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## 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, Pierosandro 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 endogen 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 mistmach 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 tools 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 $Na^+$ 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 $K_2$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 an 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 as 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® ($K_2$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+), Potassium (K+), Chloride (Cl-), Calcium (Ca++), 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:

- C0 (estimated concentration at time 0 after bolus intravenous administration): extrapolated by log linear regression of the first two plasma concentration values, to back-extrapolate C0,

- AUC0-24h (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,

- AUC0-t (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 + 0.443; 0.37 + 0.08; P<0.01), and reticulocytes (2.88 + 0.997; 1.46 + 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 ± 0.567; 0.76 ± 0.057), large unstained cells (0.19 ± 0.07; 0.08 ± 0.00), and monocytes (0.55 ± 0.186; 0.14 ± 0.028) counts, and a slightly lower reticulocytes count (2.39 ± 0.656; 1.39 ± 0.354) was still observed in females, associated with a decrease of LUC (0.11 ± 0.05; 0.04 ± 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 ± 0.980; 35.58 ± 5.109; P<0.01; F: 26.62 ± 0.972; 31.26 ± 1.547; P<0.05) in both genders and urea (4.5 ± 0.68; 7.2 ± 1.77; P<0.01) concentrations in male in the group treated with 125 mg/kg/day, and a higher glucose concentration (5.76 ± 0.534; 7.75 ± 1.041; P<0.05), a lower triglyceride concentration (0.51 ± 0.151; 0.21 ± 0.037; P<0.01) and a higher ALAT activity (39 ± 4.3; 59 ± 6.4; P<0.01) in males group. On day 43, were still evidenced an increase of glucose (*M*: 6.16 ± 0.855; 8.74 ± 0.184; *F*: 6.42 ± 0.255; 8.69 ± 1.57) and urea concentrations *M*: 6.16 ± 0.855; 8.74 ± 0.184; *F*: 6.42 ± 0.255; 8.69 ± 1.57) in both genders, while in males a lower triglyceride concentration (0.82 ± 0.339; 0.18 ± 0.071) and a higher creatinine concentration (28.40 ± 1.563; 35.58 ± 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

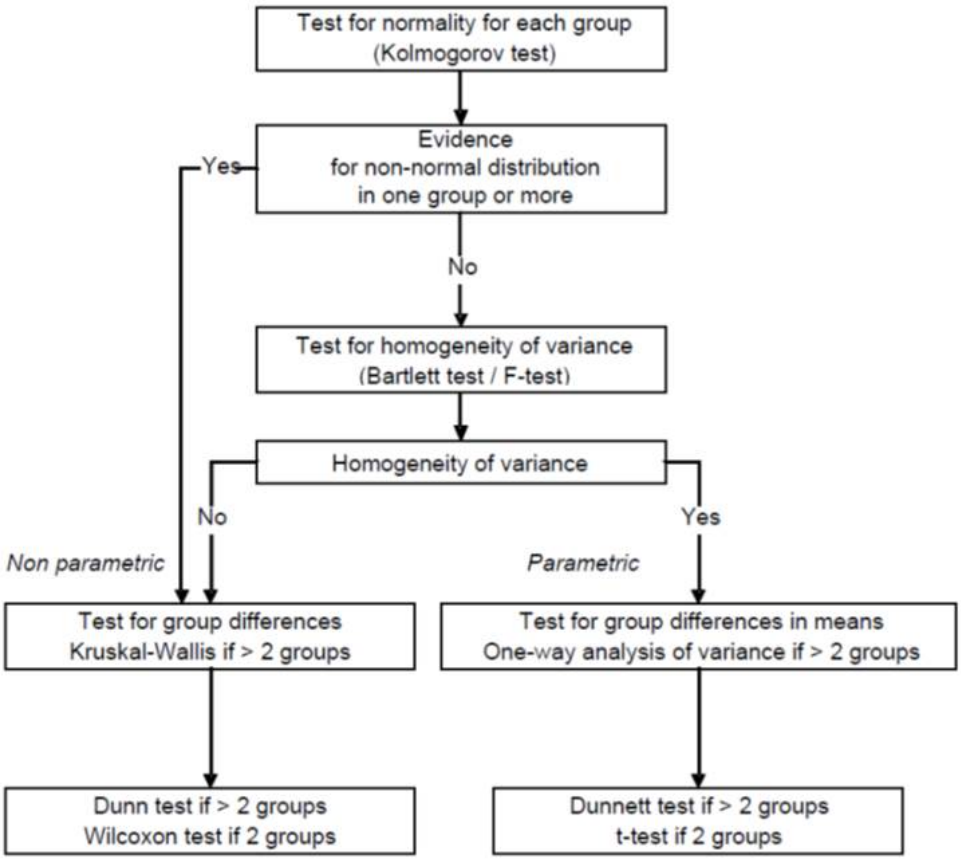
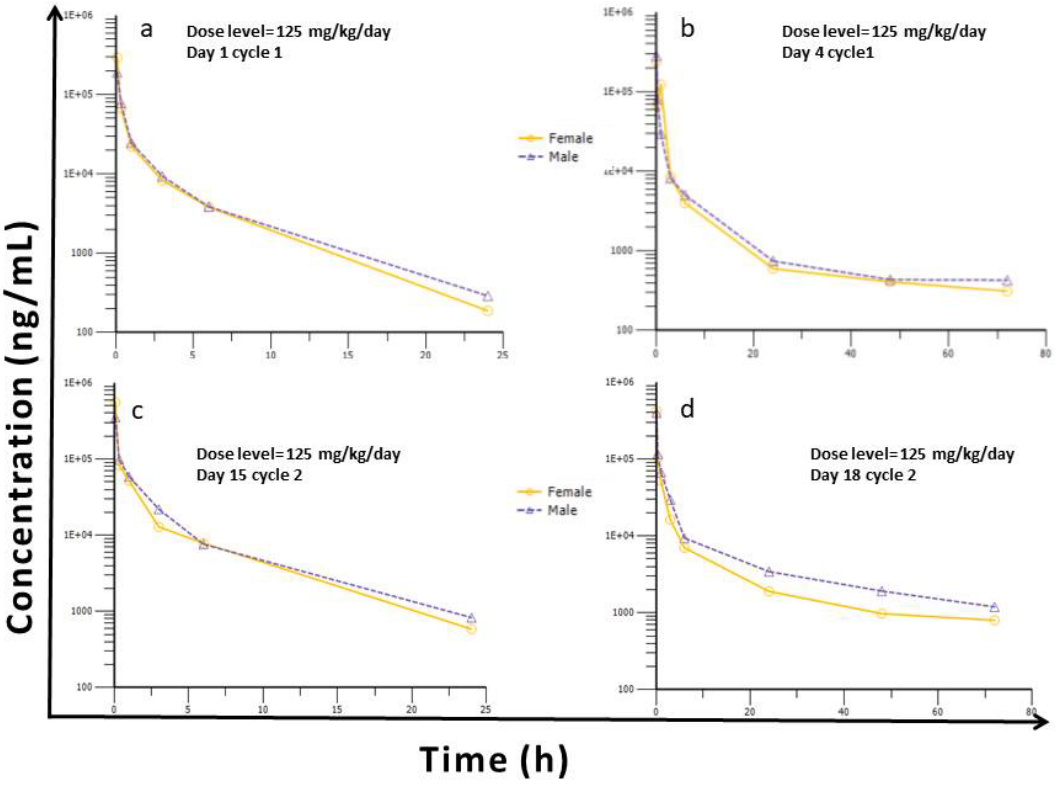


**Figure S1:** A) LNA-i-miR-221 plasma concentration-time profiles and toxicokinetic parameters. LNA-i-miR-221 plasma concentration (mg/mL)-time (h) profiles following single intravenous bolus administration at 125 mg/kg/day to male (purple dot line) and female (orange line) Sprague-Dawley rats on day 1 (a), 4 (b), 15 (c),18 (d) plotted as semi logaritmic scale. B) LNA-i-miR-221 toxicokinetic parameters (C0, AUC0-t, AUC0-24h) 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 and18.

**(A)**



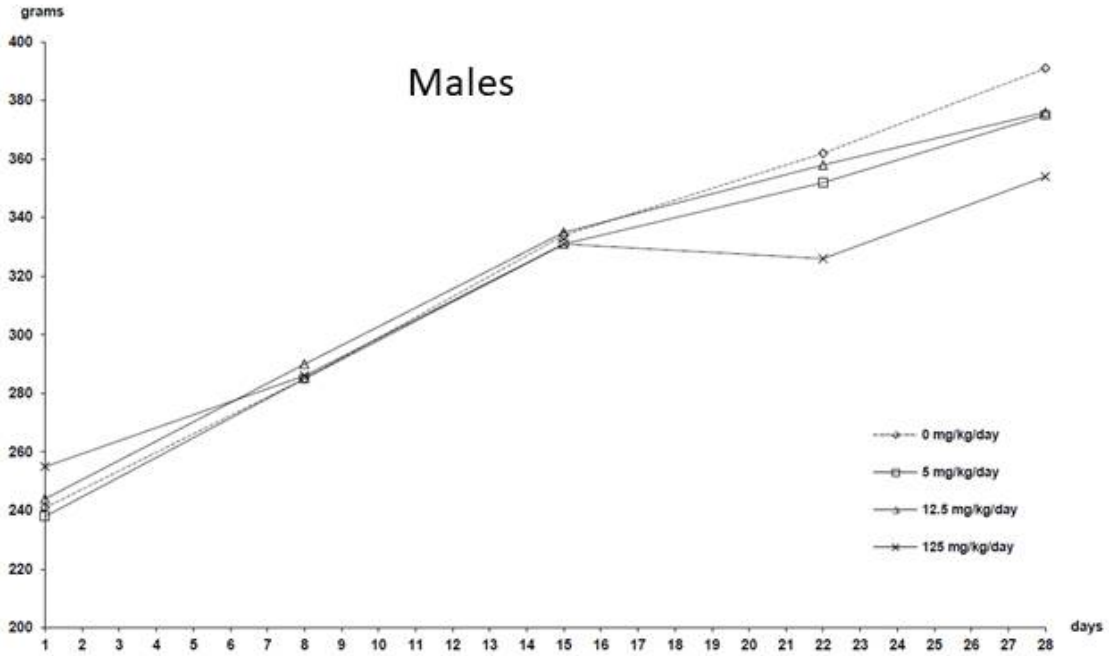

**(B)**

**LNA-i-miR-221 toxicokinetic parameters**

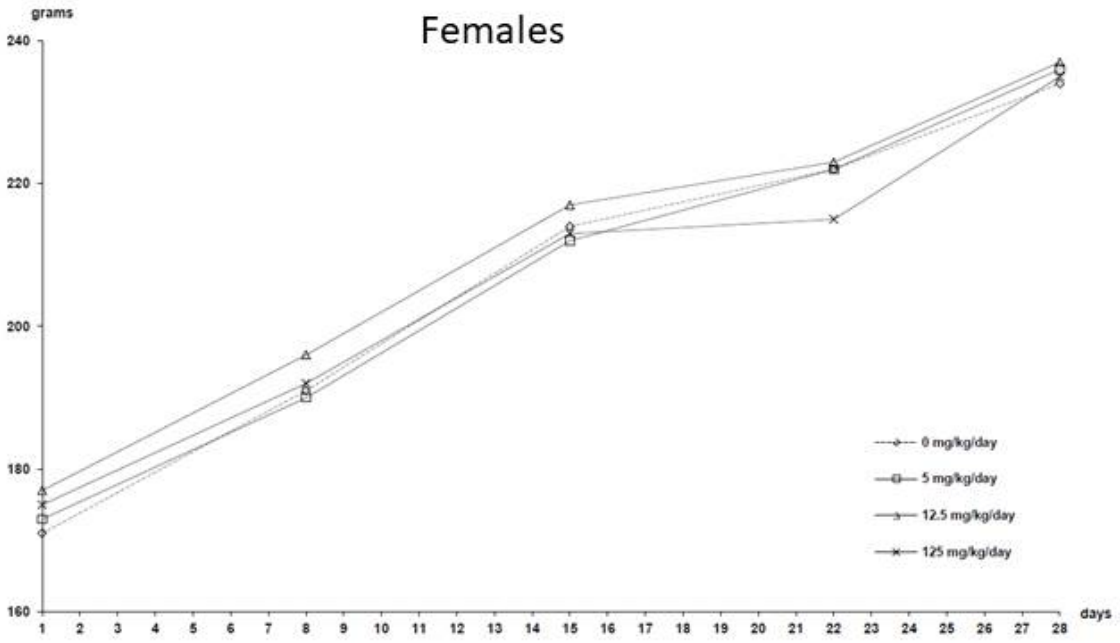
| **Period** | **Sex** | **$C_0$** (ng/mL) | **$AUC_{0-t}$** (h*ng/mL) | **$AUC_{0-24h}$** (h*ng/mL) |
|---|---|---|---|---|
| Day 1 | Female | 467000 | 190000 | 190000 |
| | Male | 254000 | 177000 | 177000 |
| Day 4 | Female | 363000 | 339000 | 318000 |
| | Male | 424000 | 245000 | 220000 |
| Day 15 | 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.

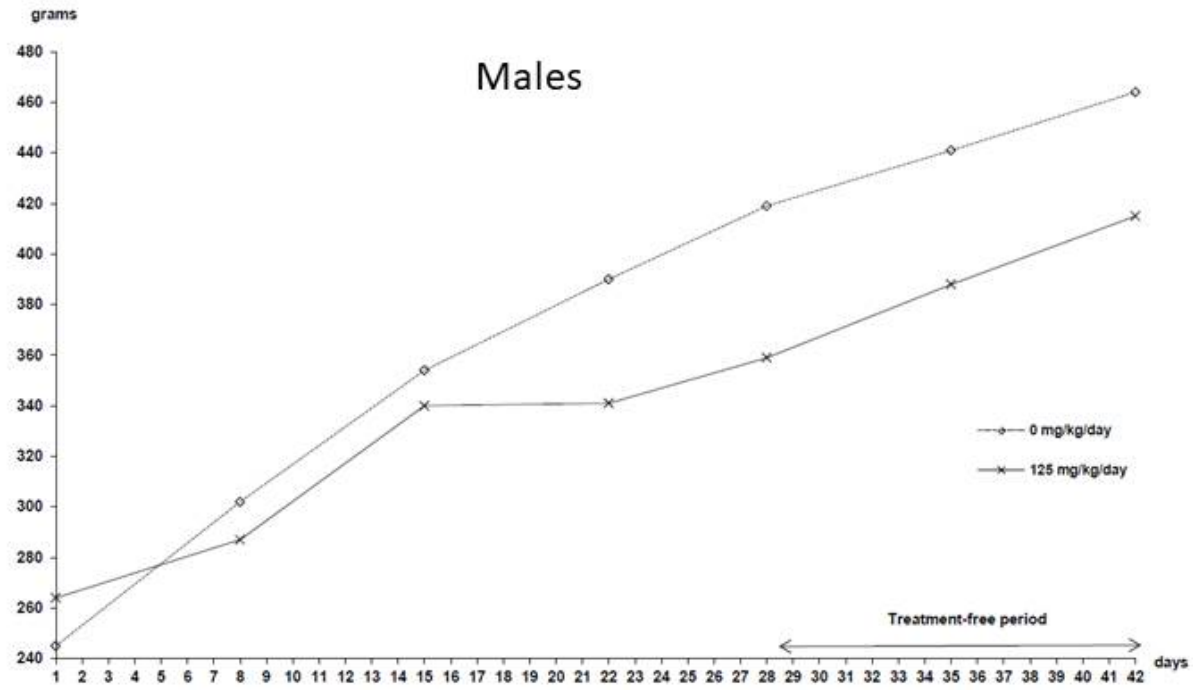
A


grams
Males
400
380
360
340
320
300
280
260
240
220
200
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28
days
0 mg/kg/day
5 mg/kg/day
12.5 mg/kg/day
125 mg/kg/day

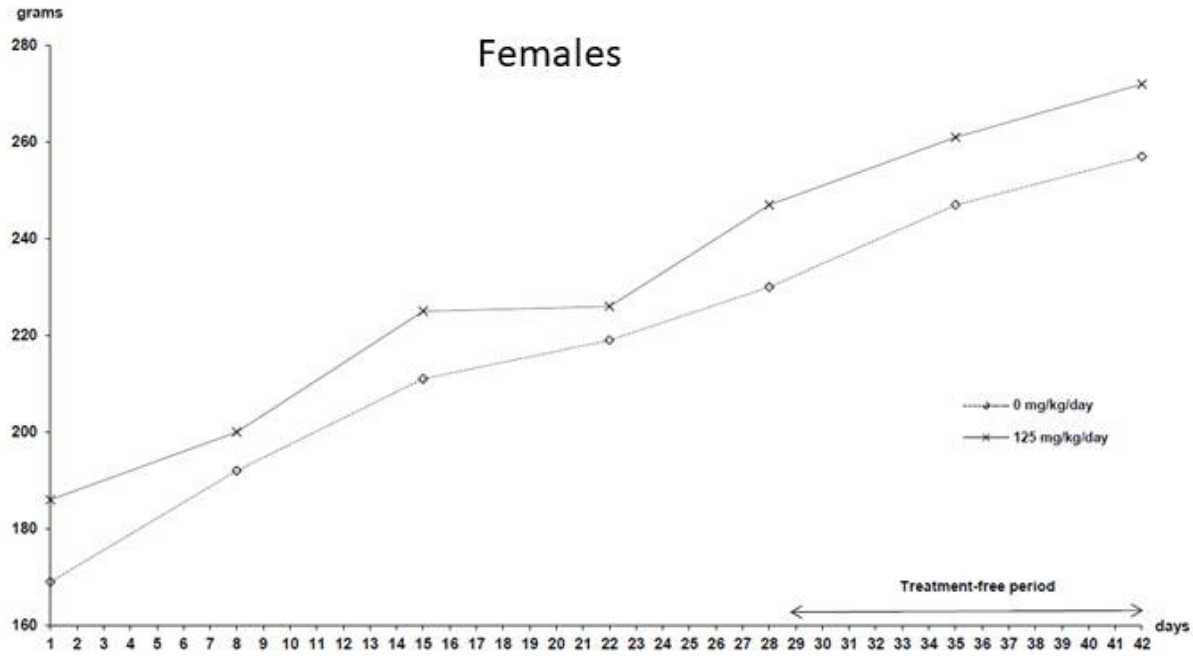
B


grams
Females
240
220
200
180
160
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28
days
0 mg/kg/day
5 mg/kg/day
12.5 mg/kg/day
125 mg/kg/day

C


grams
Males
480
460
440
420
400
380
360
340
320
300
280
260
240
0 mg/kg/day
125 mg/kg/day
Treatment-free period
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42
days

D


grams
Females
280
260
240
220
200
180
160
0 mg/kg/day
125 mg/kg/day
Treatment-free period
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42
days

## 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** | | |
|---|---|---|---|---|---|---|
| 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 |
| *- Final body weight* | +4 | +4 | -1 | 0 | -1 | -5 |
| *- Liver* | | | | | | |
| . absolute | +6 | +5 | +27** | +1 | +2 | +12 |
| . relative | +2 | +2 | +28** | +2 | +3 | +18 |
| *- Kidneys* | | | | | | |
| . absolute | +8 | +9 | +10 | +6 | -2 | +11 |
| . relative | +4 | +5 | +11 | +6 | 0 | +17* |
| *- Spleen* | | | | | | |
| . absolute | +12 | +7 | +23 | -5 | +5 | -9 |
| . relative | +9 | +4 | +25 | -5 | +6 | -4 |
| *- Testes* | | | | | | |
| . 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** | |
|---|---|---|---|---|
| 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 |
| *- Final body weight* | 411.6-449.6 | **366.0-399.5 (-11%)** | 215.6-246.9 | 241.1-241.9 (+5%) |
| *- Liver* | | | | |
| . absolute | 10.617-14.189 | 10.165-11.612 | 5.724-7.126 | **8.407-10.030 (+46%)** |
| . relative | 2.579-3.156 | 2.777-2.907 | 2.655-2.886 | **3.475-4.160 (+39%)** |
| *- Kidneys* | | | | |
| . absolute | 2.732-2.899 | 2.493-**2.940** | 1.444-1.775 | **2.123-2.170 (+35%)** |
| . relative | 0.635-0.686 | 0.681-**0.736** | 0.651-0.758 | **0.878-0.900 (+29%)** |
| *- Spleen* | | | | |

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

**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 |
| *Kidneys* | | | | | | | | |
| - 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 | - | - | - | - |
| - Dilation; 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[$] | 3 |
| *Mesenteric lymph node* | | | | | | | | |
| - Foamy/granular macrophages | | | | | | | | |
| Slight (grade 2) | - | | | - | - | | | 1 |
| Moderate (grade 3) | - | | | - | - | | | 1 |
| Marked (grade 4) | - | | | 5 | - | | | 1 |
| *Mandibular lymph node* | | | | | | | | |
| - Foamy/granular macrophages | | | | | | | | |
| Slight (grade 2) | - | | | - | - | | | 1 |
| Moderate (grade 3) | - | | | 4 | - | | | 2 |
| Marked (grade 4) | - | | | 1 | - | | | - |
| *Liver* | | | | | | | | |
| - Foamy/granular Kupffer cells | | | | | | | | |
| Minimal (grade 1) | - | | | - | - | | 1 | - |
| Moderate (grade 3) | - | | | 5 | - | | - | 3 |

-: no findings. [$]: 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 |
| *Spleen* | | | | | | | | |
| - 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 $AUC_{0-24}$ calculated for each dose increase.

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

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<br>Control | Principal* | 5 | 5 | 0 | 0 |
| | Recovery** | 5 | 5 | | |
| | Satellite (PK) | 2 | 2 | | |
| 2<br>Low dose | Principal* | 5 | 5 | 5 | 5 |
| | Satellite (PK) | 3 | 3 | | |
| 3<br>Medium dose | Principal* | 5 | 5 | 12.5 | 12.5 |
| | Satellite (PK) | 3 | 3 | | |
| 4<br>High dose | Principal* | 5 | 5 | 125 | 125 |
| | Satellite/Recovery** (PK) | 3 | 3 | | |

*The sacrifice was done on day 28

**The sacrifice was done on day 42