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Intra-tracheal administration increases gallium availability in lung: implications for antibacterial chemotherapy

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Abstract

The emergence of pan-resistant strains in nosocomial settings underscores the urgent need of novel therapies targeting vital bacterial functions. Bacterial iron metabolism is a fascinating target for new antimicrobials. Iron mimetic metal Ga(III) has been repurposed as an antimicrobial drug, in pre-clinical studies and recent clinical studies have raised the possibility of using Ga(III) for the treatment of *P. aeruginosa* pulmonary infection. Ga(III) has been approved by FDA for the treatment of cancer, autoimmune and bone resorption disorders. However, some critical issues affect the therapeutic schedule of Ga(III), principally the intravenous (i.v.) administration, and the nephrotoxicity caused by prolonged administration. Ga(III) aerosolization could represent a viable alternative for treatment of lung infections, since delivery of antimicrobial agent to the airways maximizes drug concentration at the site of infection, improves the therapeutic efficacy, and alleviates systemic toxic effects.

We demonstrate the advantage of inhaled *vs* i.v. administered Ga(III), in terms of bio-distribution and lung acute toxicity, by using a rat model. *In vivo* results support the use of Ga(III) for inhalation since, intra-tracheal Ga(III) delivery improved its persistence in the lung, while the i.v. administration caused rapid clearance and did not allow to attain a significant Ga(III) concentration in this organ. Moreover, local and systemic acute toxicity following intra-tracheal administration was not observed, since no significant signs of inflammation were found. At this stage of evidence, the direct administration of Ga(III) to the lung appears feasible and safe, boosting the development of Ga(III)-based drugs for inhalation therapy.

Keywords

Antibiotic; gallium; intra-tracheal route; lung; pneumonia; rat; toxicity

1. Introduction

Nosocomial pneumonia is the most common hospital-acquired infection (HAI), accounting for > 20% of all HAIs [1], including both hospital-acquired pneumonia (HAP) and ventilator-associated pneumonia (VAP) [2]. HAP and VAP pose a severe risk to hospitalized patients, a challenge to clinicians, and a burden to the healthcare system due to high mortality [3], therapeutic complexity, prolonged hospitalization of patients, and consequently huge socio-economic costs [4]. The poor prognosis of HAP and VAP is often associated with unsuccessful antibiotic therapy, consequent to infection by multidrug-resistant (MDR) bacterial pathogens. Methicillin-resistant *Staphylococcus aureus* (MRSA) and MDR Gram-negative pathogens, including *Acinetobacter baumannii*, *Enterobacteriaceae*, and *Pseudomonas aeruginosa* are the most common etiological agents in HAP and VAP [5,6]. Chronic lung infection by MDR *P. aeruginosa* is also the leading cause of morbidity and mortality in patients suffering from cystic fibrosis (CF), an autosomal recessive genetic disorder characterized by mutations of the CF transmembrane conductance regulator (CFTR) gene [7]. In CF patients, the obstruction of the distal airways with viscous secretions weakens bacterial clearance, thereby inducing a cycle of infection, inflammation, and mucus impaction, which inexorably leads to deterioration of lung function [8].

Irrespective of its classification, pneumonia caused by MDR pathogens poses a serious challenge to clinicians since the available antibiotic armamentarium is often ineffective. Recently, a report from EU [9], argue that the antimicrobial resistance has been estimated as responsible for 25,000 deaths per year in the EU alone and 700,000 deaths per year globally. Thus, new antibiotics and repurposing of existing ones, identification of new druggable bacterial targets, antimicrobial-like drugs and drug adjuvants are urgently needed [10-12]. A promising strategy to counteract bacterial pneumonia is the development of unconventional antibacterial drugs that exploit nutritional vulnerabilities of bacteria to inhibit their growth.

Iron is a key nutrient for pathogenic bacteria, being a cofactor for many redox enzymes involved in critical cellular functions [13]. The lung is typically an iron-poor environment where bacteria struggle

with the host's iron withholding capacity [14]. Given the essential role of iron in bacterial physiology and pathogenicity, disruption of bacterial iron metabolism represents a promising approach for the development of new antibacterials [15,16]. In this context, the post-transition metal gallium [Ga(III)] has successfully been repurposed as an iron-mimetic antimicrobial agent [17-19]. The chemical properties of Ga(III) are very similar to those of Fe(III), allowing Ga(III) to replace Fe(III) in the prosthetic group of several redox enzymes. However, differently from Fe(III), Ga(III) cannot be reduced under physiological conditions and, therefore, it cannot take part in redox reactions, ultimately impairing a number of essential functions [20, 21]. Work from our and other groups has shown that Ga(III) is endowed with both anti-bacterial and anti-biofilm activities on a wide range of pathogenic bacteria, including those responsible for nosocomial pneumonia [18, 22-25]. To date, some gallium compound, including gallium nitrate, gallium chloride, gallium maltolate and gallium citrate, have been developed in preclinical as antimicrobial drugs to evaluate the activity and the pharmacokinetics by oral, intraperitoneal, intramuscular and intra nasal route [22, 25-30]. The fascinating possibility of developing Ga(III)-based therapies to treat bacterial lung infection is corroborated by ongoing clinical trials (ClinicalTrials.gov identifiers NCT01093521; NCT02354859; NCT04294043; NCT03669614). In particular, the therapeutic use of Ga(III) is successfully supported by the results from completed clinical trials testing the pharmacokinetics, safety, tolerability, and efficacy of a pharmaceutical formulation of citrate-buffered gallium nitrate (GaN) by intravenous (i.v.) route in CF patients affected by *P.aeruginosa* lung infection (NCT01093521 and NCT02354859) [25]. It should be considered, however, that patients enrolled in these trials received a continuous infusion of GaN for 5 days, *via* the application of an i.v. catheter, an invasive procedure that causes patient's distress and requires specialized assistance at the patient site. At present, i.v. administration and the potential nephrotoxicity of long-term (> 5 days) Ga(III) administration are the major limitations to the practicability of Ga(III)-based antimicrobial therapy. Direct drug delivery to the respiratory tract is a viable approach to overcome these limitations [31, 32]. In this regard, there is an ongoing phase 1/2a clinical trial (NCT03669614) aimed at investigating the safety and

pharmacokinetics of gallium citrate, administered via inhalation, in healthy adult and *P. aeruginosa* infected cystic fibrosis subjects. No data are so far available. (https://www.clinicaltrials.gov/ct2/show/study/NCT03669614?term=NCT03669614&draw=2&rank=1).

It must be emphasized that drug delivery to the respiratory tract has become of choice for the treatment of CF lung infections. This route of administration not only improves the therapeutic efficacy by increasing drug concentration at the site of infection, but also limits side effects and improves patients' adherence to therapy [31-35]. Regarding nosocomial pneumonia, there are no inhaled antibiotics approved by the US Food and Drug Administration (FDA) for the treatment of HAP and VAP [36]. However, guidelines for the treatment nosocomial pneumonia recommend both inhaled and systemic antibiotics administration, rather than systemic antibiotics alone, for patients with VAP caused by aminoglycosides- or polymyxins- susceptible Gram-negative pathogens [2]. Adjunctive inhaled antibiotics can also be considered as a last resort in patient not responding to intravenous antibiotics [2].

To exploit the potential of gallium as an antibacterial agent, in this work we have performed *in vivo* experiments in rats to provide evidence supporting the opportunity to administrate GaN by aerosolization. In particular, we have investigated the potential advantages of using a solution of GaN administered intra-tracheally instead of intravenously, in terms of bio-distribution and acute toxicity.

2. Materials and Methods

2.1 Animals

Male Wistar rats (200-220 g, Charles River, Calco, Italy) were housed in the animal care facility of the Department of Pharmacy, University of Naples, in a controlled environment (temperature 21 ± 2 °C and humidity 60 ± 10 %) and provided with standard rodent chow and water *ad libitum*. All animals were allowed to acclimate for seven days prior to the experiments and were subjected to 12 h–12 h light-dark schedule. The experimental procedures, conformed to the guidelines of Italian and European Council low for animal care (EU Directive 2010/63/ EU and DL 26/2014), were approved by the Animal Ethics Committee of the University of Naples Federico II and by the Italian Ministry of Health that comply with the ARRIVE guidelines [37]. Rats were randomly divided in groups and differently treated, depending on protocols described below.

2.2 Treatments

Briefly, rats were anesthetized by an intraperitoneal injection (i.p.) of Zoletil (30 mg/kg) and xylazine (5 mg/kg) The amount of GaN (4 mg/kg) equivalent to 1 mg/kg of Ga(III) for all the treatments, or vehicle solution were intra-tracheally delivered (100 µl/rat) by a Microsprayer from PennCentury, as previously reported [38, 39]. A 2 mg/ml Ga(III) solution was prepared by dissolving GaN hydrate (8 mg/ml on anhydrous basis) in aqueous sodium citrate (9.2 mg/ml). After anesthesia, the cannula of the tracheal dispositive was inserted directly into the trachea through the rat mouth and at scheduled time intervals from intra-tracheal administration, treated and untreated groups were euthanized and used for the following evaluations. The differences in distribution were evaluated by comparison of data obtained from intra-tracheal (i.t.) and i.v. administration, at the same dose and time of treatments. All the reagents were purchased by Sigma Aldrich, Italy.

2.3 Ga(III) distribution upon intra-tracheal or intra venous administration

Ga(III) distribution was evaluated in different organs and biological fluids after either i.t. or i.v. administration. Briefly, after euthanasia, bronchoalveolar lavage fluid (BALF), bronchoalveolar cells (BALC), lung, kidney and liver were collected at scheduled time intervals (5, 30, 60 and 180 min

after treatments with vehicle or GaN). Briefly to obtain the bronchoalveolar lavage (BAL) the trachea was cannulated with a polyethylene tube (1 mm inner diameter) and lungs were washed once by flushing 4 ml of sterile ice-cold PBS. The BAL was centrifuged at 1200 rpm for 10 min at 14 °C to separate BALC from BALF. No significant difference among the volume (ml) of BAL harvested from each animal was observed, as reported in the supplementary figure S1. Peripheral blood was also collected and treated with solution of citrate (in the ratio 1 ml of 3.8 % citrate plus 9 ml of blood) to obtain plasma at different time points (0-180 min). Urine samples were collected from the start of treatment up to 30, 60 or 180 min.

The Ga(III) concentration was estimated by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) analysis, using an ICP-OES 710 Varian Spectrometer (Agilent Technologies). Samples were digested with low-metal-content concentrated HNO_3 (Sigma-Aldrich) at 90°C for at least one hour. Organ digestion was considered complete when the sample became clear. Each sample was diluted in double-distilled H_2O to lower the HNO_3 to 5% and subjected to ICP-OES measurements. The ICP-OES instrument was calibrated using a standard Ga(III) solution (TraceCERT®, Sigma-Aldrich), as previously described [40].

2.4 Systemic and local evaluations of Ga(III) acute toxicity after intra-tracheal administration

Acute toxicity was assessed by using male Wistar rats (as above described), and the GaN or vehicle solution were intra-tracheally administered after anesthesia. Animals were euthanized at scheduled time intervals (2, 3 and 14 days). In this case, BAL was obtained by lung washing (three times) by flushing 4 ml of sterile ice-cold PBS. The number and profile of cells from BALC were determined by Turk solution staining [41]. Lung homogenate was used to evaluate the protein expression of cyclooxigenase-2 (COX-2; 1:2000; code:610204, BD Transduction Laboratories), inducible nitric oxide synthase (iNOS; 1:1000; code:NB300-605, Novus), and MMP-9 (1:1000; code:Ab76003, Abcam) (a matrix metallopeptidase that plays important functions within neutrophil action, such as degrading extracellular matrix, activation of IL-1ß, and cleavage of several chemokines), by Western

blot analysis, as previously described [42]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:5000; code:G9545, Sigma Aldrich), was used as housekeeping protein to normalize protein expression. Sample of peripheral blood for plasma and serum collection was withdrawn to evaluate the following parameters: hematocrit, serum aspartate transaminase (AST), alanine transaminase (ALT) and creatinine as markers of hematic, hepatic, and kidney toxicity, respectively. Lactate dehydrogenase (LDH) was also measured as a marker of inflammation. Moreover, as general health index, the body weight of rats treated with vehicle or GaN was evaluated from the day of treatment up to 14 days.

2.5 Statistical analysis

Statistical analysis was performed using Graph Pad Prism (GraphPad Software, San Diego, CA). Data are expressed as mean ± S.E.M. and analyzed by one way ANOVA, followed by Bonferroni as post test. A p value < 0.05 was considered statistically significant.

3. Results

3.1 GaN distribution following intra-tracheal or intra venous administration

To evaluate whether the i.t. administration of GaN could increase Ga(III) localization in the lung (*i.e.* the target organ) compared with i.v. administration, GaN [1 mg/kg of Ga(III)] was administered to rats, either i.t. or i.v., and Ga(III) biodistribution was measured at different time points (*i.e.*, 5, 30, 60 and 180 min) by means of ICP-OES. Similar levels of Ga(III) were observed either in BALF or in lung homogenates for up to 180 min after i.t. administration (Figure 1A and B), suggesting a sort of equilibrium between the lung parenchyma and the alveolar lining fluid. Conversely, the i.v. administration of GaN resulted in very low Ga(III) levels either in BALF or in lung homogenates at any observation time (Figure 1 A and B). In fact, the concentration of Ga(III) detected after i.v. administration in both BALF and lung homogenates was lower than 1µg/ml at all time points. Remarkably, the amount of Ga(III) measured in BALF and in lung homogenates after i.t. administration, was significantly higher both at 5 and 30 min, compared to the i.v. administration (Figure 1A and B).

Ga(III) was also measured in the systemic circulation. As expected, high Ga(III) levels were measured in plasma immediately after i.v. administration, though it could also be detected after i.t. delivery (Figure 2A). A significant concentration of Ga(III) in plasma was already found 5 min after i.t. delivery, though it was undetectable immediately after administration (time 0). Ga(III) kinetics after i.t. delivery showed that the highest concentration was reached at 5 min (***$p<0.001$ *versus* 0 min) and decreased thereafter (Figure 2A). Much higher Ga(III) levels were observed in plasma immediately after i.v. administration (time 0), compared with i.t. delivery (Figure 2A; ***$p<0.001$ *versus* i.t. 0 min). Plasmatic Ga(III) was rapidly cleared 5 min after i.v. injection; Ga(III) concentrations determined at 5, 30, 60 and 180 min were significantly reduced compared to 0 min (°°°$p<0.001$), attaining similar concentrations as those observed after i.t. administration. Moreover, a lower concentration of Ga(III) was observed in plasma, compared with the concentrations reached in BALF and lung after i.t. administration (Figure 1 A and B, Figure 2A).

A time-course analysis of the total amount of Ga(III) excreted in urine is reported in Figure 2B; at 180 min the amount of Ga(III) eliminated in urine was significantly higher after i.v. administration than after i.t. administration ($^{\#}p<0.05$ i.v. *vs* i.t. at 180 min). A very low Ga(III) concentration (<0.5 µg/ml) was observed in the liver, after either i.t. or i.v. administration, at all observation times (Figure 3B). These results indicate that, irrespective of the administration mode, Ga(III) is eliminated primarily by the renal route. In fact, a significant increase of Ga(III) concentration in the kidney was observed at 60 and 180 min after i.v. administration, compared with i.t. administration (Figure 3A; *p<0.05 and °p<0.01 *vs* 60 and 180 min i.t., respectively).

To evaluate the persistence/distribution of Ga(III) in different organs after i.t. and i.v. administration routes, results were also expressed as total amount of Ga(III) in the lungs, kidneys and liver at each observation time (Figure 4). The total amount of Ga(III) measured in the lungs after i.t. administration confirmed the significant higher level and prolonged persistence of Ga(III), compared with measurements observed after i.v. administration (Figure 4A). Ga(III) was also detectable in BALC after i.t. administration (0.48 ± 0.06, 0.19 ± 0.11, 0.16 ± 0.026 and 0.75 ± 0.24 µg per BALC at 5, 30, 60 and 180 min, respectively), while it was undetectable after i.v. administration during the whole observation period.

Interestingly, the amount of Ga(III) in the kidneys was lower after i.t. than i.v. administration. In particular, at 60 and 180 min after i.t. administration the renal levels were significantly lower compared to the i.v administration (Figure 4B; $^{\S}p<0.05$ and °p<0.05 *versus* 60 and 180 min i.t., respectively).

Lastly, the amount of Ga(III) in the liver was extremely low (2-10 µg per organ) after both i.t. and i.v. administration (Figure 4C).

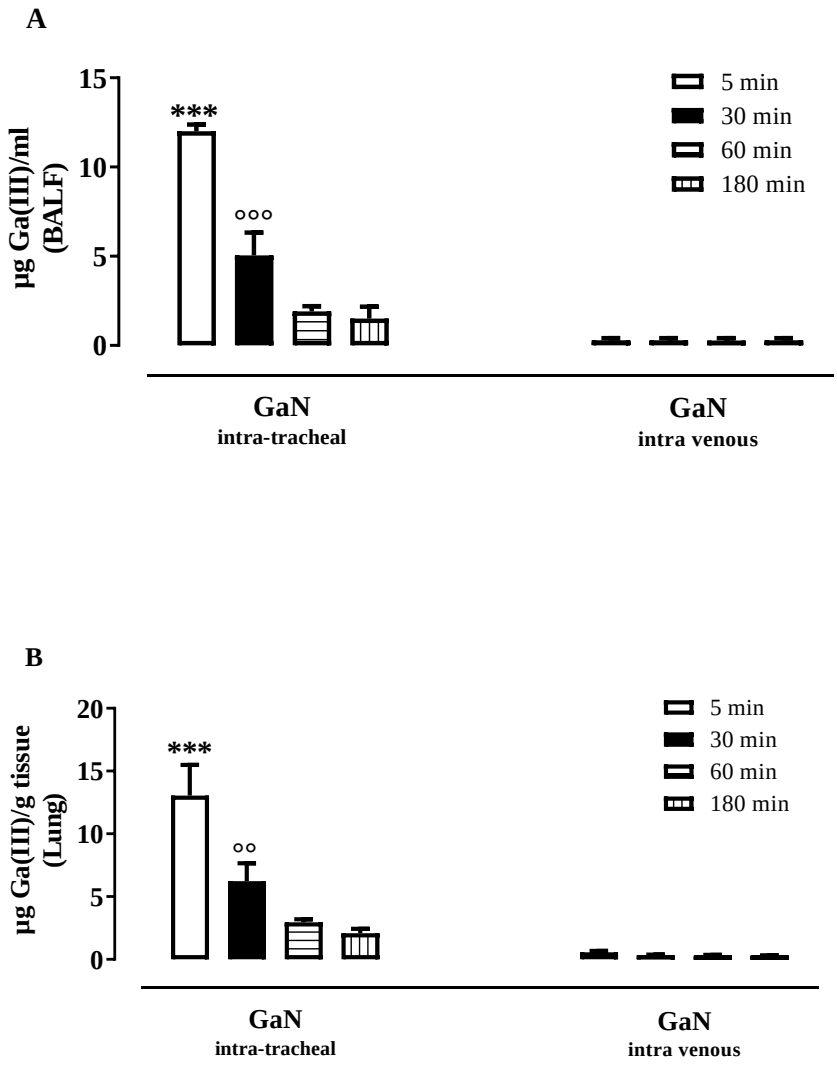


Figure 1. Ga(III) levels in BALF and lung. Ga(III) was measured at 5, 30, 60 and 180 minutes (min) after intra-tracheal (i.t.) or intra venous (i.v.) administration**. (A)** Ga(III) concentration in BALF was significantly higher at 5 and 30 min after i.t. compared to the i.v. administration at the same time points (***p<0.001 *vs* 5 min i.v; °°°p<0.001 *vs* 30 min i.v.). Data, expressed as µg Ga(III)/ml, are reported as mean ± SEM (n=3 rats in each treatment group). **(B)** Ga(III) concentration in the lung was significantly higher at 5 and 30 min after i.t. compared to the i.v. administration at the same time points (***p<0.001 *vs* 5 min i.v; °°p<0.01 vs 30 min i.v.). Data, expressed as µg Ga(III)/g tissue, are reported as mean ± SEM (n=3 rats in each treatment group).

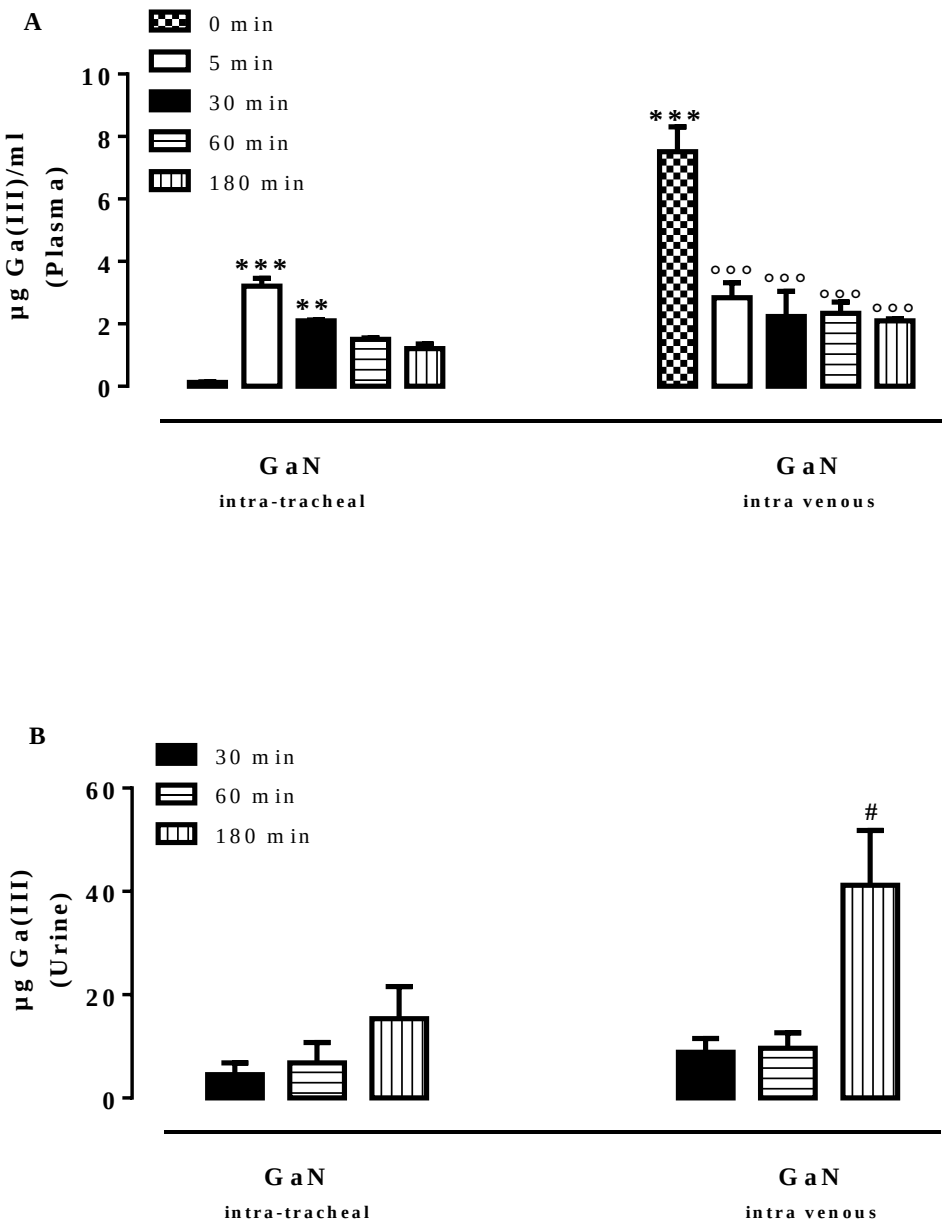


Figure 2. Ga(III) levels in plasma and urine. (A) Ga(III) concentration was evaluated in plasma at 0, 5, 30, 60 and 180 minutes (min) after intra-tracheal (i.t) or intravenous (i.v.) administration. Ga(III) concentration was significantly higher at 5 and 30 min after i.t. administration compared to time 0 (***$p<0.001$ and **$p<0.01$). Ga(III) detected at time 0 after i.v. administration was significantly higher than the value observed at time 0 after i.t. administration (***$p<0.001$). After 5, 30, 60 and 180 min from i.v. administration, Ga(III) concentration was significantly reduced compared to time

0 (***p<0.001, **p<0.01 vs 0 min i.t; °°°p<0.001 *vs* 0 min i.v.). Data, expressed as µg Ga(III)/ml, are reported as mean ± SEM (n=3 rats in each treatment group). **(B)** Ga(III) levels were evaluated in urine at 30, 60 and 180 min after i.t. or i.v. administration. Ga(III) detection was significantly higher at 180 min after i.v. administration, compared to i.t. administration at the same time point ($^{\#}$p<0.05 vs 180 min i.t). Data, expressed as µg Ga(III), are reported as mean ± SEM (n=3 rats for 30 min, n=5 rats for 60 min and n=6 rats for 180 min).

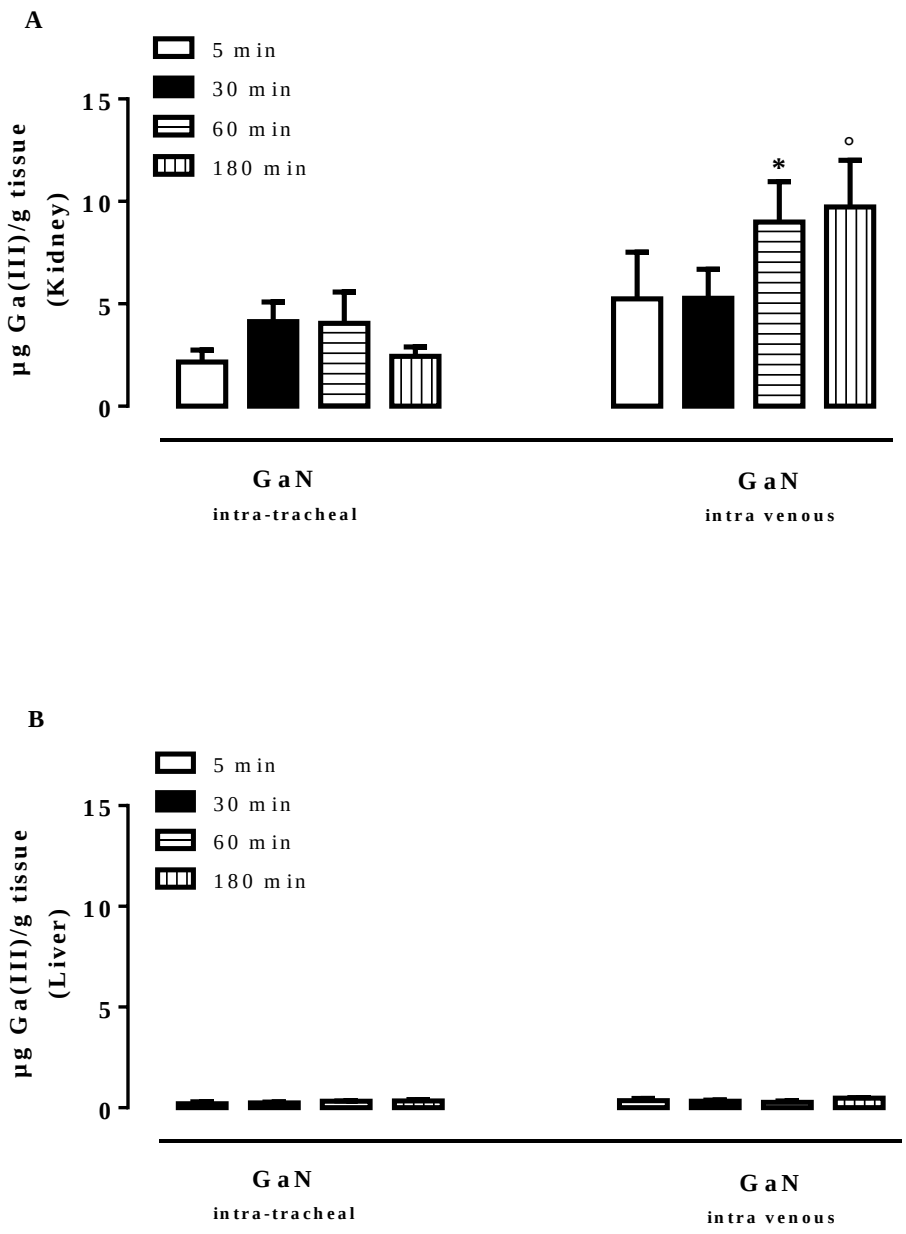


Figure 3. Ga(III) levels in kidney and liver. Ga(III) was measured at 5, 30, 60 and 180 minutes (min) after intra-tracheal (i.t) or intravenous (i.v.) administration. **(A)** Ga(III) concentration in the kidney was significantly higher at 60 and 180 min after i.v. compared to the i.t. administration at the same time points (*$p<0.05$ *vs* 60 min i.t; °$p<0.05$ *vs* 180 min i.t.). Data, expressed as µg Ga(III)/g tissue, are reported as mean ± SEM (n=3 rats in each treatment group). **(B)** Ga(III) determination in liver was very low at all considered time point after both i.v, or i.t. administration. Data, expressed as µg Ga(III)/g tissue, are reported as mean ± SEM (n=3 rats in each treatment group).

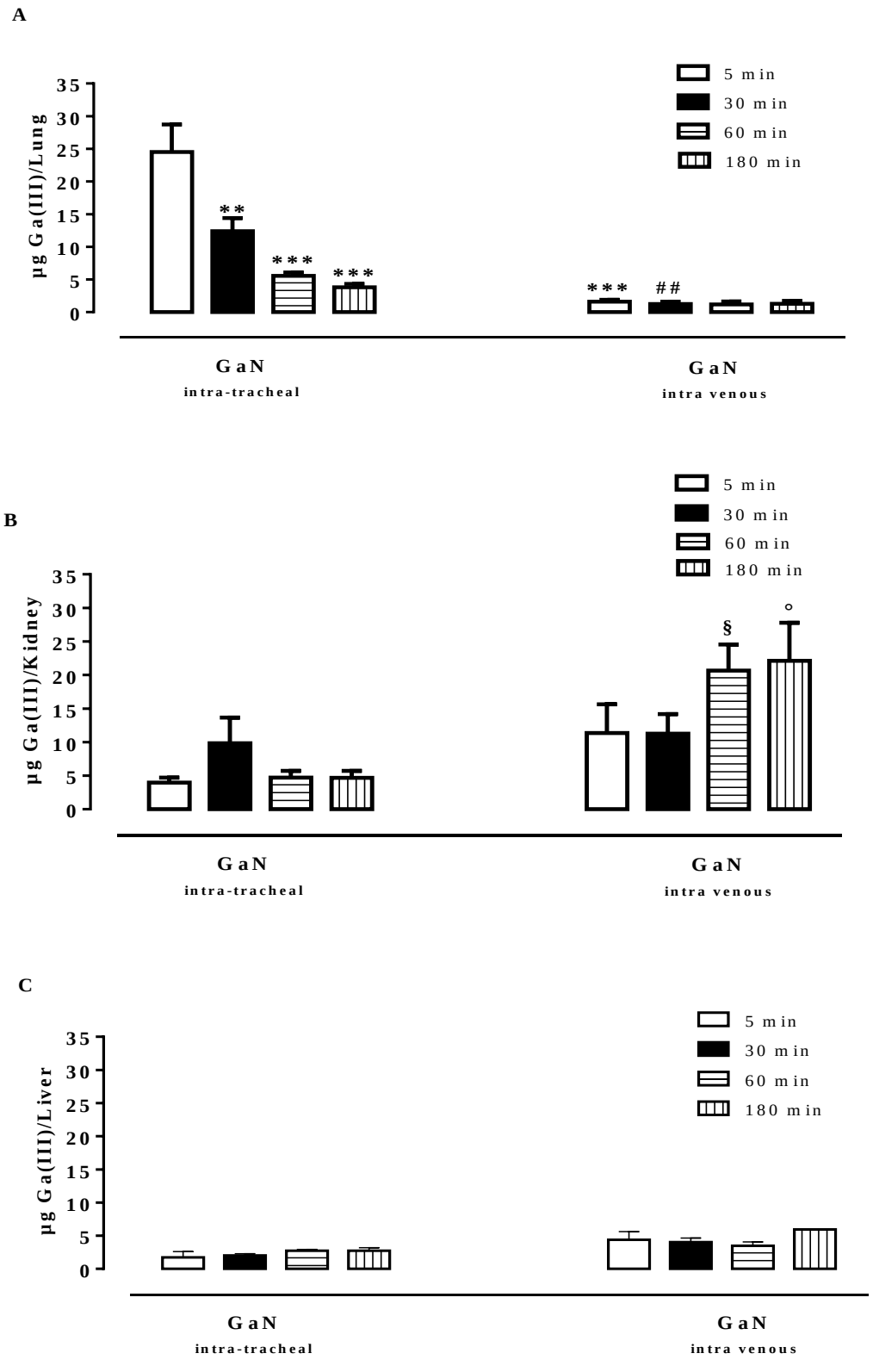

A
µg Ga(III)/Lung
35
30
25
20
15
10
5
0
5 min
30 min
60 min
180 min
**
***
***
***
##
GaN
intra-tracheal
GaN
intra venous
B
µg Ga(III)/Kidney
§
°
C
µg Ga(III)/Liver

Figure 4. Total amount of Ga(III) in lung, kidney and liver. Ga(III) was measured at 5, 30, 60 and 180 minutes (min) after intra-tracheal (i.t.) or intravenous (i.v.) administration**. (A)** Ga(III) level in the lung was significantly reduced at 30, 60 and 180 min after i.t. administration compared with the level observed at 5 min. The total amount of Ga(III) after i.v. administration was significantly lower at 5 and 30 min compared to the level observed after i.t. administration at the same time points (**p<0.01, ***p<0.001 vs 5 min i.t; ##p<0.01 vs 30 min i.t.). Data, expressed as µg Ga(III)/lung, are reported as mean ± SEM (n=3 rats in each treatment group). **(B)** The total amount of Ga(III) detected in the kidneys was significantly higher at 60 and 180 min after i.v. compared to the i.t. administration at the same time points (§p<0.05 vs 60 min i.t.; °p<0.05 vs 180 min i.t.). Data, expressed as µg Ga(III)/kidney, are reported as mean ± SEM (n=3 rats in each treatment group). **(C)** Total Ga(III) determination in liver was extremely low at all time points after both i.v. or i.t. administration. Data, expressed as µg Ga(III)/liver, are reported as mean ± SEM (n=3 rats in each treatment group).

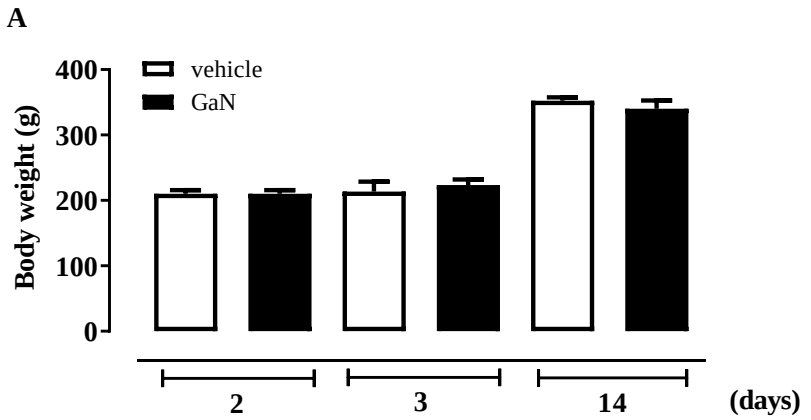
3.2 Local and systemic assessment of Ga(III) toxicity after intra-tracheal administration

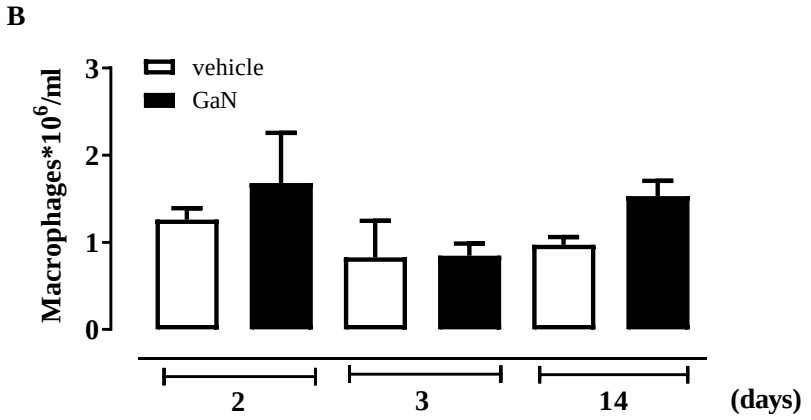
The acute toxicity of Ga(III) was evaluated after a single i.t. administration by monitoring the rat body weight, the expression of inflammation markers, and systemic toxicity parameters for up to 14 days.

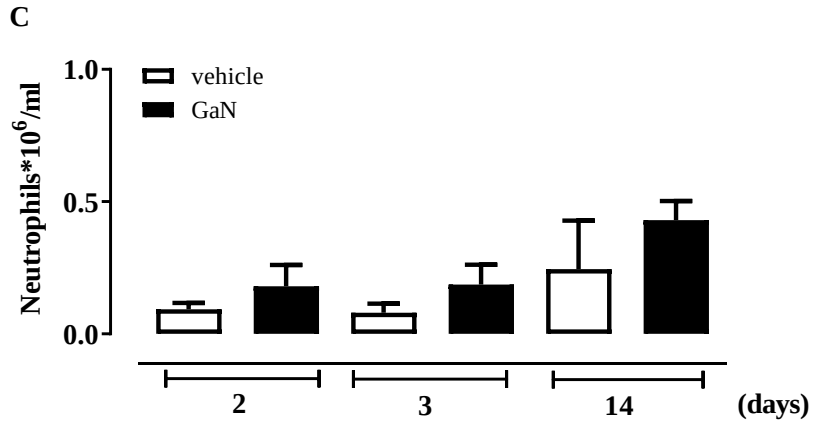
No significant change in rat body weight was observed for the whole duration of the experiment (Figure 5A). Local toxicity within the lung was investigated by inflammatory cell count and no significant changes in macrophage and neutrophil numbers were observed in BAL of animals treated with GaN, compared with the vehicle (Figure 5B and 5C).

To gain further insights into the safety of i.t. administrated Ga(III), the expression of inflammation markers including COX-2, iNOS and MMP9 was investigated in the lung homogenates by Western blotting (Figure 6A). No significant difference in protein expression between Ga(III)-treated and untreated rats was observed. The expression of COX-2 (Figure 6B), iNOS (Figure 6C), and MMP-9 (Figure 6D) proteins did not appear significantly increased after i.t. Ga(III) delivery for up to 14 days, compared with the vehicle.

To investigate any systemic toxicity caused by a single dose of i.t. GaN administration, several biomarkers were evaluated. No hemolysis was observed since neither the red blood cell (RBC) number nor the haemoglobin (Hgb) content showed significant differences between vehicle- and GaN-treated rats for up to 14 days (Figure 7). Moreover, hematocrit levels (Hct) were unchanged between vehicle and GaN groups (Figure 7), as a general index of health. Also, the levels of LDH, an enzyme released during tissue damage and considered a marker of injury and disease, were undistinguishable between GaN- and vehicle-treated groups (Figure 7). This result agrees with the roughly similar number of circulating white blood cells (WBC), *e.g.*, lymphocytes (LY), monocytes (MO), and granulocytes (GR) in the two groups (Figure 7). Both hepatic and kidney acute toxicity can be excluded since AST, ALT, creatinine and urea levels were comparable in GaN- and vehicle-treated rats (Figure 7).


A
vehicle
GaN
Body weight (g)
400
300
200
100
0
2
3
14
(days)


B
vehicle
GaN
Macrophages*10^6/ml
3
2
1
0
2
3
14
(days)


C
vehicle
GaN
Neutrophils*10^6/ml
1.0
0.5
0.0
2
3
14
(days)

301

Figure 5. Acute toxicity of Ga(III) intra-tracheal treatment after 2, 3 or 14 days from administration.

(A) Ga(III) did not alter the body weight compared to the vehicle, at all time points. Data expressed as grams (g), are reported as mean ± SEM (n=3 rats for vehicle and GaN at 2 days; n=3 rats for vehicle and GaN at 3 days; n=4 rats for vehicle and GaN at 14 days). **(B)** The concentration of macrophages was similar in rats treated with Ga(III) or with vehicle. Data reported as macrophages*10^6/ml, are expressed as mean ± SEM (n=3 rats for vehicle and GaN at 2 days; n=3 rats for vehicle and GaN at 3 days; n=4 rats for vehicle and GaN at 14 days). **(C)** The concentration of neutrophils did not increase after Ga(III) treatment compared to the vehicle. Data reported as neutrophils *10^6/ml, are expressed as mean ± SEM (n=3 rats for vehicle and GaN at 2 days; n=3 rats for vehicle and GaN at 3 days; n=4 rats for vehicle and GaN at 14 days).

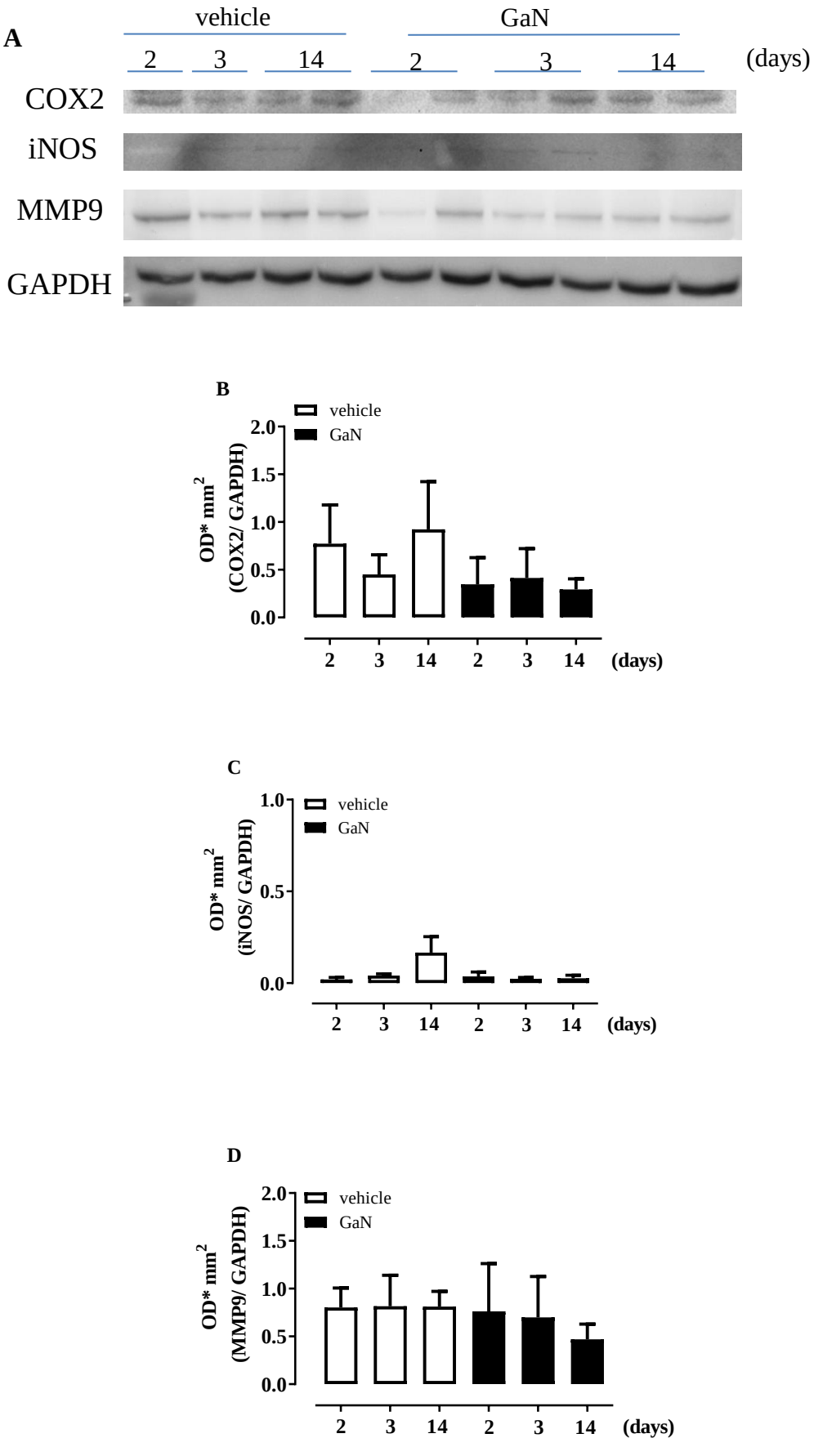

A
vehicle
GaN
2
3
14
2
3
14
(days)
COX2
iNOS
MMP9
GAPDH
B
vehicle
GaN
OD* mm2
(COX2/ GAPDH)
2.0
1.5
1.0
0.5
0.0
2
3
14
2
3
14
(days)
C
vehicle
GaN
OD* mm2
(iNOS/ GAPDH)
1.0
0.5
0.0
2
3
14
2
3
14
(days)
D
vehicle
GaN
OD* mm2
(MMP9/ GAPDH)
2.0
1.5
1.0
0.5
0.0
2
3
14
2
3
14
(days)

Figure 6. Western blot analysis in lung homogenates of rats treated by intra-tracheal route with vehicle or Ga(III) for 2, 3 or 14 days. (A) A representative Western blot for COX2, iNOS, MMP9 expression analysis. GAPDH was used as housekeeping protein. **(B)** COX2 is weakly expressed after treatment with both vehicle or Ga(III) at all time points. **(C)** the expression of iNOS is feeble in all groups analyzed. **(D)** MMP9 is faintly expressed in lung homogenates of rats treated with both vehicle or Ga(III). Results are normalized against GAPDH as housekeeping protein. Data reported as mean ± SEM are expressed as OD*mm^2. (n=3 rats for vehicle and GaN at 2 days; n=3 rats for vehicle and GaN at 3 days; n=4 rats for vehicle and GaN at 14 days).

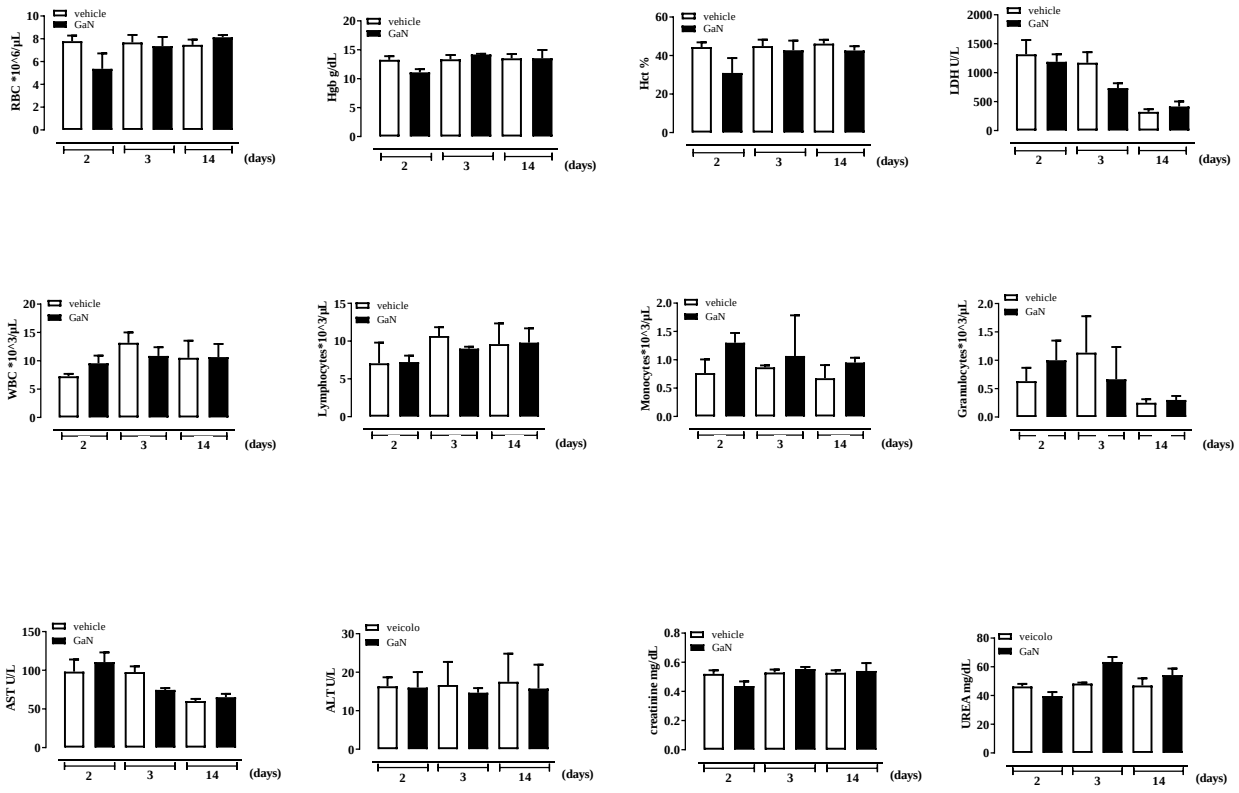


Figure 7. Hematologic and serum biochemical analyses of rats treated by intra-tracheal route with vehicle or Ga(III) for 2, 3 or 14 days. Red blood cell (RBC) numbers (expressed as RBS*10^6/µL), hemoglobin content (Hgb, expressed as g/dL), hematocrit levels (Hct, expressed as %) as well as LDH (expressed as U/L) were not statistically different between vehicle- and Ga(III)-treated rats at each time of observation. Treatment with Ga(III) did not cause significant changes in levels of circulating white blood cells (WBC, expressed as WBC*10^3/µL), lymphocytes (LY, expressed as lymphocytes*10^3/µL), monocytes (MO, expressed as monocytes*10^3/µL) as well as granulocytes (GR, expressed as granulocytes*10^3/µL), relative to the vehicle-treated group. Similarly, AST (expressed as U/L), ALT (expressed as U/L), creatinine (expressed as mg/dL) and urea (expressed as mg/dL) were not significantly different after treatment with Ga(III). Data are reported as mean ± SEM (n=3 rats for vehicle and GaN at 2 days; n=3 rats for vehicle and GaN at 3 days; n=4 rats for vehicle and GaN at 14 days).

4. Discussion

Ga(III) has extensively been used as a therapeutic agent for the treatment of cancer, autoimmune diseases and bone resorption disorders [43,44]. A commercial formulation of GaN has been approved by the FDA to treat hyper-calcemia, a disorder often associated to various types of cancer. The approved posology of GaN is in the range of 100-200 mg/m^2 daily for 5 consecutive days by i.v. route. The therapeutic schedule is a consequence of the unfavorable pharmacokinetics of Ga(III). The average plasma clearance of Ga(III) is 0.15 L/h *per* kg. Ga(III) is not metabolized in the liver or the kidney, but it is significantly excreted in urine, since more than half of the administered dose of Ga(III) is generally excreted within 24 h [45]. Nephrotoxicity is the most serious among the side effects associated to Ga(III)-based therapy [46]. GaN treatment causes proteinuria, characterized by an increase in blood urea nitrogen and serum creatinine, and a consequent decrease in creatinine clearance. To mitigate side effects, a slow i.v. infusion of Ga(III) is recommended, since a precipitation of the metal following rapid infusion was observed in the kidney, representing the primary cause of Ga(III) toxicity. Another dose-related toxicity is the microcytic hypochromic anemia, which is generally mild (< 3 g/dl decrease in hemoglobin) and shows clinically important signs only at high bolus i.v. doses [45]. Thus, it is evident that lowering of the systemic absorption is a key factor to reduce the renal concentration, hence toxicity. The scheme of treatment for the use of GaN as an anti-*Pseudomonas* drug in CF patients enrolled in the clinical trials was the same approved by the FDA for the treatment of hyper-calcemia [25]. However, subjects were also instructed to consume 2 liters of fluids above their normal intake during the infusion period [25]. While hydration helps to avoid nephrotoxicity, it certainly reduces Ga(III) concentration in the lung, i.e. the target organ for antibacterial activity.

To overcome Ga(III)-related toxic effects, local lung treatment could have undisputed advantages, since it is a non-invasive route which aids the drug to concentrate in the target organ, thereby avoiding or limiting the well-known systemic side effects. To corroborate this hypothesis, we have investigated the advantages of using a solution of GaN administered i.t. instead of i.v., in terms of bio-distribution

and potential toxicity. Our *in vivo* results prove the possibility to use Ga(III) for inhalation therapy since, at least after a single dose and up to 14 days, no sign of severe toxicity was observed either locally (in the lung) or systemically. By comparison with the untreated animals, the rat body weight, regarded as general index of health, was unmodified by i.t. Ga(III) administration during the 14 days observation. Inflammation markers were unaffected by i.t. GaN administration and no obvious signs of lung toxicity were observed, given that both neutrophil and macrophage counts in BAL were similar between treated and untreated animals, at all the scheduled time intervals. Moreover, in lung homogenates the expression of canonic proteins involved in acute inflammatory process *i.e.*, COX-2 and iNOS, were unmodified compared with the untreated control. MMP9, along with elastase, is a regulatory factor in neutrophil migration across the basement membrane, and plays several important functions within neutrophil action, such as degradation of the extracellular matrix, activation of IL-1β, and cleavage of several chemokines. Of note, the expression of MMP9 was unmodified in lung homogenates of rat treated with an i.t. GaN solution, supporting the non-toxic effect of i.t.-administered Ga(III). Overall, our data also argue against systemic toxicity, particularly renal and hematic toxicity, the most common side effects associated with Ga(III) treatment in humans. Most importantly, we have shown that Ga(III) concentration in the lung is significantly higher when GaN is administered i.t. than i.v.

Interestingly, recent results from the clinical trial on the efficacy of GaN as an anti-*Pseudomonas* agent in CF patients showed that the concentration of GaN in human sputum was less than 1 µg/ml after 5 days of continuous treatment [25]. This GaN concentration can easily be achieved by local treatment, while it is far to be reached by the i.v. route, as demonstrated by our results. Moreover, the improvement in respiratory function (FEV) observed in GaN-treated CF patients was not accompanied by a significant change in the *P. aeruginosa* load in lungs 14 or 28 days after the onset of treatment [25]. Indeed, using the dosage approved by FDA, the mean and the median *P. aeruginosa* density even if declined in treated patients, was non statistically significant (5.5 million and 1.8 million CFU/g between days 0 and 14 and by 29.8 million and 3.9 million CFU/g between days 0 and

28 [25]. Due to the limited number of patients included in this clinical study it is not yet possible to determine whether gallium significantly reduces the bacteria load, in this context the possibility to increase gallium at the target organ might favor this effect. This latter finding could be related to the low concentration of Ga(III) that can be reached in the lung after i.v. administration of GaN. Our data showed a significantly higher concentration of Ga(III) after i.t. administration, and this could result in stronger inhibition of *P. aeruginosa* and other bacterial pathogens causing HAP. Active uptake and accumulation of some antibiotics by alveolar macrophages have been demonstrated to improve the antimicrobial activity [47]. We revealed detectable amount of Ga(III) also in BALC after i.t. administration, while Ga (III) was undetectable in BALC after i.v. administration. These data could contribute a more significant antibacterial effect of Ga(III) by using inhaled route instead of the conventional i.v. route.

In essence, our results confirmed the unfavorable kinetics of i.v.-administered Ga(III), since low concentrations of Ga(III) were measured in the BAL and lung homogenates after i.v. injection, together a high concentration in urine and in kidneys. Conversely, plasma levels of Ga(III), 5 min after i.t. administration, were significantly lower compared with i.v. administration. A fast drop in Ga(III) plasma levels was observed 5 min after i.v. injection, confirming the short half-life of circulating Ga(III). Our data highlight the therapeutic advantages of i.t. Ga(III) administration; besides persisting in the lung, the total amount of Ga(III) measured after 180 min in urine was significantly lower compared to the i.v. administration. A similar profile was also observed at the renal level, in line with the notion that Ga(III) tends to concentrate and precipitate in the kidney [48, 49], *i.e.* the target organ for toxicity. The pharmacokinetics of gallium has already been addressed by using different gallium compounds and routes of administration. Choi et al. (2018) showed the antimicrobial activity Ga(III) tetraphenylporphyrin and its nanoparticle GaNP towards non-tuberculous mycobacteria upon intra peritoneal and intramuscular injection. Interestingly, the intraperitoneal route resulted more effective than the intramuscular route in delivering gallium to the lung [27]. It was also shown by the same group that a combination of $Ga(NO_3)_3$ and Ga porphyrin

(GaPP) exhibited synergistic inhibitory activity against the growth of different mycobacteria with highest *in vitro* and *in vivo* activity against *M. abscessus*. Of note, the intranasally administered $Ga(NO_3)_3$/GaPP combination showed significant antimicrobial activity in mice infected with *M. abscessus* with an increase of Ga(III) level in lung compared to spleen [28]. These results are in line with previous data showing potent activity of intranasally administered $Ga(NO_3)_3$ towards *P. auruginosa* [22].

Thus, taking together our results on the intratracheal route of administration with the previous data on intra peritoneal, intramuscular, oral and intranasal administration of gallium compounds, it is clear that the unfavorable and well-known kinetics of Ga(III) by i.v route encourages the use of alternative administration routes, e.g. i.t. administration, to maximize its antibacterial properties. In conclusion, all the advantages expected by using the i.t. administration instead of the i.v. are easily understandable by comparing the total Ga(III) content in different organs. A high and probably therapeutically effective level of Ga(III) within the lung (the drug activity target) and a low level in the kidney (the drug toxicity target) are achieved.

5. Conclusions

Ga(III) is emerging as a last-resort drug to combat lung infections sustained by otherwise untreatable pan-resistant bacteria. The use of the i.t. route of administration could improve Ga(III) therapeutic effects, by minimizing the related risks. At this stage of evidence, the direct administration of Ga(III) to the lung appears feasible and safe. The safety in humans seems to be confirmed for healthy patients (press release by the company) enrolled in the ongoing clinical trial on inhaled gallium citrate (NCT03669614) although, to date, the results have not yet been disclosed on ClinicalTrials.gov. The possibility to further modify the kinetics of Ga(III) by i.t. route with a specific formulation for aerosol treatment of bacterial pneumonia is even more desirable and deserves further studies.

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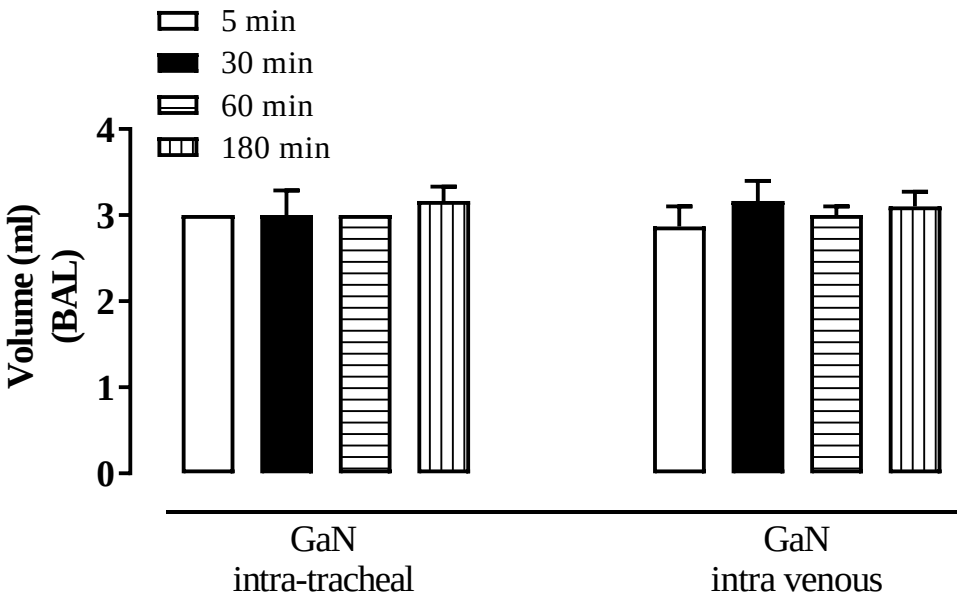
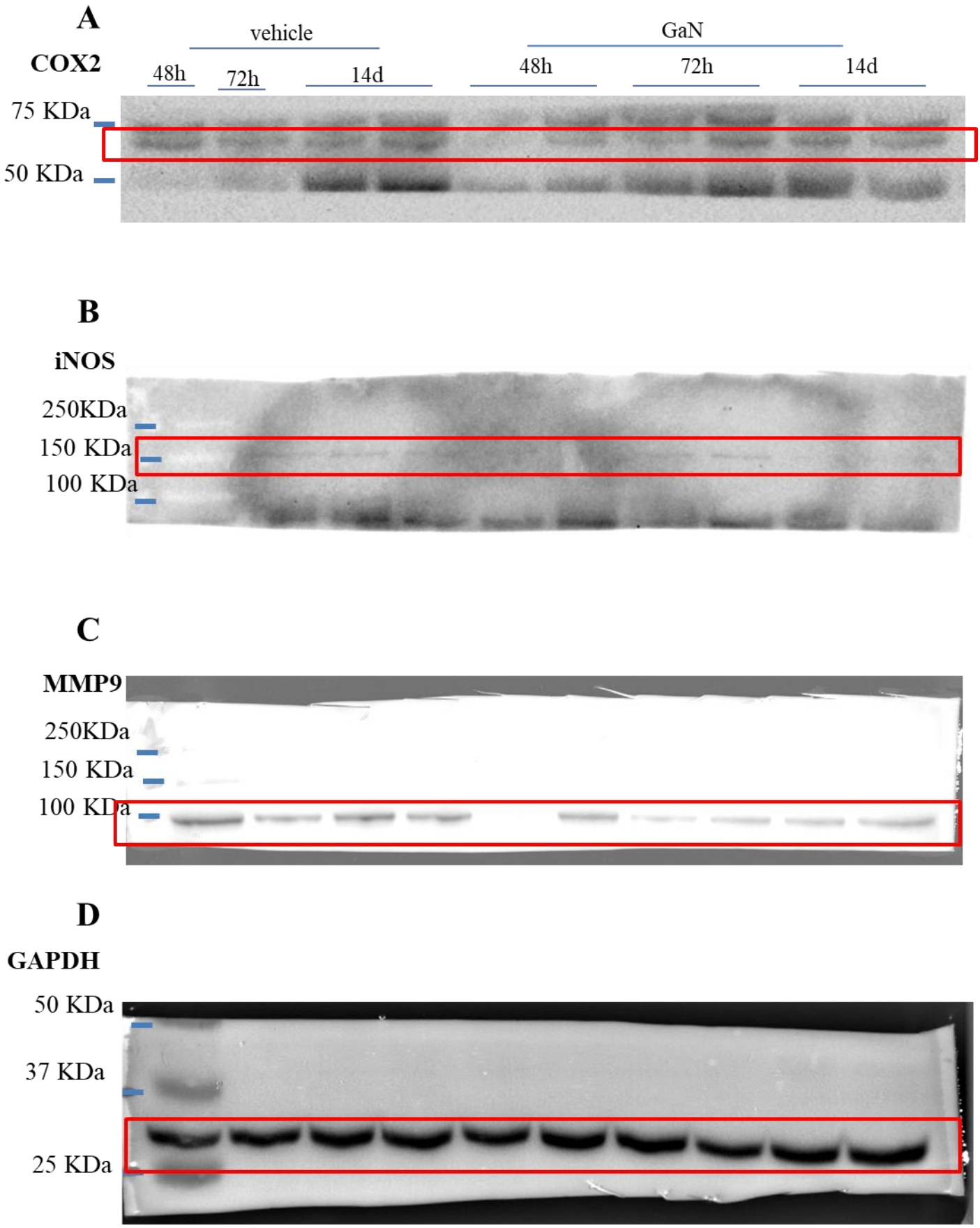
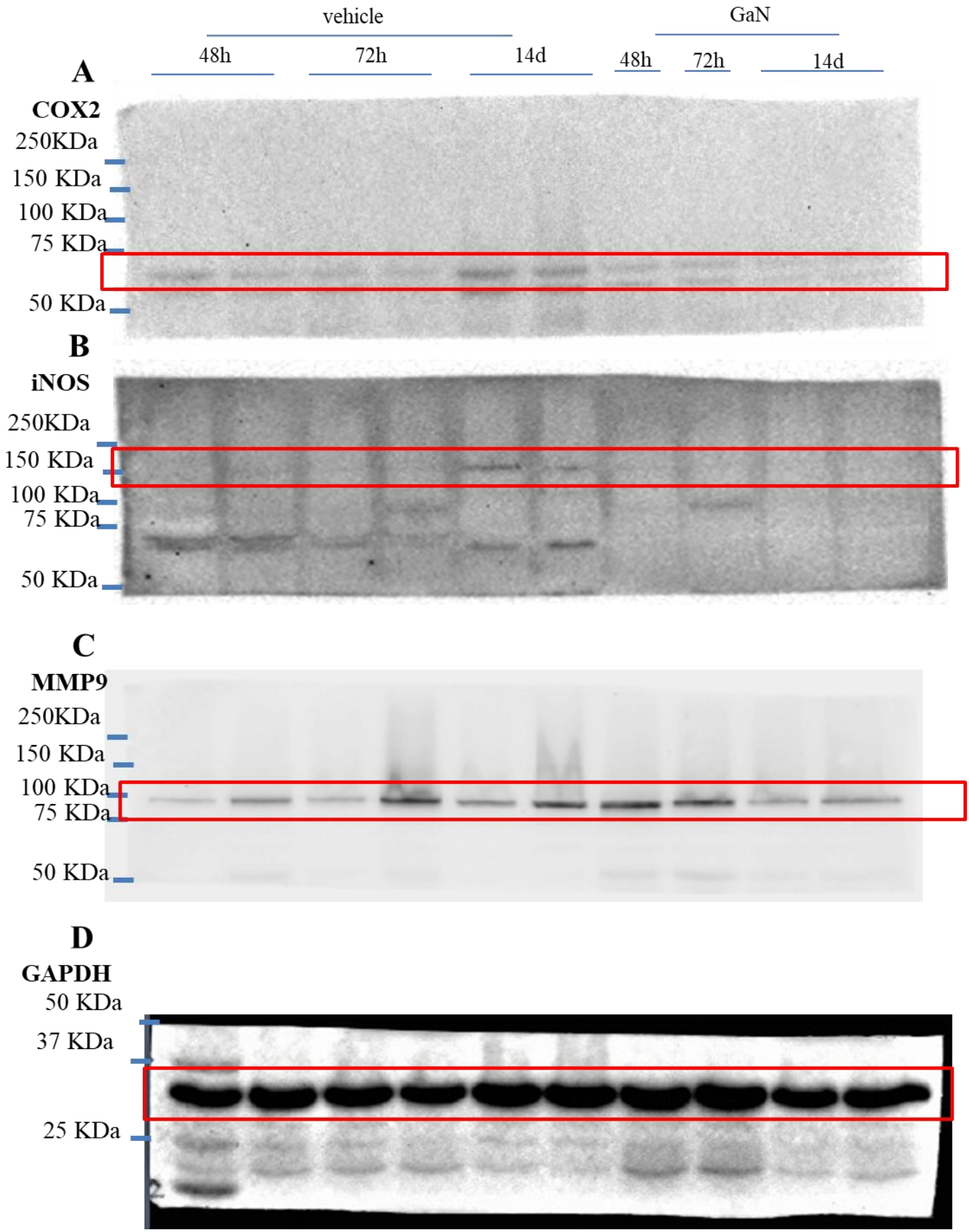


Supplementary Figure S1. BAL volume. BAL volume was measured at 5, 30, 60 and 180 minutes (min) after intra-tracheal (i.t.) or intra venous (i.v.) administration**.** The volume was similar at all time point considered after i.t. or i.v. administration. Data, expressed as volume (ml) of BAL, are reported as mean ± SEM (n=3 rats in each treatment group).



Supplementary Figure S2



Supplementary Figure S3

Figure legend

Figure 2 and 3: Uncropped western blot. In order to visualize all pattern of proteins on the same samples, the membrane was cut at 50 kDa. The upper slice was incubated with COX2 antibody (Figure 2A and 3A) and lower part was incubated with GAPDH (Figure 2D and 3D). After development the upper slice was cut again between 100kDa and 75 kDa; the upper part was incubated with iNOS antibody(Figure 2B and 3B) and lower with MMP9 antibody (Figure 2C and 3C).