Therefore thrombocytopenia induced by COMBI-1 and COMBI-0.75 was considered as light. This result was not surprising as CIS-DPI-ET alone did not induce any form of thrombocytopenia. Moreover, considering all the mouse groups and all the blood parameters, total WBC counts ranged between $2.9 \pm 0.2 \times 10^3$ and $10 \pm 2 \times 10^3$ cells/mm^3 and PLT counts between $507 \pm 38 \times 10^3$ and $958 \pm 33 \times 10^3$ cells/mm^3. These values are still considered as normal for BALB/c mice, as described in the literature (WBC: $\sim$ 3–13 $\times 10^3$ cells/mm^3 and PLT: $\sim$ 400–1600 $\times 10^3$ cells /mm^3) [61,62]. This showed that the significant fluctuations from the vehicle-IV groups do not describe major toxicities that should prevent these combinations from being considered as potential therapeutic options.

Overall, the co-administration of CIS-DPI-ET and IV doublets, regardless of the regimen and doses, did not induce any major bw losses or pulmonary, renal or haematological toxicities. Therefore these groups should be considered as possible therapeutic options as they were all associated with higher pulmonary cytotoxicity. It is probable that the strategies of dose reduction by 25% or a delay in administrations would hinder the full potential of CIS-DPI-ET and CARB-PTX-IV combinations. Indeed, a 24 h-delay would further give to the tumour the opportunity to grow (*i.e.* as it is not exposed to cisplatin locally) than if both administrations (CIS-PDI-ET and CARB-PTX-IV) were performed the same day. In a similar way, a 25%-reduced IV dose would lead to a lower efficacy than a total IV dose, as there is a clear established dose-response relationship for cytotoxic drugs. However, even considering both of these strategies, a therapeutic intensification is expected, in comparison to the well-spaced conventional chemotherapy cycles [4]. Indeed, this successful strategy was demonstrated in our previous preclinical study that combined a 25%-reduced CIS IV dose and CIS-DPI-ET, administered 24 h later [24]. Besides a similar strategy of dose re-arrangement was assessed by Zarogoulidis et al., and demonstrated a significant increase in survival for NSCLC patients treated by a CARB total dose that was fractionated between ET (1/3 of total CARB dose) and IV (2/3 of total CARB dose), in comparison to patients only treated by a total IV dose (3/3 of total CARB dose) [63].

However, these groups demonstrated several differences. Indeed, COMBI-1 induced significantly higher BALF cell counts, in part due to the recruitment of the NT-GRA proportion in BALF in comparison with CARB-PTX-IV. This was related to higher MON, LYM and GRA in blood than CARB-PTX-IV. Moreover, regenerative anaemia (polycythaemia and macrocytosis) was observed one week earlier than with CARB-PTX-IV. Although thrombocytopenia was detected for this group (COMBI-1), this was not significantly different from its occurrence with CARB-PTX-IV. Consequently, except for a recruitment of pro-inflammatory cells in BALF and inducement of regenerative anaemia earlier than the IV chemotherapy, COMBI-1 did not induce any other toxicity than with CARB-PTX-IV. Furthermore, the strategy of reducing the CARB-PTX dose by 25% (CARB-PTX-0.75) also increased the proportion of NT-GRA in BALF in comparison with IV doublets, in a lower proportion than COMBI-1, with no major WBC, RBC or PLT count differences with CARB-PTX-0.75. However, the strategy of delaying the administrations by 24 h (COMBI-1–24 h) seemed to avoid pro-inflammatory cell (mostly NT-

GRA) recruitment in BALF as well as WBC, RBC and PLT differences in comparison with CARB-PTX-IV. Moreover, none of these groups demonstrated higher AKI in comparison to IV doublets. Consequently, all these combination groups seemed interesting to be investigated in future efficacy studies.

5. Conclusion

The administration of a controlled-release CIS-based DPI formulation in the lungs for 2 consecutive weeks, did not lead to any sign of myelotoxicity, acute kidney injury or pulmonary inflammation except for a transient NT-GRA increase. The addition of CIS-DPI-ET to CARB-PTX-IV on the same day at their MTD induced a higher WBC and BALF cell counts, a higher NT-GRA proportion in BALF and earlier regenerative anaemia than with CARB-PTX-IV alone. The strategy of IV dose reduction by 25% and the separation of CIS-DPI-ET and CARB-PTX-IV administration by 24 h avoided regenerative anaemia and/or BALF and WBC increase when compared to their respective IV doublets. Interestingly, all the combination groups induced non-reversible higher cytotoxicity (total protein concentration and LDH activity) than the non-treated groups. These combination strategies were overall well-tolerated and should be considered in lung cancer therapy development. Future studies will assess the efficacy of these regimens on murine lung cancer models.

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K. Amighi, N. Wauthoz, and R. Rosière are holders of patents related to some of the 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 with the Unit of Pharmaceutics and Biopharmaceutics. The authors have no 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 other than those disclosed.

Conflict of interest statement

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.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2021.111716.

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