

# Dose-Finding Study and Pharmacokinetics Profile of the Novel 13-Mer Antisense miR-221 Inhibitor in Sprague-Dawley Rats

Maria Teresa Di Martino,<sup>1</sup> Mariamena Arbitrio,<sup>2</sup> Daniele Caracciolo,<sup>1</sup> Francesca Scionti,<sup>1</sup> Pierosandro Tagliaferri,<sup>1</sup> and Pierfrancesco Tassone<sup>1</sup>

<sup>1</sup>Department of Experimental and Clinical Medicine, Magna Graecia University, Catanzaro, Italy; <sup>2</sup>CNR-Institute for Biomedical Research and Innovation, Catanzaro, Italy

**miR-221 is overexpressed in several malignancies where it promotes tumor growth and survival by interfering with gene transcripts, including p27Kip1, PUMA, PTEN, and p57Kip2. We previously demonstrated that a novel 13-mer miR-221 inhibitor (locked nucleic acid [LNA]-i-miR-221) exerts antitumor activity against human cancer with a pilot-favorable pharmacokinetics and safety profile in mice and non-naive monkeys. In this study, we report a non-good laboratory practice (GLP)/GLP dose-finding investigation of LNA-i-miR-221 in Sprague-Dawley rats. The safety of the intravenous dose (125 mg/kg/day) for 4 consecutive days, two treatment cycles, was investigated by a first non-GLP study. The toxicokinetics profile of LNA-i-miR-221 was next explored in a GLP study at three different doses (5, 12.5, and 125 mg/kg/day). Slight changes in blood parameters and histological findings in kidney were observed at the highest dose. These effects were reversible and consistent with an *in vivo* antisense oligonucleotide (ASO) class effect. The no-observed-adverse-effect level (NOAEL) was established at 5 mg/kg/day. The plasma exposure of LNA-i-miR-221, based on C<sub>0</sub> (estimated concentration at time 0 after bolus intravenous administration) and area under the curve (AUC), suggested no differential sex effect. Slight accumulation occurred between cycles 1 and 2 but was not observed after four consecutive administrations. Taken together, our findings demonstrate a safety profile of LNA-i-miR-221 in Sprague-Dawley rats and provide a reference translational framework and path for the development of other LNA miR inhibitors in phase I clinical study.**

## INTRODUCTION

RNA therapeutics is an emerging field and a provocative challenge for the next decade.<sup>1,2</sup> Among novel strategies that may provide successful use of RNA-based therapeutics, the targeting of non-coding RNAs is creating a new valuable opportunity to be investigated in early clinical trials. MicroRNAs (miRNAs) are a class of highly conserved endogenous small non-coding RNAs (19–23 nt) that regulate gene expression by translational repression, mRNA cleavage, and mRNA decay, which translates in the modulation of several crucial cell pathways. In cancer, the role of miRNAs has been well depicted, since they may act as oncogenes, promoting tumor development by inhibiting

tumor suppressor genes, or as tumor suppressors by regulating oncogenes and/or genes that control cell differentiation.<sup>3–14</sup>

miR-221, a member of the miR-221/222 cluster gene, is located on the X chromosome and acts as an oncomiR in many human solid and hematologic malignancies. In these diseases, overexpression of miR-221 influences a large set of gene transcripts involved mainly in cell proliferation and apoptosis. We previously developed an original 13-mer oligonucleotide miR-221 inhibitor, named locked nucleic acid (LNA)-i-miR-221, which is a second-generation phosphorothioate (PS) antisense oligonucleotide (ASO), and takes advantages from LNA technology and PS backbone chemistry in terms of increased affinity to the target and resistance to nucleases. In the PS oligonucleotide, the sulfur atom replaces one of the non-bridging oxygen atoms in the internucleoside phosphate, increasing nuclease stability. We recently reported on the biological effects induced by LNA-i-miR-221<sup>15,16</sup> by specific inhibition of miR-221 and consequent modulation of its canonical targets, including p27Kip1, PUMA, PTEN, and p57Kip2, regulators of the cell cycle and apoptosis.<sup>17</sup> *In vitro* and *in vivo* studies demonstrated that LNA-i-miR-221 exerts strong anti-tumor activity, providing the first evidence of its efficacy against multiple myeloma (MM)<sup>15</sup> and other tumors.<sup>18</sup> Moreover, detectability of LNA-i-miR-221 in animals and tumor tissues as well as in plasma and urine specimens was demonstrated<sup>19</sup> together with a favorable pilot pharmacokinetics profile and rapid wide tissue distribution in mice and non-naive monkeys.<sup>20</sup>

In the translational aim toward a first-in-human study, we investigated the suitability of LNA-i-miR-221 for clinical use by a non-GLP as well as a GLP dose-finding investigation of this new agent in Sprague-Dawley rats. Our data provide a formal framework for the definition of the optimal pharmacokinetics and safety profile of

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**Correspondence:** Maria Teresa Di Martino, PhD, Department of Experimental and Clinical Medicine, Magna Graecia University, Viale Europa, 88100 Catanzaro, Italy.  
**E-mail:** [teresadm@unicz.it](mailto:teresadm@unicz.it)

**Correspondence:** Pierfrancesco Tassone, MD, Department of Experimental and Clinical Medicine, Magna Graecia University, Viale Europa, 88100 Catanzaro, Italy.  
**E-mail:** [tassone@unicz.it](mailto:tassone@unicz.it)



**Table 1. Main Organ Weights**

	Males	Females
Group	2	2
Dose level (mg/kg/day)	125	125
Examined animals	3	3
Final body weight	−2	−7
Adrenal Glands		
Absolute	−22	−13
Relative	−20	−8
Kidneys		
Absolute	+9	+10
Relative	+11	+18
Liver		
Absolute	+20	+20
Relative	+22	+29
Ovaries		
Absolute	NA	−21
Relative	NA	−16
Spleen		
Absolute	+12	−11
Relative	+13	−5

The differences in percent between treated and control animals are mentioned from 8% onward. The organs were weighed wet as soon as possible after dissection. The ratio of organ weight to body weight recorded immediately before sacrifice was calculated. There was a low number of animals per group (n = 3) for statistical analysis, and thus the significance of the organ weight changes was considered to be irrelevant. NA, not applicable.

LNA-i-miR-221, which is essential to move to a phase I clinical study (EudraCT: 2017-002615-33). Our findings also provide a reference translational path for the clinical development of other LNA miR inhibitors.

## RESULTS

### Rat Pilot Non-GLP Study

Rat toxicity studies were designed with the aim to evaluate the potential toxicity of LNA-i-miR-221. In a non-GLP study, LNA-i-miR-221 was administered at a high dose level of 125 mg/kg/day. This dose level was selected based on a previous monkey study and corresponds to the rat equivalent of maximum tolerated dose (MTD) of 8.75 mg/kg,<sup>20</sup> where, however, no toxicity was observed. The intravenous (i.v.) route of injection was selected since it is the intended mode of injection in the first-in-human clinical study. As shown in Table 1, treatment with LNA-i-miR-221 changed the ratio of main organ weights as compared to controls, where they are noted from 8% onward. In particular, increased weight in male kidney, spleen, and liver ranged from 9% to 20% of absolute values, while decreased weight in female spleen, adrenals, and ovaries ranged from −11% to −21% of absolute values. Despite the low number of animals per group, a relationship to LNA-i-miR-221 could not be excluded in these organs. A complete

macroscopic post-mortem examination performed on all principal animals (sacrificed on day 28) revealed only a tan discoloration in the kidneys from all treated males and two out of three females. This finding may correlate with the increased organ weights and has been related to LNA-i-miR-221 administration. No significant clinical signs have been related to the LNA-i-miR-221 administration. Under the experimental conditions of the study, the no-observed-adverse-effect level (NOAEL) has not been established. In addition, in this study an animal group was allocated only for pharmacokinetics (PK) investigations to evaluate the systemic exposure for sex differences and time course of the LNA-i-miR-221, following i.v. bolus administration at a dose level of 125 mg/kg/day during 4 consecutive days, for two cycles separated by a 10-day washout period. LNA-i-miR-221 was quantifiable in all plasma samples collected by blood sampling in both sexes. A low to moderate inter-animal plasma concentration variability was observed, with coefficient of variation (CV) values ranging from 4% to 40% and from 2% to 65% in males and females, respectively. LNA-i-miR-221 plasma concentration time profiles and all pharmacokinetics parameters are shown in Figure S1. The plasma exposure of LNA-i-miR-221, based on C<sub>0</sub> and AUC values, after multiple administrations at the highest dose (125 mg/kg/day), showed in males an apparent trend vs higher exposure, due to potential LNA-i-miR-221 accumulation. Nevertheless, as no descriptive statistic was applicable in these cases, no definitive conclusion can be given regarding these trends. Accumulation was observed between days 1 and 18 in both sexes, but no clear accumulation was observed after four consecutive administrations (between days 1 and 4 or between days 15 and 18).

### Pivotal GLP Rat Toxicity Study

The formal GLP rat toxicity study was designed on the perspective of the proposed clinical trial of LNA-i-miR-221 in advanced cancer patients. Three groups of five principal and three satellite animals per sex (male and female Sprague-Dawley rats) received LNA-i-miR-221, during 4 consecutive days for two cycles with a 10-day washout, by i.v. bolus injection at dose levels of 5, 12.5, and 125 mg/kg/day. The rationale of two cycles is within our project to combine miR-221/222 inhibition with melphalan, which results in restoring of cell sensitivity to alkylating agents, in accordance with to our *in vitro* and *in vivo* findings in preclinical models of MM.<sup>21</sup> Recently a renewed scientific interest on melphalan is emerging, and major efforts have been devoted to delineate the mechanisms underlying primary or acquired melphalan resistance. These efforts have already led to the design of novel regimens to overcome melphalan resistance or to improve its anti-tumor activity.<sup>22</sup> With the aim of providing the rationale for clinical trials investigating LNA-i-miR-221 plus melphalan in drug-refractory MM, our study recapitulates the melphalan treatment schedule. We designed a protocol with at least two treatment cycles to translate in the clinical setting for a minimum chance of exploring not only safety but also induction of the clinical response. The dose levels were selected based on the results of previous studies.<sup>15,20,21</sup> Based on the MM mouse xenograft models, we reported tumor growth inhibition at 25 mg/kg.<sup>15</sup> This dose has been selected as a therapeutic

**Table 2. Blood Parameter Changes**

	Day 19		Day 43	
	Vehicle $\pm$ SD	125 mg/kg/day $\pm$ SD	Vehicle $\pm$ SD	125 mg/kg/day $\pm$ SD
<b>Hematologic Parameter</b>				
NeutroHephil	0.95 $\pm$ 0.443* F	0.37 $\pm$ 0.08 F	1.81 $\pm$ 0.567 M	0.76 $\pm$ 0.057 M
Reticulocytes	2.88 $\pm$ 0.997** F	1.46 $\pm$ 0.219 F	2.39 $\pm$ 0.656 F	1.39 $\pm$ 0.354 F
Monocytes			0.55 $\pm$ 0.186 M	0.14 $\pm$ 0.028 M
LUC			0.19 $\pm$ 0.07 M 0.11 $\pm$ 0.05 F	0.08 $\pm$ 0.00 M 0.04 $\pm$ 0.00 F
Eosinophils	0.08 $\pm$ 0.032	0.14 $\pm$ 0.045		
<b>Biochemistry Parameter</b>				
Creatinine	22.32 $\pm$ 0.980 M 26.62 $\pm$ 0.972 F	35.58 $\pm$ 5.109 * M 31.26 $\pm$ 1.547 ** F	28.40 $\pm$ 1.563 M	35.58 $\pm$ 1.450 M
Urea	4.5 $\pm$ 0.68 M 4.9 $\pm$ 0.40 F	7.2 $\pm$ 1.77 * M 6.4 $\pm$ 0.61 F	4.5 $\pm$ 0.52 M 4.7 $\pm$ 0.38 F	6.9 $\pm$ 0.71 M 7.1 $\pm$ 1.27 F
Glucose	5.76 $\pm$ 0.534 M	7.75 $\pm$ 1.041** M	6.16 $\pm$ 0.855 M 6.42 $\pm$ 0.255 F	8.74 $\pm$ 0.184 M 8.69 $\pm$ 1.57 F
Triglyceride	0.51 $\pm$ 0.151 M	0.21 $\pm$ 0.037* M	0.82 $\pm$ 0.339 M	0.18 $\pm$ 0.071 M
ALAT	39 $\pm$ 4.3 M	59 $\pm$ 6.4* M	47 $\pm$ 10.2 M	34 $\pm$ 3.5 M

Blood parameter changes were observed at the end of the treatment period (day 19, n = 5/sex/group) and at the end of the recovery period (day 43, n = 3/sex/group) as measured in the animal group treated with 125 mg/kg/day of LNA-i-miR-221 or in the control (vehicle) group. M, male; F, female. \*p < 0.01, \*\*p < 0.05.

dose and used to identify the relative equivalent species doses for our formal toxicity studies, according to the Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals S6 (R1), Parent Guideline, 1997.<sup>23</sup> In the light of providing, with our findings, support for phase I clinical trial approval, the study was drawn with the same schedule established for the first-in-human study.

### Clinical Examination Results

The appearance of treatment-related clinical signs was monitored in both sexes at all treatment doses. Clinical signs limited to hematoma on the tail, chromodacryorrhea, or alopecia as well as soft pale feces were considered due to the administration procedure, since it was observed in the control group and in both sexes unrelated to LNA-i-miR-221 treatment. At 5 and 12.5 mg/kg/day, in both sexes, there were no changes in mean body weight, when compared to control group, over the whole study period. At 125 mg/kg/day, a moderate lower mean body weight was observed in males from day 22 to day 42 (i.e., 37 g difference versus controls on day 28, reaching statistical significance,  $p < 0.05$ ), whereas in females no relevant changes in mean body weight were observed. This decrease can be considered related to the LNA-i-miR-221 treatment but is of minor toxicological significance, and because it occurred in the absence of a decrease in food consumption and feeding efficiency, it did not occur in the female group, and it is not associated with signs of poor clinical condition (Supplemental Materials and Methods; Figure S2). Moreover, no changes were observed on breathing or on central nervous system (CNS) activity, and no ocular findings were noted at all doses. Detailed information about evaluations of clinical examinations are available in Supplemental Materials and Methods.

### Laboratory Investigation Results

In two time points during the study, on days 19 and 43, blood sampling was taken for analytical evaluations, including hematology and biochemistry.

**Hematology.** No relevant changes in hematology parameters were observed at the end of the treatment (day 19) in animals exposed to doses of 5 and 12.5 mg/kg/day. In the group treated at the highest dose, statistically significant dose-related 49%–71% decreases of neutrophil and reticulocyte counts were observed in females. At the end of the recovery period (day 43), in males we observed 64%–78% decreases of neutrophils, large unstained cells (LUCs), and monocyte counts, and a slightly lower reticulocyte count was still observed in females, associated with a 64% decrease of LUCs. In a single animal a decrease in eosinophil counts occurred, which was considered as incidental. These changes were considered related to the LNA-i-miR-221, but they were of minor significance and small magnitude. No changes were seen in white and red blood cells, and no clinical conditions were associated. Moreover, no changes in coagulation parameters were observed at all doses in both sexes. Detailed data are reported in Table 2 and Supplemental Materials and Methods.

**Biochemistry.** Regarding biochemistry on day 19, no relevant changes were observed in both sexes at 5 and 12.5 mg/kg/day, while at 125 mg/kg/day, increases of creatinine and urea concentrations, correlating with microscopic findings in the kidney (see below), were noted in both sexes with statistical significance. Specifically, changes in biochemistry included 1.2- to 1.6-fold higher creatinine levels in both sexes and a 1.6-fold higher urea concentration, a 1.4-

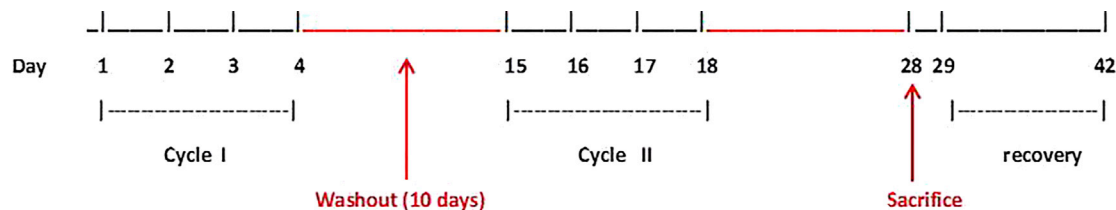
fold higher glucose concentration, a 0.4-fold lower triglyceride concentration, and 1.5-fold higher alanine aminotransferase (ALAT) activity in males. Furthermore, biochemistry at the end of the recovery period (day 43) still evidenced an increase of glucose and urea concentrations in both sexes, while in males a lower triglyceride concentration and a higher creatinine concentration were detected. However, the creatinine concentration in females and the ALAT activity in males returned to normal levels (Table 2). These changes were considered LNA-i-miR-221 related but were not adverse, taking into account the slight magnitude of changes. The urinary parameters did not change either at the end of the treatment (day 19) or at the end of the recovery period (day 43), at all doses and in both animal sexes.

### Pathology

**Organ Weights.** Measures of organ weight performed for all principal animals at the end of the second washout period (day 28) demonstrated that the LNA-i-miR-221 induced organ weight changes in liver, kidney, and spleen. Specifically, when compared with controls, minimal or slight increases in the mean absolute (+27;  $p < 0.01$ ) and relative (+28;  $p < 0.01$ ) weights of liver were seen in males and females at the highest dose. This increase correlated with foamy/granular Kupffer cells at microscopic examination. In addition, a minimal increase in the mean absolute and relative weights of kidney in males (+10; +11) and females (+11; +17), respectively, at the highest dose was recorded, even if not statistically significant. These increases correlated with microscopic tubular changes (basophilic granules, regeneration). Similarly, the slight increase of the absolute mean and weight of spleen in males (+23; +25) and decrease in females (−9; −4) at the highest dose correlated with the increased development of germinal centers observed microscopically. Finally, there were minimal increases in the mean absolute and relative weights of testes (+10; +12) in males treated at 125 mg/kg/day that correlated with the presence of interstitial granular macrophages observed at microscopic examination. Other organ weight changes were not considered to be related to LNA-i-miR-221, as they were small in amplitude, had no gross or microscopic correlates, and/or were not dose related in magnitude. Significant changes in mean absolute and relative organ weights in treated groups at the end of the second washout period are summarized in Table S1. Pathology examinations performed at the end of the recovery period in males treated at 125 mg/kg/day showed a minimal decrease of terminal body weight (−11% for the mean weight) as compared to controls, which could be considered related to LNA-i-miR-221. In females, a moderate increase of the absolute and relative weights of liver (+46%) and kidneys (+35%) were detected (Table S2). Only one male animal had a highest kidney weight (2.940 g) compared to controls, while in females a minimal increase in the absolute (+22%) and relative (+16%) weights of the spleen was noticed. In males, the slight increase in the mean relative organ weights for brain, epididymides, spleen, testes, and thyroids were considered to be due to reductions in body weight and not due to LNA-i-miR-221-related organ toxicity. Other differences in organ weights were minor and reflected the usual range of individual variations.

**Macroscopic Examination.** Macroscopic examination of organs performed at the end of the first washout period did not show macroscopic findings. At the end of the second washout period, LNA-i-miR-221 induced tan discoloration of the kidneys at 12.5 and 125 mg/kg/day doses, as well as enlargement of lymph nodes in one animal at the highest dose group. Tan color of the kidneys was observed in three out of five males and two out of four females at 12.5 mg/kg/day, and in four out of five males and in all three females at a 125 mg/kg/day dose treatment. This finding correlated microscopically, as below reported, with the presence of basophilic granules, indicative of the LNA-i-miR-221 accumulation, along with tubular regenerative and/or vacuolar lesions at the highest dose level. At the lower dose of 5 mg/kg/day, tan color of the kidneys was also noted in two out of five males and one out of four females, but in the absence of microscopic correlates, so this could be considered incidental and unrelated to the LNA-i-miR-221 treatment. Enlargement of the iliac lymph nodes was noted in one out of five males at the highest dose and correlated with microscopic examination, which showed LNA-i-miR-221-related foamy/granular macrophages. A scab was noted at the tip of the tail (i.e., injection site) and correlated with serocellular crust and deep ulcer evidenced at microscopic examination. The other macroscopic findings had no histologic correlates or correlated with common histologic findings in control rats, and were considered to be incidental. Finally, at the end of the recovery period, tan discoloration of the kidneys was found in one out of two females at the highest dose treatment. The meaning of this finding remained unclear because no microscopic examinations were performed on the recovery animals group. The few other macroscopic findings noted at the end of the recovery period are commonly recorded in the rat, and none can be considered related to the LNA-i-miR-221 treatment.

**Microscopic Findings.** Microscopic observation performed at the end of the second washout period showed events related to the administration of LNA-i-miR-221 in the kidneys, liver, and lymph nodes mainly, and were considered consistent with findings observed after systemic administration of single-stranded oligonucleotides.<sup>24</sup> These effects involved degeneration/regeneration and basophilic granules in the kidneys, as well as foamy/granular macrophages in most organs and tissues. Specifically, in the kidneys at doses of 12.5 and 125 mg/kg/day, basophilic granules, vacuolization, degeneration/necrosis, and regeneration of proximal tubular cells were found. Basophilic granules were characterized by variable sizes of dark blue/gray cytoplasmic granules located in proximal tubular cells, and they were generally associated with tubular cytoplasmic vacuolization (clear vacuoles) that was most prominent in females. Tubular regeneration was characterized by tubules lined by basophilic enlarged cells, with karyomegaly (i.e., large nuclei) and prominent nucleoli, and occasional mitotic figures. Degeneration/necrosis consisted of scattered necrotic cells and/or sloughed cells/debris in tubules, with rare casts. In addition, an occasional tubular dilation was observed. Incidence and severity of selected microscopic findings in the kidneys at the end of the second washout period are summarized in Table S3. Infiltrates of foamy and/or granular macrophages (i.e., with vacuolar and/or basophilic granular cytoplasm) were noted at the highest dose



**Figure 1. Rat Treatment Timeline**

LNA-i-miR-221 was administered for 4 consecutive days in two cycles: four administrations on days 1, 2, 3, and 4 for the first cycle, and four administrations on days 15, 16, 17, and 18 for the second cycle (eight administrations in total). A period of 10 days washout between the two cycles was considered. The day of sacrifice was on day 28. Day 1 corresponds to the first day of the treatment period. A recovery period ran from day 29 to day 42.

in most organs and tissues of LNA-i-miR-221-treated animals. The most pronounced changes were seen in the lymph nodes and in the liver (Table S4). Basophilic granular cytoplasm was characterized by small dark gray granules similar to those seen in renal proximal tubular cells. In all lymph nodes, large vacuolated and/or granular macrophages were seen in the sinusoids. In the liver, Kupffer cells were enlarged and had vacuolated and granular cytoplasm. There were also some aggregates of foamy/granular macrophages in portal tracts or around central veins. In two of three females, this was associated with a minimal increase in mitotic figures. Minimal or slight granular macrophages were seen in many other organs/tissues, e.g., interstitium of the bone marrow, testes and epididymides, ovaries, uterus, vagina, heart, adrenals, pancreas, salivary glands, Harderian glands, and joints. Finally, the spleen showed an increased development (i.e., increased numbers and size) of germinal centers (i.e., center of secondary lymphoid follicles, Table S5) in males and females at 125 mg/kg/day dose treatment when compared with controls. The few microscopic findings noted at the injection sites were considered to be related to the injection procedure and not to LNA-i-miR-221. Of note, at 125 mg/kg/day, one of three females showed minimal diffuse hypertrophy of the thyroid follicular cells. This isolated finding was considered to be most probably incidental and unrelated to LNA-i-miR-221. Other microscopic findings noted in treated animals were considered incidental changes, as they also occurred in controls, were of low incidence, had no dose relationship in incidence or severity and/or are common background findings for the rat.

Consequently, under this experimental conditions and based on the above-described evidence, the NOAEL was established at 5 mg/kg/day, which is the safety dose that ensures no adverse event.

### Pharmacokinetics

In the pivotal rat GLP study, systemic exposure was evaluated for sex difference and time course of LNA-i-miR-221 at low (5 mg/kg/day), middle (12.5 mg/kg/day), and high (125 mg/kg/day) doses following bolus injection for 4 consecutive days for two cycles with a 10-day washout (Figure 1). In addition, a recovery period ran from day 29 to day 42 to investigate the exposure of rats to LNA-i-miR-221. The dose levels were selected based on the results of previous equivalent monkey doses (2.5, 6.25, and 8.75 mg/kg) and non-GLP rat

(125 mg/kg) studies. LNA-i-miR-221 was not quantifiable in the plasma from control animals, whereas in treated groups LNA-i-miR-221 was quantifiable from 0.08 to 24 h post-administration in all treated groups, except for the group treated with the lower dose, specifically three males on day 1 and two females on day 4, where LNA-i-miR-221 was quantifiable up to 3 h post-administration. After multiple administrations at the highest dose (125 mg/kg/day), based on AUC and  $C_0$ , exposure increased slightly more than dose proportionally between 5 and 125 mg/kg/day in both sexes. The volume of distribution (Vd) and clearance (Cl) decreased slightly as the dose increased, especially between 12.5 and 125 mg/kg/day in both sexes (Table 3). No significant time effect pattern was observed, but a trend toward higher exposure in both sexes was observed at the highest dose level on days 15 and 18. Nevertheless, as no descriptive statistic was applicable in these cases, no definitive conclusion can be given regarding these trends. Certainly, the slight accumulation was observed in animals between cycles 1 and 2, with no clear accumulation during the four consecutive administrations (between the first day and the fourth day of administration of the first cycle or between the first day [day 15] and the fourth day [day 18] of the second cycle of administration). Specifically, on day 1 the  $AUC_{0-24h}$  (h·ng/mL) in males was 177,070 and in females 190,000, while on day 18 the  $AUC_{0-24h}$  (h·ng/mL) in males was 436,000 and in females 344,000. Logarithmic scale representations of LNA-i-miR-221 plasma concentration versus time profiles following single i.v. administration to male and female Sprague-Dawley rats at different days and dose are shown in Figures 2 and 3. LNA-i-miR-221 toxicokinetics (TK) parameters in plasma following i.v. (bolus) administration at a nominal dose level of 5, 12.5, and 125 mg/kg/day to male and female Sprague-Dawley rats are reported in Table 3. In the pivotal rat study, a comparison of the mean values of males and females within each dose group showed higher exposure to LNA-i-miR-221 in males, especially at higher doses. As reported in Table 3, on day 1 the increase of dose from 5 to 12.5 mg/kg (2.5-fold) established an  $AUC_{0-24h}$  increase of ~2.5-fold, as calculated by the mean values of combined females and males in each dose group. The increase of the dose from 5 to 125 (25-fold) and from 12.5 to 125 mg/kg (10-fold) established an increase in  $AUC_{0-24h}$  of 39.5- and 15.9-fold, respectively. On day 18 (cycle 2), the  $AUC_{0-24h}$  increase, from 5 to 12.5 mg/kg of dose, was ~3.4-fold, while from 5 to 125 and 12.5 to 125 mg/kg it was

**Table 3. LNA-i-miR-221 Toxicokinetics Parameters**

Period	Sex	C <sub>0</sub> (ng/mL)	AUC <sub>0-t</sub> (h · ng/mL)	AUC <sub>0-24h</sub> <sup>a</sup> (h · ng/mL)	Vd (mL/kg)	Cl (mL/h/kg)	t <sub>1/2</sub> ± SD (h)
5 mg/kg/day							
Day 1	F	21,485	11,221	12,787	1,879 ± 1,393	457 ± 112	3.05 ± 2.14
	M	18,150	6,325	9,382	658 ± 132	782 ± 166	0.585 ± 0.014
Day 4	F	18,112	7,856	9,801	1,612 ± 1,815	662 ± 220	1.91 ± 2.35
	M	20,541	9,862	9,862	3,553 ± 861	514 ± 104	4.76 ± 0.217
Day 15	F	18,707	9,234	9,234	3,296 ± NA	537 ± NA	4.26 ± NA
	M	14,246	8,083	8,083	4,396 ± NA	612 ± NA	4.97 ± NA
Day 18	F	19,490	10,398	10,398	3,545 ± NA	469 ± NA	5.25 ± NA
	M	24,469	12,157	12,157	3,193 ± NA	417 ± NA	5.25 ± NA
12.5 mg/kg/day							
Day 1	F	51,467	26,620	26,620	2,597 ± 445	473 ± 66.4	3.80 ± 0.168
	M	60,092	27,016	27,016	2,654 ± 583	473 ± 93.5	3.187 ± 0.099
Day 4	F	57,188	31,388	31,388	2,456 ± 531	402 ± 70.8	4.22 ± 0.168
	M	67,055	31,571	31,571	2,495 ± 362	395 ± 47.0	4.37 ± 0.206
Day 15	F	45,333	27,599	27,599	2,606 ± 316	454 ± 63.4	3.98 ± 0.142
	M	65,562	32,812	32,812	2,539 ± NA	379 ± NA	4.64 ± NA
Day 18	F	55,025	29,363	29,363	2,998 ± NA	423 ± NA	4.94 ± NA
	M	95,080	45,242	45,242	2,053 ± NA	271 ± NA	5.21 ± NA
125 mg/kg/day							
Day 1	F	79,9291	420,966	420,966	1,510 ± 169	298 ± 36.2	3.51 ± 0.035
	M	63,3578	432,082	432,082	1,565 ± 307	290 ± 37.8	3.71 ± 0.241
Day 4	F	77,9878	450,242	450,242	1,916 ± 356	274 ± 29.2	4.82 ± 0.529
	M	74,0797	470,791	470,791	1,613 ± 676	272 ± 63.0	4.15 ± 1.41
Day 15	F	69,0764	462,730	462,730	1,571 ± NA	269 ± NA	4.04 ± NA
	M	97,4027	694,482	694,482	1,210 ± NA	181 ± NA	4.57 ± NA
Day 18	F	80,2382	620,686	620,686	1,474 ± NA	200 ± NA	5.07 ± NA
	M	1,150,506	900,137	900,137	1,180 ± NA	132 ± NA	6.20 ± NA

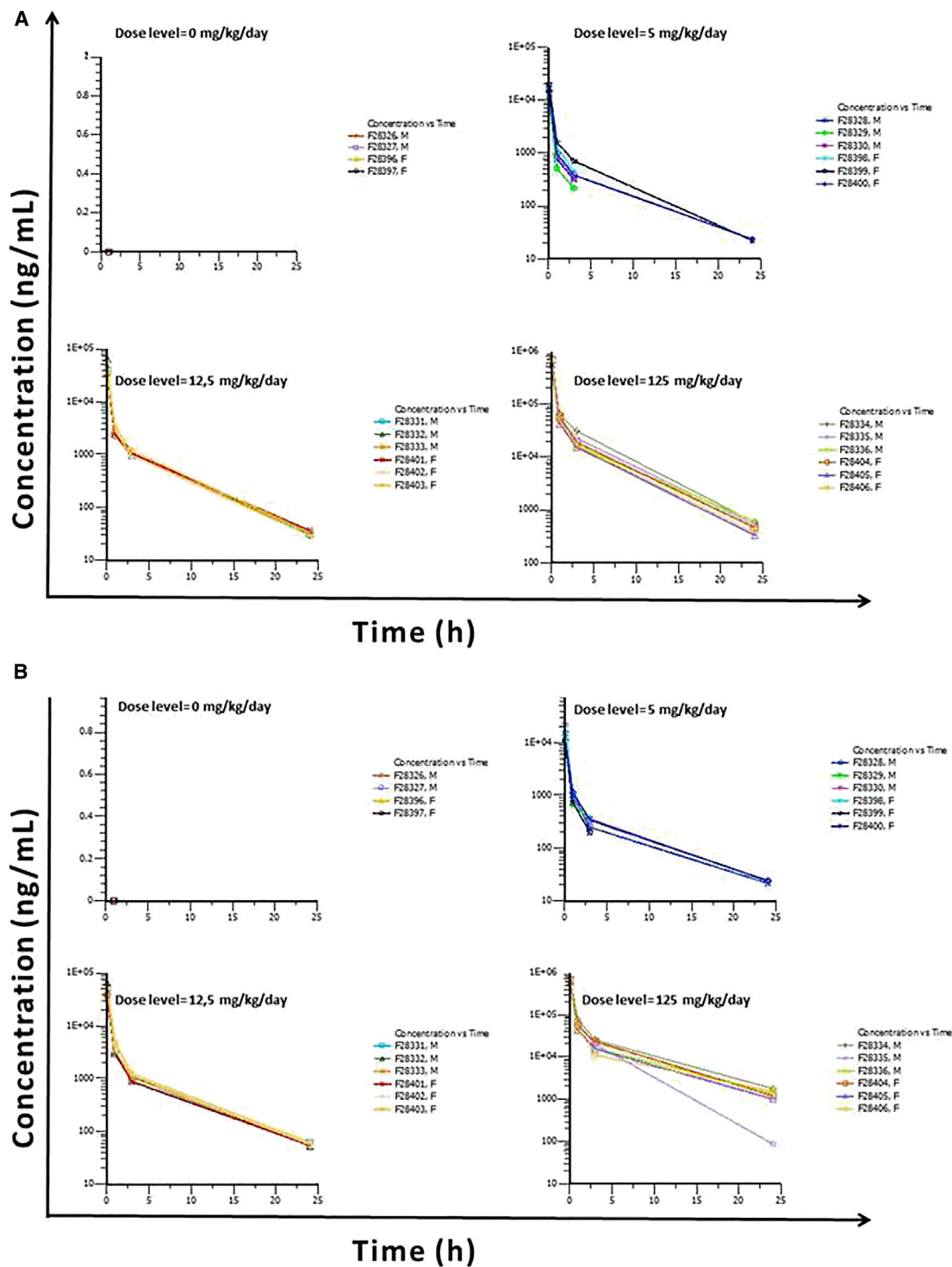
LNA-i-miR-221 toxicokinetics parameters in plasma following intravenous (bolus) administration at a nominal dose level of 5, 12.5, and 125 mg/kg/day to male and female Sprague-Dawley rats. SD was not calculated (NA [not applicable]) for groups with reduced animals (fewer than two). Vd, volume of distribution; Cl, clearance; AUC<sub>0-t</sub>, AUC from hour 0 to the time point of the last quantifiable concentration; t<sub>1/2</sub>, half time.

<sup>a</sup>Assumed to be equal to AUC<sub>0-∞</sub>.

68.9- and 20.5-fold, respectively. In conclusion, the AUC<sub>0-24h</sub> increment from day 1 to 18 was similar in the different dose range evaluated: 1.4-fold from 5 to 12.5 mg/kg, 1.7-fold from 5 to 125 mg/kg, and 1.3-fold from 12.5 to 125 (10-fold) mg/kg. Similar results have been obtained by sex, as reported in Table S6, including that the male/female ratio was not significant. Based on these data, we conclude that, at the highest dose after multiple injections, in males, there is an apparent trend versus higher exposure, due to the potential LNA-i-miR-221 accumulation. Accumulation was limited to a 2-fold increase in the high-dose males between day 1 and day 18; no other accumulation was observed. Moreover, the estimated clearance of LNA-i-miR-221 decreased while the plasma half-life (generally ranged from 3.05 to 6.20 hours) increased with dose enhancement and time (day 18 versus day 1). Based on our findings, males appear to be more exposed than females to LNA-i-miR-221.

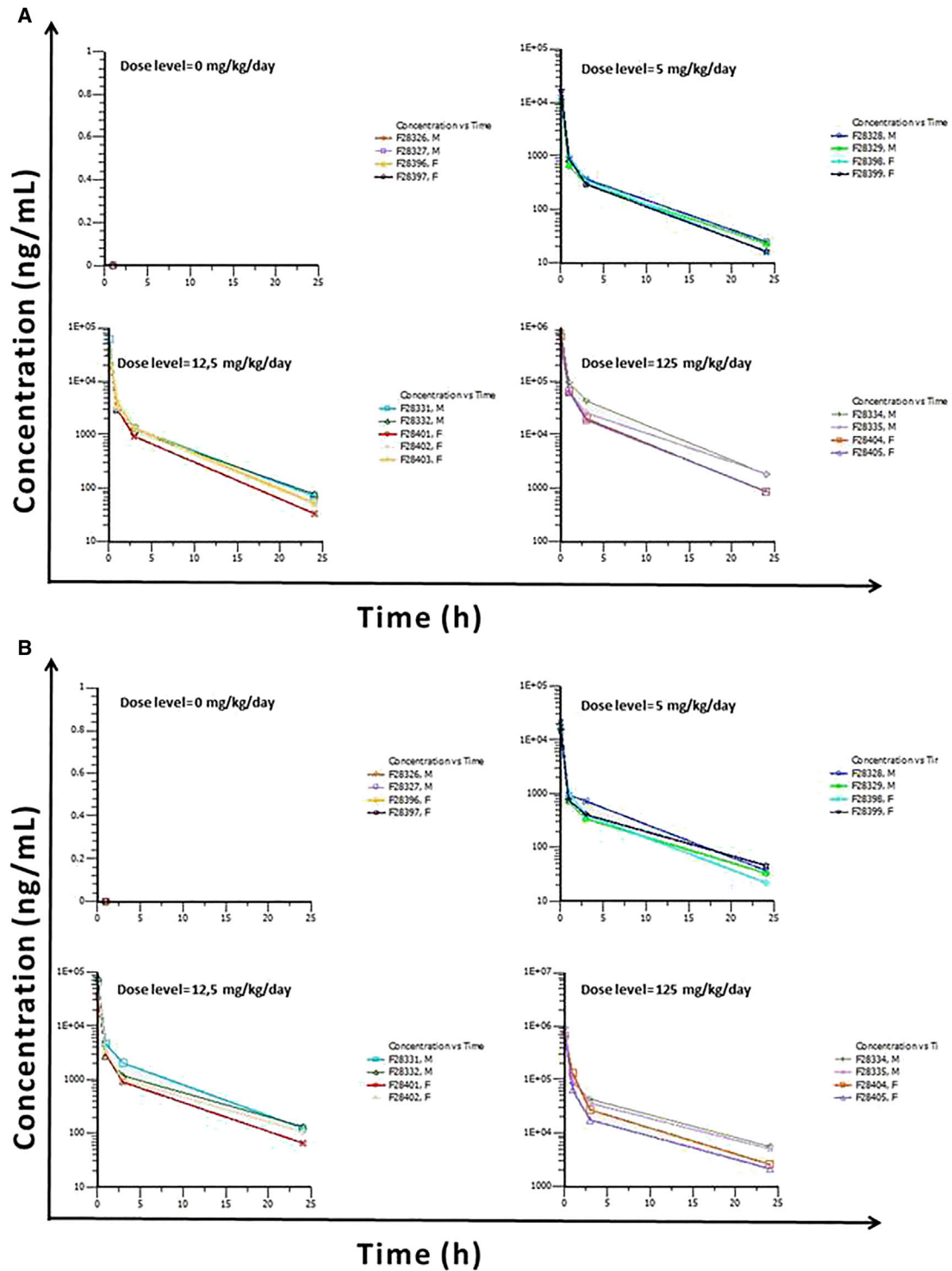
## DISCUSSION

It is well established that miR-221 promotes tumorigenesis by inhibiting tumor suppressor genes involved in cancer hallmarks, including growth, resistance to cell death, invasion, metastasis, and immune escape. The cell cycle regulator p27kip1 has been identified as the main miR-221 target in different tumors. It has been shown that in prostate carcinoma expression of p27kip1 and miR-221 is inversely correlated.<sup>25</sup> Two target sites for miR-221/222 were identified in the 3' UTR of p27 mRNA, which explains p27 downregulation following ectopic miR-221 expression and enhanced proliferation and G<sub>1</sub> to S cell cycle phase transition. These results were also confirmed in neurological tumors,<sup>26,27</sup> breast cancer,<sup>28</sup> hepatocellular carcinoma (HCC)<sup>29</sup>, lung cancer,<sup>30</sup> and myeloma.<sup>17</sup> In liver, CDKN1C/p57 was also described as a direct target of miR-221, suggesting that miR-221 has oncogenic function in HCC through negative regulation



**Figure 2. LNA-i-miR-221 Plasma Concentrations and Time Profiles on Days 1 and 4**

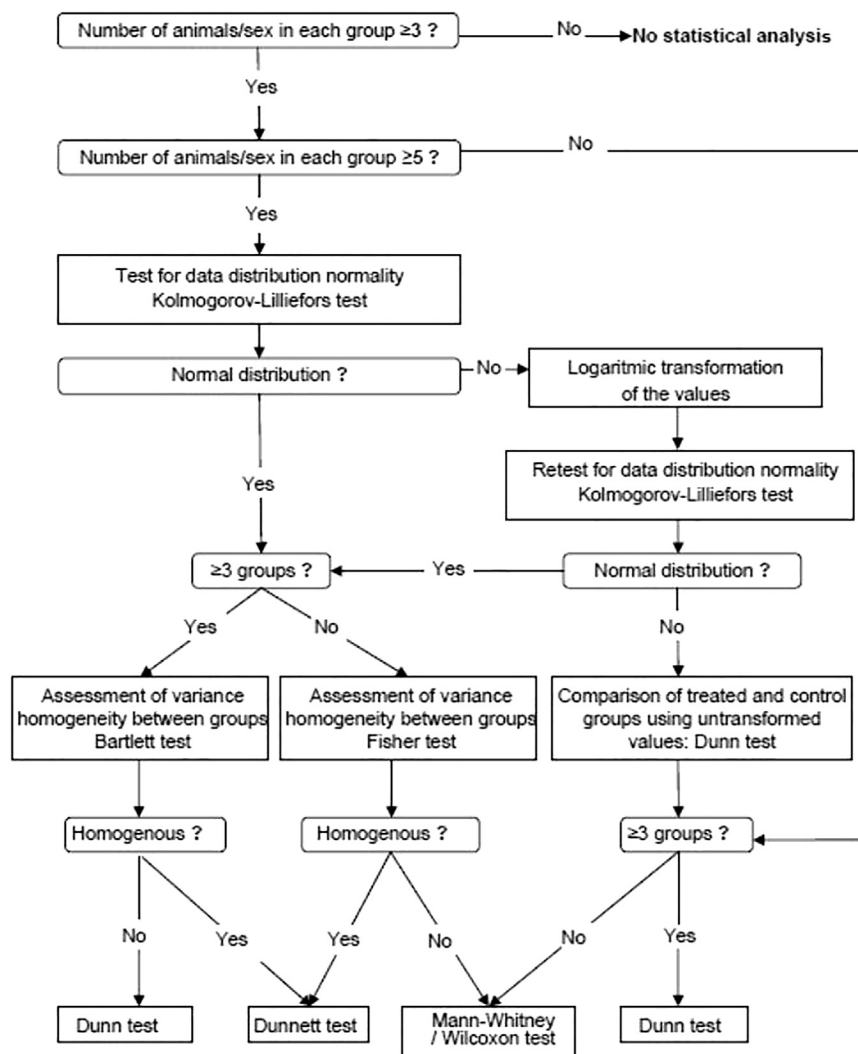
(A and B) LNA-i-miR-221 plasma concentrations (mg/mL)/time (h) profiles following a single intravenous bolus administration at 0, 5, 12.5, and 125 mg/kg/day to males and females on day 1 (A) and on day 4 (B) plotted on a semi-logarithmic scale are shown.



**Figure 3. LNA-i-miR-221 Plasma Concentrations and Time Profiles on Days 15 and 18**

(A and B) LNA-i-miR-221 plasma concentrations (ng/mL)/time (h) profiles following a single intravenous bolus administration at 0, 5, 12.5, and 125 mg/kg/day to males and females on day 15 (A) and on day 18 (B) plotted on a semi-logarithmic scale are shown.





**Figure 4. Statistical Analyses Flowchart**

GiToxLAB software was used to perform the statistical analyses of body weight, food consumption, hematology, blood biochemistry, and urinalysis data; different software was applied according to the sequence depicted.

strating drug-like biologic properties of LNA oligonucleotides together with very low systemic toxicity, therefore suggesting their suitability for human use. So far, however, to our knowledge, no advanced formal GLP pre-clinical pharmacokinetics investigation for targeting oncogenic miRNAs or clinical attempts in cancer patients has been reported.

Using an original 13-mer LNA inhibitor, named LNA-i-miR-221, we previously demonstrated that direct miR-221 inhibition upregulates canonical miR-221 targets, including p27kip1, *in vitro* and *in vivo*, translating as significant anti-tumor activity.<sup>15,21</sup> This new agent was also associated with a favorable PK profile in pilot studies in mice and monkeys,<sup>20</sup> making it a promising molecule for clinical translation. Toward this aim, we presented herein a non-GLP and a GLP dose-finding investigation in Sprague-Dawley rats as a crucial step to move into a first-in-human study. The rat species was chosen as a relevant toxicology investigation species based on the sequence retention of miR-221<sup>43</sup> and as rodent species accepted by regulatory authorities for this type of study.

Our formal rat GLP study was designed to evaluate the potential toxicity of LNA-i-miR-221

of cell cycle progression inhibitors.<sup>31</sup> Moreover, miR-221 antagonizes PTEN expression leading to activation of AKT, indicating that inhibition of miR-221 might play an important therapeutic role by PTEN upregulation in cancer cells.<sup>32</sup> Finally, downregulation of proapoptotic PUMA/BBC3 has been associated with drug resistance,<sup>33,34</sup> while its upregulation induced by miR-221/222 inhibition translates to sensitizing activity to temozolamide<sup>35</sup> and melphalan.<sup>21</sup>

So far, while *in vitro* and *in vivo* findings demonstrated therapeutic activity of miRNA inhibition, no clinical attempts have been made in cancer patients to evaluate the potential activity of non-coding RNA inhibitors. Among available anti-miRNAs strategies, LNA antisense oligonucleotides may represent one of the most promising paths for their chemistry features<sup>36–41</sup> and have been successfully used to efficiently inhibit endogenous non-coding RNAs. Recently, systemic injection of LNA-miR-122 inhibitor (miravirsen, Santaris Pharma/Roche) has been investigated in patients carrying hepatitis C virus (HCV) infection up to a phase II clinical trial,<sup>42</sup> demon-

with three different doses (low, middle, high). A recovery group of animals was also included to determine the exposure during the period of treatment. In the principal animal group, according to experimental design, the observation until 10 days after the last treatment did not demonstrate any clinical signs or effects on food consumption. The slightly lower mean body weight recorded only in the male group treated with the highest dose was considered a minor side effect, since the mean food consumption and clinical conditions were not affected. In this group, slight changes in hematology parameters, including a decrease of neutrophils and reticulocytes counts, were considered related to LNA-i-miR-221 with minor toxicological significance, since they were not associated with changes in the absolute number of white or red blood cells or in clinical conditions. The increases of creatinine in both sexes and urea concentration in males were correlated with microscopic findings in kidneys and, consistent with other reports,<sup>44</sup> were considered as class effects induced by systemic administration of single-stranded oligonucleotides. Macrophage infiltrates, following treatment with the highest dose, correlated

with an increase in liver weight and enzyme concentrations. Moreover, the granular and/or foamy cytoplasm observed in macrophages was considered to be due to the accumulation of oligonucleotides as well as cellular activation and cytokine production, as already described.<sup>44</sup> In addition, the minimal increase in mitotic figures associated with foamy/granular Kupffer cells could be related to the release of cytokines by the activated Kupffer cells, while the basophilic granules are reflective of accumulation of drug-related material and/or lysosomal degradation products. Finally, the increased development of splenic germinal centers was suggestive of lymphoid stimulation.

The PK profiles defined by both non-GLP and GLP rat studies indicate no sex differences for the plasma exposure of LNA-i-miR-221, with similar  $C_0$  and AUC values in males and females. No accumulation (defined as a  $\geq 2$ -fold increase in AUC) was observed between cycle 1 and cycle 2. However, accumulation was observed between day 1 and day 18 at 125 mg/kg/day. This effect can be due to inter-individual variability and may not be clearly sex related, taking into account that other ASOs do not show sex-related effects.<sup>45</sup> LNA-i-miR-221 decreased in rat plasma following biphasic elimination kinetic, with a rapid tissue distribution phase, and low to moderate inter-individual variability of concentrations. Exposure increases slightly more than dose proportionally between 5 and 125 mg/kg/day in both sexes.

Certainly, the PK profile was similar in all animal species tested<sup>20</sup> and to other oligonucleotides with a PS backbone.<sup>46</sup> The bioavailability of LNA-i-miR-221 likely relies on the systemic distribution and retention by tissues after a process of internalization and excretion favored by surface protein interactions and endocytosis similarly to the other PS ASO class of molecules.<sup>24,47</sup> We can speculate that, similar to other ASOs, LNA-i-miR-221 binds to plasma proteins and transfers rapidly from blood to tissues, with a short distribution half-life and a low urine recovery, as previously reported.<sup>20</sup> To predict an acceptable human plasma clearance applying appropriate multiple allometric interspecies scaling approaches,<sup>48</sup> we developed a PK model by non-compartmental analysis methods. This allowed us to identify that measured exposure AUC in rat at the NOAEL and the predicted human exposure at human equivalent dose (HED) are comparable and consistent with safe exposure in rats and monkeys. To draw inferences about safe human plasma levels in the absence of prior human data, we then applied this approach to predict LNA-i-miR-221 clearance in humans by the use of HEDs.<sup>49</sup> The geometric mean of the different estimates was finally used to predict the clearance of LNA-i-miR-221 and its exposure in humans, according to rat NOAEL conversion in HED calculations recommended in the US Food and Drug Administration (FDA) guidance.<sup>50</sup>

Finally, we can speculate that LNA-i-miR-221 was not associated with clinical changes or irreversible alterations in rats. Pathological findings were dose-dependent and detected at the middle dose and high dose by tissue examination (Table S3). All of these effects are chargeable to ASO class and considered reversible based on available studies.<sup>24</sup> Our work defined the NOAEL at 5 mg/kg/day, which is

relevant for establishment of the first dose in the subsequent first-in-human study, according to guidelines (EMA/CHMP/SWP/28367/07).<sup>23</sup> In detail, after the conversion of NOAEL to HED, on the basis of body surface area and correction factor ( $K_m$ ), we considered that the use of the conventional safety factor (10-fold de-escalation) would lead to a starting dose far from the presumable therapeutic dose, which does not fulfill our ethical standard. In fact, taking into consideration the therapeutic dose in mice (25 mg/kg), which is equivalent to 2.02 mg/kg in humans, a low starting dose (0.078 mg/kg = 1/10 of NOAEL/HED) would lead to the need to explore several escalating dose levels before approaching the potential therapeutic window, according to the conventional Fibonacci escalation flow. Therefore, we decided on a starting dose at 0.5 mg/kg, equivalent to 64.1% of the HED (0.78 mg/kg). Modeling of PK data strongly supports this dose selection, demonstrating that the predicted human exposure is consistent with safe exposure in rats and monkeys. In fact, the measured exposure AUC in rats at the NOAEL (females at 10,398 h·ng/mL and males at 12,157 h·ng/mL on day 18) and the predicted human exposure at HED (9,849 h·ng/mL) are comparable. According to the different allometric scaling approaches used, the predicted exposure at HED (0.78 mg/kg) ranged from 6,741 to 12,686 h·ng/mL. These data were also supported by the inclusion of a plasma protein binding correction that did not modify the prediction.<sup>49</sup> Moreover, as previously demonstrated,<sup>20</sup> vital organs, including liver and bone marrow, are sites of major LNA-i-miR-221 uptake. This pattern of distribution may represent an advantage for the treatment of solid tumors of a primary or secondary involvement of these sites as well as hematopoietic malignancies, such as MM. In conclusion, our formal rat study indicates the suitability of LNA-i-miR-221 for clinical use and provides a reference translational framework and path for the development of LNA-miRNA-based therapeutics in human cancers.

## MATERIALS AND METHODS

### LNA-i-miR-221 and Vehicle

A unique GLP batch 178722 (Exiqon, Sweden)–236504 (BioSpring, Germany) of LNA-i-miR-221 was used for our study. Chemical analysis of the formulation was performed at Aptuit (Verona, Italy). The formulation batch achieved the acceptance criteria and was used for animal treatments. Prior to the first administration, the pH, density, and osmolality were determined for the LNA-i-miR-221 formulation and vehicle. More details on the materials are reported on [Supplemental Materials and Methods](#).

### Rat Pilot Non-GLP Study

A pilot non-GLP rat study was first designed for the evaluation of the potential toxicity of LNA-i-miR-221, following 4 consecutive days for two cycles i.v. administration (bolus injection) with a washout period of 10 days (total of eight injections) (Figure 1). A total of 42 rats (21 males and 21 females) were used at CiToxLAB (France). The strain of rats was Sprague-Dawley, Crl CD (SD) IGS BR (Charles River Laboratories, Italy). The LNA-i-miR-221 dose injected to each animal was adjusted according to the body weight and in a steady volume of 1.0 mL/kg. Control animals received 0.9% NaCl as vehicle, under

the same conditions (Table S7). The study also included a group intended as satellite animals treated with the same dose (125 mg/kg/day) and treatment schedule of the principal group, for pharmacokinetic investigation only (see also Supplemental Materials and Methods).

### Pivotal GLP Rat Toxicity Study

The pivotal study was performed according to International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guideline M3 to evaluate the toxicity of LNA-i-miR-221. A total of 72 Sprague-Dawley rats (36 males and 36 females) were used for this GLP study. The treatment schedule repeated the non-GLP study (Figure 1). In total, three groups of five principal and three satellite animals per sex received LNA-i-miR-221 at 5, 12.5, and 125 mg/kg/day (Table S8). Each animal was checked for clinical signs, body weight, food consumption, ophthalmology status, breathing, and CNS activity using the functional observation battery (FOB) and biochemical and hematological parameters.

Laboratory analyses were performed on animals at the end of the second treatment period (day 19) and at the end of the recovery period (day 43). The histological tissue preparation was performed in compliance with GLP procedures at Novaxia (France). Tissues were preserved in 10% buffered formalin, except for the eyes with optic nerves and Harderian glands, and the testes and epididymides, which were fixed in modified Davidson's fixative. Tissue peer review was performed for at least 30% of the histological slides from the highest dose group per sex and on an adequate number of slides from identified target organs to confirm that findings recorded by the study pathologist were consistent and accurate. More details on this study are available in Supplemental Materials and Methods.

### TK Evaluation

LNA-i-miR-221 quantification was performed using the rat plasma by LC-MS/MS analysis, as previously described.<sup>19,20</sup> The TK evaluation was performed using non-compartmental analysis on Phoenix WinNonlin software, version 6.4 (Pharsight, Mountain View, CA, USA) at CiToxLAB. TK parameters were determined from the mean concentration of the matrix samples collected from different animals at each time point (sparse sampling model). A separate TK analysis was performed for each sex and sampling occasion. The standard deviation (SD) and CV were calculated to assess inter-individual variability. The absence of quantifiable levels of LNA-i-miR-221 at pre-dose (before the first administration) and in control animals was evaluated (see also Supplemental Materials and Methods).

### Statistical Analysis

Statistical analysis of body weight, food consumption, hematology, blood biochemistry, and urinalysis data were performed at CiToxLAB according to the sequence illustrated in Figure 4. PathData software was used to perform the statistical analysis of organ weight data (level of significance, 0.05 or 0.01) according to the sequence illustrated in Figure S3.

### Chemical Analysis of the Dose Formulations

Analysis was performed at Aptuit to determine the concentration of the LNA-i-miR-221. For each determination, two samples of 1 mL per formulation were taken from control and LNA-i-miR-221 dose solution formulations on day 1 and kept at  $-80^{\circ}\text{C}$  and protected from light. The analytical method as well as stability data were validated at Aptuit prior to dose formulation analysis. Acceptance criteria were fixed on the measured concentration equal to nominal concentration  $\pm 10\%$ . Prior to the first administration, the pH, density, and osmolality were determined for the vehicle and the LNA-i-miR-221 formulations.

### SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.omtn.2020.01.036>.

### AUTHOR CONTRIBUTIONS

M.T.D.M., P. Tagliaferri, and P. Tassone designed the experimental work and wrote the manuscript; M.T.D.M. and M.A. evaluated results; F.S. and D.C. revised results.

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**OMTN, Volume 20**

## **Supplemental Information**

### **Dose-Finding Study and Pharmacokinetics**

#### **Profile of the Novel 13-Mer Antisense**

#### **miR-221 Inhibitor in Sprague-Dawley Rats**

**Maria Teresa Di Martino, Mariamena Arbitrio, Daniele Caracciolo, Francesca Scionti, Piersandro Tagliaferri, and Pierfrancesco Tassone**

## SUPPLEMENTARY DATA

### *LNA-i-miR-221 off-target genes analysis*

LNA-i-miR-221 has a complementary base sequence to the strand of endogenous mature miR-221. Theoretically, a short oligonucleotide sequence could anneal to different nucleic acid sites. To evaluate if aspecific annealing might occur for miR-221 sequence homology, computational based analysis has been performed. Blastn suite (<https://blast.ncbi.nlm.nih.gov>) analysis in miRBase against both mature and immature human miRNA transcripts was performed to evaluate off-target effect of LNA-i-miR-221. Likely, the only miRNA target of LNA-i-miR-221 is miR-221-3p that is the specific target of LNA-i-miR-221 oligonucleotide based on Watson and Crick annealing and no mismatch annealing were detected. Furthermore, a Blastn analysis in Ensembl against cDNA and ncRNA was performed. This search in Ensembl reveals only targets with perfect complementary sequence, so unlike miRBase no potential off-targets with one or more mismatches could be disclosed. The analysis identifies only the following potential off-target: CELP-003 30 ENST00000624767.1: carboxyl ester lipase pseudogene [Source: HGNC Symbol; Acc: HGNC:1849]. It is a tandemly arranged CEL pseudogene that lacks exon 2-7 of CEL and harbours a stop codon in its second exon (i.e. exon 8), otherwise the two genomic sequences are highly similar. It has been described that different recombination events take place in the intron 9-exon 11 region of the CEL-CELP locus and hybrid variant of CEL are generated that are correlated for influencing the risk of chronic pancreatitis. Such events have no functional correlation with LNA-i-miR-221 annealing. There might be lncRNA off-targets annealing that are not yet indexed in Ensembl; it is not possible to predict such annealing by computational analysis. It is important to consider that in the case of eventual binding to other RNA molecules (which has high probability to occur), it does not specifically activate nucleases and would not impair translation which occurs only by annealing to specific binding sites at mRNA 3'UTR. Oligonucleotides may bind proteins which is also a source of unintended off-target effects, but there is no tool for predicting this event. Bioinformatic predictive tool evaluation let us to conclude that LNA-i-miR-221 is an oligo with a pretty clean off-target profile.

### *LNA-i-miR-221 and vehicle*

24 g of white powder as Na<sup>+</sup> salt, with molecular weight of 4385 Da, were received in Citoxlab from BioSpring, in plastic tube, covered by aluminium bag and stored at temperature of -20°C, protected from light and humidity. The purity evaluated by IP-UHPLC was equal to 90,2%, but any correction factor was applied to calculate the dose formulation. The expiration date of the batch was next to the study completion. Sodium Chloride 0.9% (batch n. 5F113) was used as vehicle, and for the LNA-i-miR-221 dose preparation. For pivotal study, stock solution at 125 mg/mL was prepared for the two cycles of treatment, aliquoted for each day of treatment and then stored frozen at -80°C until use (except for aliquot used on day 1), protected from light. For formal study, the three LNA-i-miR-221 formulations for treatment at 5, 12.5 and 125 mg/mL, were prepared once for each cycle of treatment.

### *Rat pilot non-GLP study*

At the beginning of the treatment period, the animals were five weeks old. The males had a mean body weight of 167 g (range: 152 g to 190 g) and the females had a mean body weight of 133 g (range: 125 g to 142 g). Upon arrival at CiToxLAB France, the animals were given a clinical examination to ensure that they were in good condition. The animals were acclimated to the study conditions for seven days before the beginning of the treatment period. A larger number of animals than necessary were allocated into the study and acclimated, in order to permit the selection and/or replacement of individuals. During the acclimation period, the required number of animals (21 males and 21 females) were selected according to body weight and clinical condition. The animals were allocated to groups by sex using a computerized randomization procedure. At the beginning of the study, each animal received a unique CiToxLAB identity number by an implanted microchip. All animals had free access to SSNIFF R/M-H pelleted maintenance diet (see detailed content in table S3), batch No. 3044117 (SSNIFF Spezialdiäten GmbH, Soest, Germany), which was distributed weekly. The animals had free access to bottles containing tap water (filtered with a 0.22 µm filter). During periods of fasting, food, but not water, was removed. The batches of diet and sawdust were analyzed for composition and contaminant levels. Bacterial and chemical analyses of water are performed regularly to exclude the presence of possible contaminants (pesticides and heavy metals). During the study no contaminants were present in the diet, drinking water or sawdust at levels which could have been expected to interfere with, or prejudice, the outcome of the study. In order to avoid adsorption of the LNA-i-miR-221 dose on the administration devices used for the treatment, the syringe plus infusion set was pre-rinsed with an aliquot of the dose then discarded. The administration system was weighted before and after dosing in order to determine the precise quantity of LNA-i-miR-221 administered. The LNA-i-miR-221 formulation were maintained on ice and protected from light until the dosing procedure was completed. On day 28, at the end of washout period, ten days after last treatment, the animal were sacrificed and the body weight was recorded just before sacrifice. All these animals were submitted to a full macroscopic post-mortem examination. Animals were deeply anesthetized by an intraperitoneal (IP) injection of sodium pentobarbital and sacrificed by exsanguination. Designated organs specified in the Tissue Procedure Table (table S4) were weighed wet as soon as possible after dissection and preserved. The ratio of organ weight to body weight recorded immediately before sacrifice, was calculated. According to the sequence reported in figure S1, there was a low number of animals per group (n = 3) for statistical analysis, thus the significance of the organ weight changes was considered to be not relevant. A complete macroscopic post-mortem examination were performed on all principal animals and including the examination of the external surfaces, all orifices, the cranial cavity, the external surfaces of the brain, the thoracic, abdominal, pelvic cavities and the neck with their associated organs and tissues. The study included also a group intended as satellite group animals treated with the same dosage (LNA-i-miR-221 at 125 mg/kg/day) and treatment schedule of principal group, included the controls, allocated only for pharmacokinetic investigations. In these animals approximately 0.3 / 0.4 mL of venous blood was withdrawn, from the tail vein into a tube containing K<sub>2</sub>EDTA. The collected samples were immediately placed on ice and centrifuged within 30 minutes after sampling at 3000g, at +4°C for 10 minutes. Before sampling, animals were placed into a warming cabinet at +37°C in order to allow time for vasodilatation of the tail veins. Then, animals were placed into a restraint tube and sites of injection were disinfected with 70% ethanol. The satellite animals were sacrificed after the last sampling and no tissues were



preserved. For calculation purposes, values below the Limit Of Quantification (LOQ < 10.0 ng/mL of LNA-i-miR-221) were considered as zero and no AUC was calculated if there was less than three quantifiable time-points. LNA-i-miR-221 was quantifiable in plasma samples collected on all blood sampling occasions (from 0.08h to 24h on days 1 and 15, from 0.08h to 72h on days 4 and 18, at pre-dose on day 14 and at 0.08h on days 2, 3, 16 and 17) in both sexes.

#### *Pivotal GLP rat toxicity study*

At the beginning of the treatment period, the animals were approximately seven weeks old. Males had a mean body weight of 247 g (range: 225 g to 271 g) and females had a mean body weight of 177 g (range: 144 g to 203 g). After clinical examination to ensure that they were in good condition, the animals were acclimated to the study conditions for a period of 12 days before the beginning of the treatment period. Eight supernumerary animals per sex were also allocated into the study and acclimated, in order to permit the selection and/or replacement of individuals. The animals were allocated to groups by sex using a computerized randomization procedure and identified by an implanted microchip. In addition, the exposure of rats to LNA-i-miR-221 was determined for the recovery period occurred from day 29 to day 42. The animal management and the LNA-i-miR-221 administration are as above described. A control group of five principal, five recovery and two satellite animals per sex, received the vehicle alone (NaCl 0.9%) under the same experimental conditions. The LNA-i-miR-221 formulations were administered, managed and maintained as above described. The vein used for the injection on each day was documented in the raw data (data not shown). Each animal was checked for mortality and clinical signs twice a day during the treatment and observation periods, at approximately the same time. Body weight was recorded once before the beginning of the treatment period, on the first day of treatment and then at least once a week until the end of the study. Food consumption was recorded on principal and recovery animals at the same occasions. In addition, detailed clinical examinations were performed once before the beginning of the treatment period and then at least once a week until the end of the study. Observations included, but were not limited to, changes in the skin, fur, eyes and mucous membranes, occurrence of secretions and excretions and autonomic activity (e.g. lacrimation, piloerection, pupil size, unusual respiratory pattern). Changes in gait, posture and response to handling, as well as the presence of clonic or tonic movements, stereotypes (e.g. excessive grooming, repetitive circling) and bizarre behavior (e.g. self-mutilation, walking backwards) were also checked. Ophthalmology examinations were performed before the beginning of the study in all principal and recovery animals and at the end of the second treatment period in control and highest dose groups, in principal and recovery animals. The pupils of the animals were dilated with tropicamide (Mydriaticum®, Laboratoires Théa, Clermont-Ferrand, France). After assessment of the corneal reflex at instillation of the tropicamide, the appendages, optic media and fundus were examined by indirect ophthalmoscopy (Oméga 500, Heine, Herrsching, Germany). Visual observations of breath were performed on days 1 and 15, in principal and recovery animals, for 30 seconds just after the treatment and then 30 minutes, 1 and 2 hours post-treatment. Evaluation of the CNS activity using the FOB were performed in four males and four females for principal animals and recovery animals once before the beginning of the treatment (week -1) and on one occasion at the beginning of the study before dosing and

2, 4 and 6 hours after dosing. FOB involved the evaluation of muscle tone, gait and equilibrium and CNS excitation, autonomic and behavioral domains (spontaneous activity, affective response and sensorial parameters), the evaluation of neurologic (muscle tone, gait and equilibrium and CNS excitation), as well as the presence of clonic or tonic movements, stereotypes (e.g. excessive grooming, repetitive circling) and bizarre behavior (e.g. self-mutilation, walking backwards). Specifically, the animals were observed (i) in the cage, (ii) in the hand and (iii) in the standard arena and the following parameters were assessed and graded: (i) in the cage "touch escape", (ii) in the hand fur appearance, salivation, lacrimation, piloerection, exophthalmos, reactivity to handling, pupil size (presence of myosis or mydriasis) and (iii) in the standard arena in 2-minute recording, grooming, palpebral closure, defecation, urination, tremors, twitches, tonic and clonic convulsions, gait, arousal (hypo- and hyper-activity), posture, stereotypic behavior, breathing, ataxia and hypotonia. Reactivity to manipulation or to different stimuli included measurements of reflexes and responses here listed were recorded: • touch response, • forelimb grip strength, • pupillary reflex, • visual stimulus, • auditory startle reflex, • tail pinch response, • righting reflex, • landing foot splay, • rectal temperature (at the end of the observation period).

Prior to blood sampling and during urine collection, the animals were deprived of food for an o.n. period of at least 14 hours. Blood samples were collected from the orbital sinus of the animals before the daily treatment, under light isoflurane anesthesia, into appropriate tubes. For urine collection, the animals were put into individual metabolism cages for an o.n. period of at least 14 hours. The urine was collected onto thymol crystals. The following parameters were determined on blood collected into BD Microtainer® (K<sub>2</sub>EDTA) tubes: Erythrocytes (RBC), Mean cell volume (MCV), Packed cell volume (PCV), Hemoglobin (HB), Mean cell hemoglobin concentration (MCHC), Red blood cell distribution width (RDW), Mean cell hemoglobin (MCH), Thrombocytes (PLT), Leucocytes (WBC), Differential white cell count with cell morphology, Reticulocytes (RTC). A blood smear for determination of the differential white cell count (with cell morphology) was prepared for each animal and stained with May Grünwald Giemsa. A blood smear (stained with blue cresyl) for determination of the reticulocyte count was prepared for each animal. Blood collected into sodium citrate tubes were used for following parameters analysis: Prothrombin time (PT), Fibrinogen (FIB), Activated partial thromboplastin time (APTT). Two bone marrow smears were prepared from the femoral bone at necropsy of each animal sacrificed on completion of the treatment and treatment-free periods, for May Grünwald Giemsa staining. The following parameters were determined using blood collected into lithium heparin tubes: Sodium (Na<sup>+</sup>), Potassium (K<sup>+</sup>), Chloride (Cl<sup>-</sup>), Calcium (Ca<sup>++</sup>), Inorganic phosphorus (PHOS), Glucose (GLUC), Urea (UREA), Creatinine (CREAT), Total bilirubin (TOT.BIL), Total cholesterol (CHOL), Triglycerides (TRIG), Alkaline phosphatase (ALP), Alanine aminotransferase (ALAT), Aspartate aminotransferase (ASAT), Total proteins (PROT), Albumin (ALB), Albumin/globulin ratio (A/G). The following parameters were determined in urine: volume, pH, specific gravity (SP.GRAV), appearance (APP), color (COLOR), proteins (PROT), glucose (GLUC), ketones (CETO), bilirubin (BILI), Nitrites (NITR) Blood hemoglobin (BLOOD), urobilinogen (UROB), Cytology of sediment.

For post-mortem analysis, the animals were deeply anesthetized by an intraperitoneal injection of sodium pentobarbital and sacrificed by exsanguination. The organs were weighted wet as soon as possible after dissection, for principal and recovery animals. The ratio of organ weight to body weight was recorded immediately before sacrifice.

#### Clinical examination

Mortality for premature or terminal sacrifice were annotated only for one female in the control group, that was found dead on day 10, but any clinical signs were associated with this premature death except that an hematoma on the tail (identified from day 4 to day 6) and a red discoloration at macroscopic examination of lung. In addition any treatment-related clinical signs were observed in both genders, at all treatment doses. Clinical signs at dose of 5 mg/kg/day were limited to hematoma on the tail in one male and to soft feces in one other male. At 12.5 mg/kg/day, hematoma was noted on tail of one male and soft feces were observed in one other male. Instead, at 125 mg/kg/day, for the first and the second cycles, in one male was observed an abnormal color on tail and a chromodacryorrhea in one other male. Furthermore, in females, clinical signs were limited to alopecia in forelimbs in one female, increase in size in left hindlimb associated to scabs in one other female and soft pale feces in one other female. During the recovery period, clinical signs were limited to alopecia on the head, scabs on the head and thinning of hair in males. Regarding other clinical signs, the animals of both genders treated with 5 or 12.5 mg/kg/day did not show relevant changes in mean body weight, when compared to control group, over the whole study period (figure S3 and S4) while at 125 mg/kg/day, a moderate lower mean body weight was observed in males from day 22 to day 42, (150 + 30.1; 100 + 21.5;  $P < 0.01$ ), reaching statistical significance,  $p < 0.01$ , whereas in females no relevant changes in mean body weight were observed at same dose. By visual observations of the breath no changes were evaluated on first day of each treatment cycle, just after the treatment and 1 and 2 hours post-treatment, in principal and recovery animals, at all doses tested.

Moreover, observations on CNS activity, performed using the FOB, involved the evaluation of neurologic, autonomic and behavioral domains, as well as the presence of clonic or tonic movements, stereotypes and bizarre behavior, evidenced any relevant finding in all treated animals. Neither ocular findings were noted in any animal treated.

#### Toxicokinetics evaluation

For toxicokinetics evaluation were calculated the following parameters:

- $C_0$  (estimated concentration at time 0 after bolus intravenous administration): extrapolated by log linear regression of the first two plasma concentration values, to back-extrapolate  $C_0$ ,
- $AUC_{0-24h}$  (area under the curve from 0 to 24 hours): calculated according to the linear trapezoidal rule with the linear/log interpolation method (sparse sampling model), using nominal sampling times,

- AUC<sub>0-t</sub> (area under the curve from 0 hour to the time-point of the last quantifiable concentration): calculated according to the linear trapezoidal rule with the linear/log interpolation method (sparse sampling model), using nominal sampling times.

### *Hematology*

Any relevant changes in hematology parameters were observed at the end of the treatment, on day 19, in all treated animals at doses of 5 and 12.5 mg/kg/day. In the group treated at the highest dose, statistically significant dose-related decrease was registered in females related to neutrophil ( $0.95 \pm 0.443$ ;  $0.37 \pm 0.08$ ;  $P < 0.01$ ), and reticulocytes ( $2.88 \pm 0.997$ ;  $1.46 \pm 0.219$ ;  $P < 0.05$ ) counts. At the end of the recovery period, on day 43, in males were noted decrease in neutrophils ( $1.81 \pm 0.567$ ;  $0.76 \pm 0.057$ ), large unstained cells ( $0.19 \pm 0.07$ ;  $0.08 \pm 0.00$ ), and monocytes ( $0.55 \pm 0.186$ ;  $0.14 \pm 0.028$ ) counts, and a slightly lower reticulocytes count ( $2.39 \pm 0.656$ ;  $1.39 \pm 0.354$ ) was still observed in females, associated with a decrease of LUC ( $0.11 \pm 0.05$ ;  $0.04 \pm 0.00$ ). In a single animal occurred the decrease in eosinophils count ( $0.08$  vs  $0.14$  G/L) and was considered as incidental (Table 2).

### *Biochemistry*

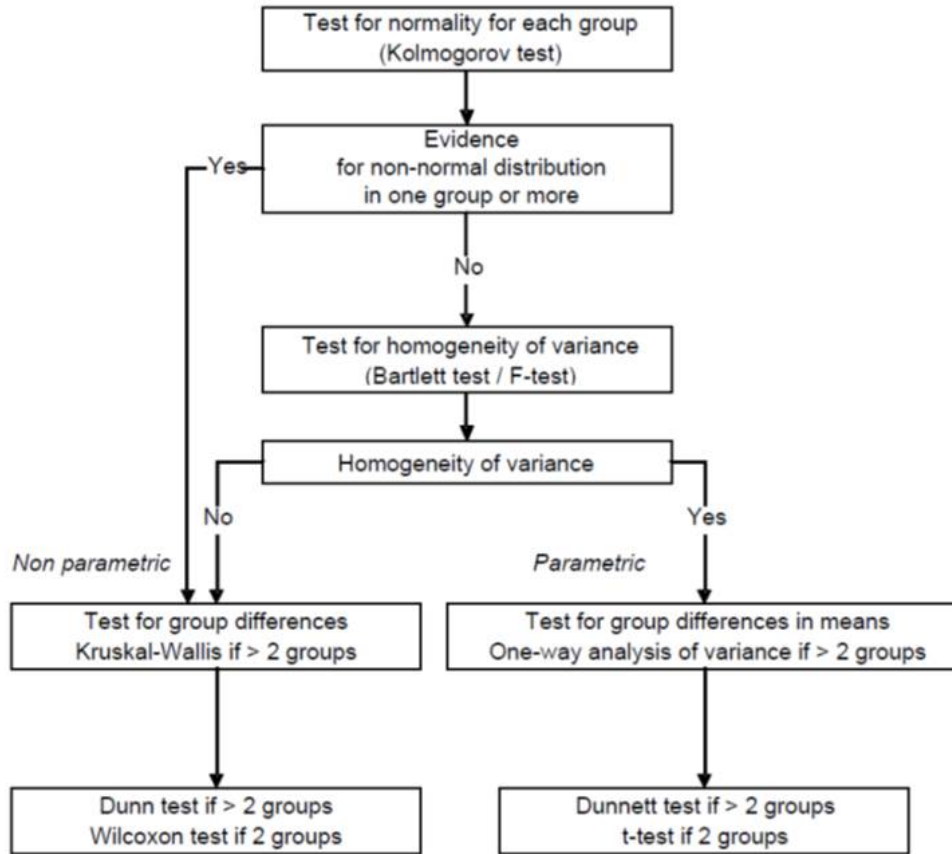
On day 19, were observed at 125 mg/kg/day, increase of creatinine and urea concentrations, correlating with microscopic findings in the kidney, in both genders with statistical significance. Specifically, changes in biochemistry parameters included higher creatinine ( $M: 22.32 \pm 0.980$ ;  $35.58 \pm 5.109$ ;  $P < 0.01$ ;  $F: 26.62 \pm 0.972$ ;  $31.26 \pm 1.547$ ;  $P < 0.05$ ) in both genders and urea ( $4.5 \pm 0.68$ ;  $7.2 \pm 1.77$ ;  $P < 0.01$ ) concentrations in male in the group treated with 125 mg/kg/day, and a higher glucose concentration ( $5.76 \pm 0.534$ ;  $7.75 \pm 1.041$ ;  $P < 0.05$ ), a lower triglyceride concentration ( $0.51 \pm 0.151$ ;  $0.21 \pm 0.037$ ;  $P < 0.01$ ) and a higher ALAT activity ( $39 \pm 4.3$ ;  $59 \pm 6.4$ ;  $P < 0.01$ ) in males group. On day 43, were still evidenced an increase of glucose ( $M: 6.16 \pm 0.855$ ;  $8.74 \pm 0.184$ ;  $F: 6.42 \pm 0.255$ ;  $8.69 \pm 1.57$ ) and urea concentrations  $M: 6.16 \pm 0.855$ ;  $8.74 \pm 0.184$ ;  $F: 6.42 \pm 0.255$ ;  $8.69 \pm 1.57$ ) in both genders, while in males a lower triglyceride concentration ( $0.82 \pm 0.339$ ;  $0.18 \pm 0.071$ ) and a higher creatinine concentration ( $28.40 \pm 1.563$ ;  $35.58 \pm 1.450$ ) were detected.

### *Pathology*

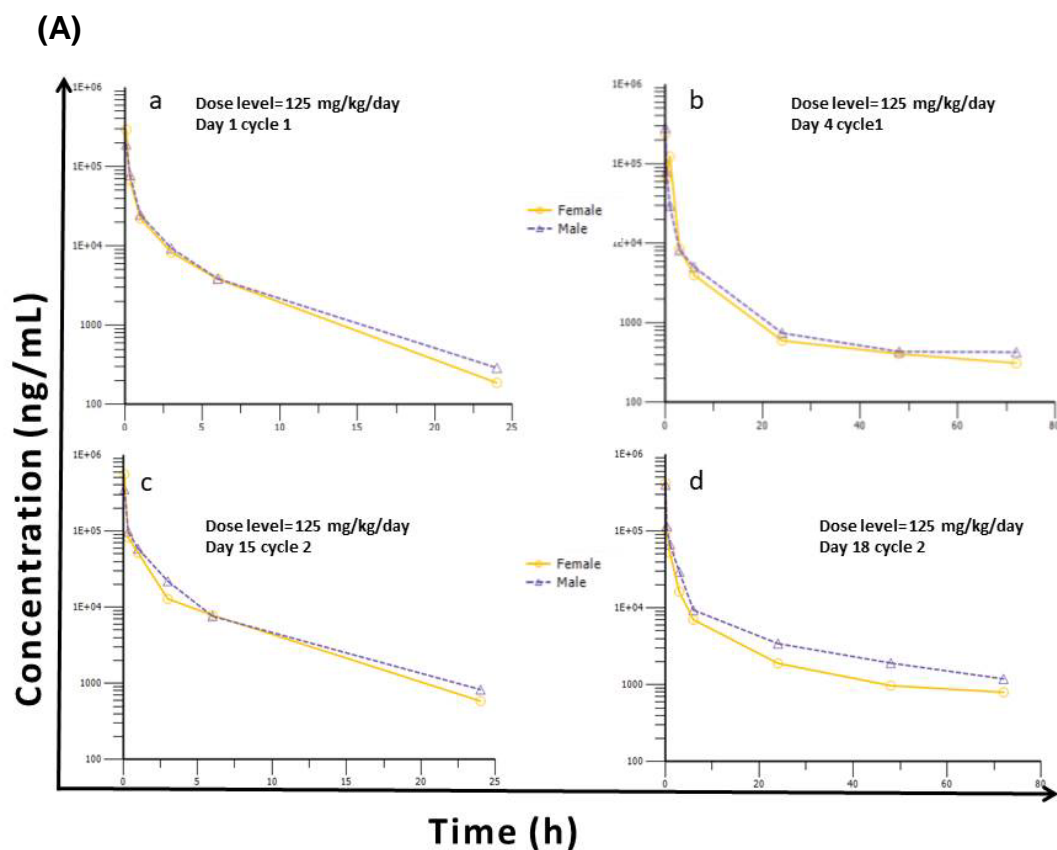
Included examination of the external surfaces, all orifices, the cranial cavity, the external surfaces of the brain, the thoracic, abdominal and pelvic cavities with their associated organs and tissues and the neck with its associated organs and tissues. On completion of the treatment and recovery periods, animals were sacrificed and principal and recovery animals were submitted to a full macroscopic post-mortem examination. This included examination of the external surfaces, all orifices, the cranial cavity, the external surfaces of the brain, the thoracic, abdominal and pelvic cavities with their associated organs and tissues and the neck with its associated organs and tissues. The organs were weighted wet, as soon as possible after dissection, and the ratio of organ weight to body weight, recorded immediately before sacrifice, was calculated.

## SUPPLEMENTARY FIGURES

**Figure S3:** PathData software was used to perform the statistical analysis of organ weight data with level of significance of 0.05 or 0.01, according to the sequence here depicted



**Figure S1:** A) LNA-i-miR-221 plasma concentration-time profiles and toxicokinetic parameters. LNA-i-miR-221 plasma concentration (mg/mL)-time (h) profiles following single intravenous bolus administration at 125 mg/kg/day to male (purple dot line) and female (orange line) Sprague-Dawley rats on day 1 (a), 4 (b), 15 (c), 18 (d) plotted as semi logarithmic scale. B) LNA-i-miR-221 toxicokinetic parameters ( $C_0$ ,  $AUC_{0-t}$ ,  $AUC_{0-24h}$ ) in plasma following intravenous (bolus) administration of LNA-i-miR-221 at a nominal dose-level of 125 mg/kg/day to male and female Sprague-Dawley rats. Evaluation was performed at days 1, 4, 15 and 18.

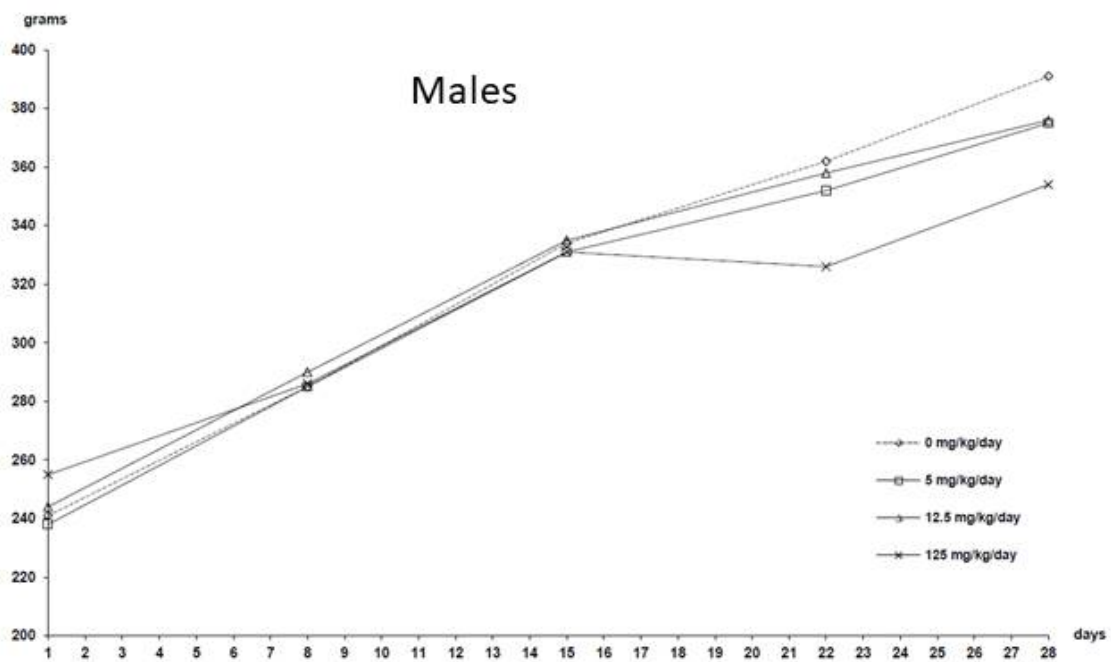


(B)  
LNA-i-miR-221 toxicokinetic parameters

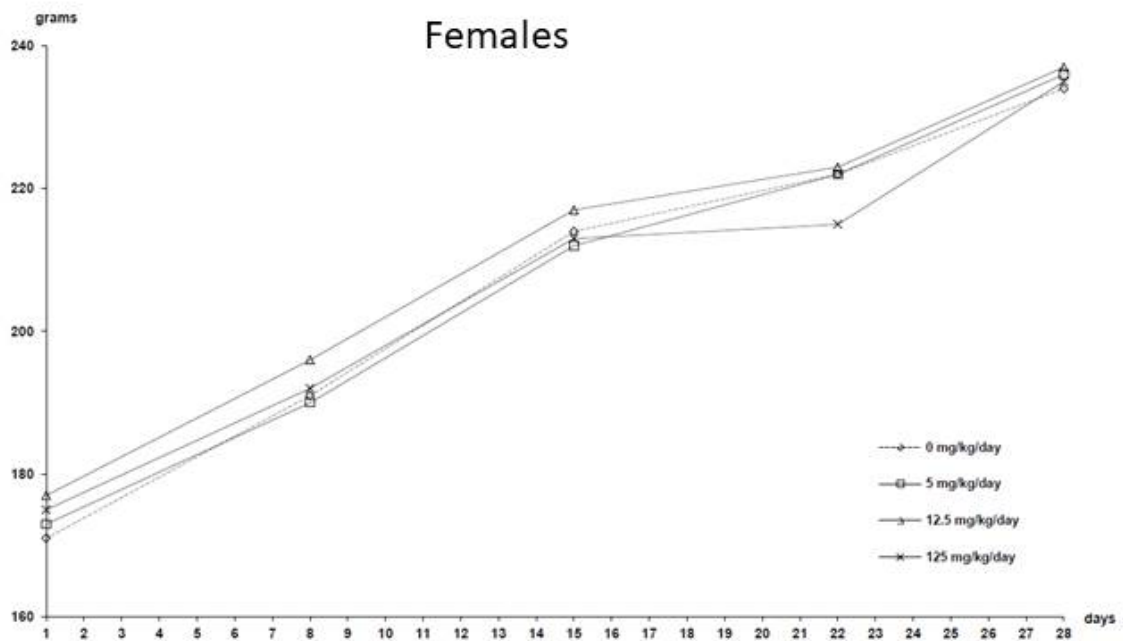
Period	Sex	$C_0$ (ng/mL)	$AUC_{0-t}$ (h*ng/mL)	$AUC_{0-24h}$ (h*ng/mL)
Day 1	Female	467000	190000	190000
	Male	254000	177000	177000
Day 4	Female	363000	339000	318000
	Male	424000	245000	220000
Day 15	Female	1030000	360000	360000
	Male	535000	346000	346000
Day 18	Female	652000	400000	344000
	Male	596000	538000	436000

**Figure S2:** Mean body weight of principal (A,B) and recovery (C,D) animal groups recorded for vehicle treatment (0 mg/kg/day), low (5 mg/kg/day), mid (12.5 mg/kg/day) and high (125 mg/kg/day) dose, male and female. Body weight was recorded once before the beginning of the treatment period, on the first day of treatment and then at least once a week until the end of the study. Mean body weight of recovery animals group were recorded at higher dose (125 mg/kg/day). A moderate lower mean body weight was observed in males from day 22 to day 42 (150 + 30.1; 100 + 21.5;  $P < 0.01$ ), reaching statistical significance,  $P < 0.01$ , whereas in females no relevant changes were observed at same dose.

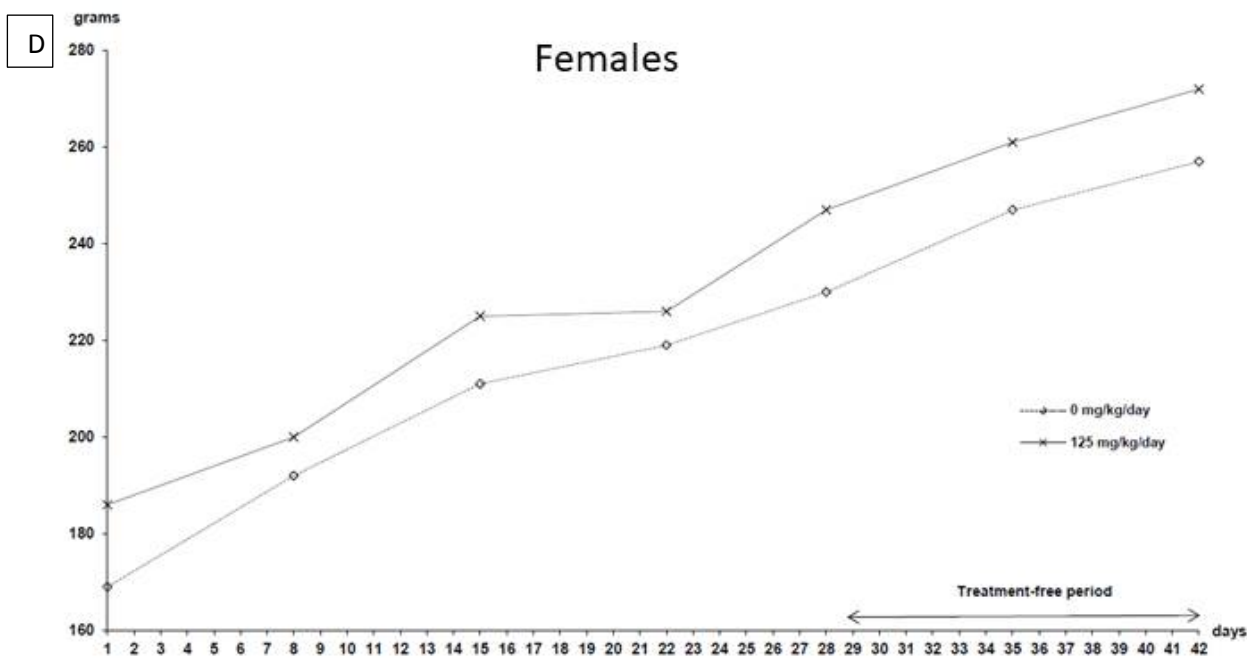
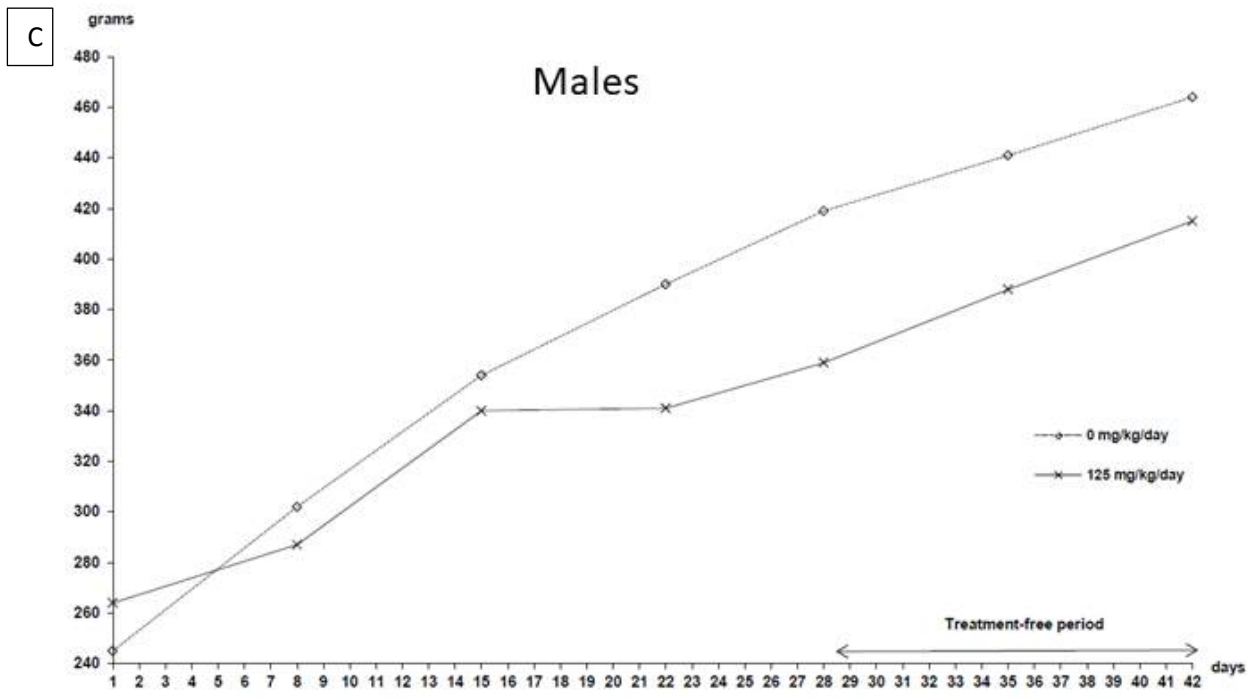
A



B







## SUPPLEMENTARY TABLES

**Table S1: Relevant changes in mean absolute and relative organ weights.** Relevant changes in mean absolute and relative organ weights in treated groups at the end of the first washout period are reported as percentages (%) values versus controls. Statistical significance was determined based on organ weights values and not for percent of changes

Sex	Male			Female		
	2	3	4	2	3	4
Group	2	3	4	2	3	4
Dose-level (mg/kg/day)	5	12.5	125	5	12.5	125
Exam. animals / Num. of animals	5/5	5/5	5/5	4/5	4/5	3/5
- <i>Final body weight</i>	+4	+4	-1	0	-1	-5
- <i>Liver</i>						
. absolute	+6	+5	+27**	+1	+2	+12
. relative	+2	+2	+28**	+2	+3	+18
- <i>Kidneys</i>						
. absolute	+8	+9	+10	+6	-2	+11
. relative	+4	+5	+11	+6	0	+17*
- <i>Spleen</i>						
. absolute	+12	+7	+23	-5	+5	-9
. relative	+9	+4	+25	-5	+6	-4
- <i>Testes</i>						
. absolute	+1	+6	+10			
. relative	-3	+3	+12			

\*: p<0.05

\*\* : p<0.01

## Table S2: Relevant changes in mean absolute and relative organ weights

Range and selected percent (%) changes compared to controls of liver, kidneys and spleen weights at the end of the recovery period. In bold the findings considered to be related to the LNA-i-miR-221 treatment

Sex	Male		Female	
	1	4	1	4
Group	1	4	1	4
Dose-level (mg/kg/day)	0	125	0	125
Exam. animals / Num. of animals	5/5	2/2	4/5	2/2
- <i>Final body weight</i>	411.6-449.6	<b>366.0-399.5</b> (-11%)	215.6-246.9	241.1-241.9 (+5%)
- <i>Liver</i>				
. absolute	10.617-14.189	10.165-11.612	5.724-7.126	<b>8.407-10.030</b> (+46%)
. relative	2.579-3.156	2.777-2.907	2.655-2.886	<b>3.475-4.160</b> (+39%)
- <i>Kidneys</i>				
. absolute	2.732-2.899	2.493- <b>2.940</b>	1.444-1.775	<b>2.123-2.170</b> (+35%)
. relative	0.635-0.686	0.681- <b>0.736</b>	0.651-0.758	<b>0.878-0.900</b> (+29%)
- <i>Spleen</i>				

. absolute	0.667-0.882	0.749-0.788	0.410-0.484	<b>0.500-0.555</b> <b>(+22%)</b>
. relative	0.151-0.204	0.197-0.205	0.179-0.196	<b>0.207-0.230</b> <b>(+16%)</b>

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**Table S3:** Incidence and severity of selected microscopic findings in the kidneys at the end of the second washout period

Sex	Male				Female			
Group	1	2	3	4	1	2	3	4
Dose-level	0	5	12.5	125	0	5	12.5	125
No. animals	5		3*	5	5	1*	2*	3
<i>Kidneys</i>								
- Basophilic granules; tubular cell								
Minimal (grade 1)	-		3	-	-	-	2	-
Slight (grade 2)	-		-	4	-	-	-	-
Moderate (grade 3)	-		-	1	-	-	-	3
- Vacuolation; tubule								
Minimal (grade 1)	-		-	5	-	-	-	-
Slight (grade 2)	-		-	-	-	-	-	1
Moderate (grade 3)	-		-	-	-	-	-	2
- Regeneration; tubule								
Minimal (grade 1)	-		3	-	-	-	2	-
Slight (grade 2)	-		-	-	-	-	-	3
Moderate (grade 3)	-		-	5	-	-	-	-
- Degeneration/necrosis; tubule								
Minimal (grade 1)	-		-	4	-	-	-	2
Slight (grade 2)	-		-	1	-	-	-	-
- Dilatation; tubule								
Minimal (grade 1)	-		-	2	-	-	-	-

-: no findings. \*: animals with gross findings.

**Table S4:** Incidence and severity of selected microscopic findings in the lymph nodes and liver at the end of the second washout period

Sex	Male				Female			
Group	1	2	3	4	1	2	3	4
Dose-level	0	5	12.5	125	0	5	12.5	125
No. animals	5			5	5		1 <sup>§</sup>	3
<i>Mesenteric lymph node</i>								
- Foamy/granular macrophages								
Slight (grade 2)	-			-	-			1
Moderate (grade 3)	-			-	-			1
Marked (grade 4)	-			5	-			1
<i>Mandibular lymph node</i>								
- Foamy/granular macrophages								
Slight (grade 2)	-			-	-			1
Moderate (grade 3)	-			4	-			2
Marked (grade 4)	-			1	-			-
<i>Liver</i>								
- Foamy/granular Kupffer cells								
Minimal (grade 1)	-			-	-		1	-
Moderate (grade 3)	-			5	-		-	3

-: no findings. <sup>§</sup>: only for liver (gross observation).

**Table S5:** Incidence and severity of germinal center development in the spleen at the end of the second washout period

Sex	Male				Female			
Group	1	2	3	4	1	2	3	4
Dose-level	0	5	12.5	125	0	5	12.5	125
No. animals	5			5	5			3
<i>Spleen</i>								
- Germinal center development								
Minimal (grade 1)	4			2	4			1
Slight (grade 2)	1			3	-			2

-: no findings.

**Table S6:** Exposure of LNA-i-miR-221 after single and repeated i.v. (bolus) administration at 5, 12,5 and 125 mg/kg/day in male and female Sprague-Dawley rats. FC calculated as ratio of mean values of AUC<sub>0-24</sub> calculated for each dose increase.

LNA-i-miR-221 mg/kg (dose increase fold)	AUC <sub>0-24</sub> male Day 1 FC	AUC <sub>0-24</sub> female Day 1 FC	AUC <sub>0-24</sub> Male / Female Day 1 ratio	AUC <sub>0-24</sub> male Day 18 FC	AUC <sub>0-24</sub> female Day 18 FC	AUC <sub>0-24</sub> Male / Female Day 18 ratio	AUC <sub>0-24</sub> Male / Female Day18/Day1 ratio
5 >12,5 (2,5)	2,87	2,01	<b>1,43</b>	4,35	2,41	<b>1,80</b>	<b>1,25</b>
12.5 >125 (10)	15,99	15,81	<b>1,01</b>	19,89	21,13	<b>0,94</b>	<b>0,93</b>
5>125 (25)	46,05	32,9	<b>1,39</b>	86,56	51,05	<b>1,69</b>	<b>1,22</b>

Table S7: Pilot non GLP study. A total of 42 animals were allocated in the study

Group		Number of animals		Dose-level (mg/kg/day)	Concentration (mg/mL)
		Males	females		
1	Principal*	3	3	0	0
	Satellite (PK)	3	3		
2	Principal*	3	3	125	125
	Satellite (PK)	12	12		

\*The sacrifice was done on day 28

Table S8: Formal GLP toxicity study. A total of 72 animals were allocated in the study

Group	Number of animals		Dose-level (mg/kg/day)	Concentration (mg/mL)
	Ma	Females		

		les			
1 Control	Principal*	5	5		
	Recovery**	5	5	0	0
	Satellite (PK)	2	2		
2 Low dose	Principal*	5	5		
	Satellite (PK)	3	3	5	5
3 Medium dose	Principal*	5	5		
	Satellite (PK)	3	3	12.5	12.5
4 High dose	Principal*	5	5		
	Satellite/Recovery** (PK)	3	3	125	125

\*The sacrifice was done on day 28

\*\*The sacrifice was done on day 42