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Dose-finding study and pharmacokinetics profile of the novel 13-mer antisense miR-221 inhibitor in Sprague Dawley rats

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Abstract

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 (LNA-i-miR-221) exerts antitumor activity against human cancer with a pilot favorable pharmacokinetics and safety profile in mice and non-naive monkeys. We here report a non-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 four consecutive days, two treatment cycles, was investigated by a first non-GLP study. 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 in vivo ASO class effect. The NOAEL was established at 5 mg/kg/day. The plasma exposure of LNA-i-miR-221, based on C0 and AUC, suggested no differential sex effect. Slight accumulation occurred between cycles one and two 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\(^1,2\). Among novel strategies that may provide successful use of RNA-based therapeutics, the targeting of non-coding RNAs is raising as 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 or\(^3-14\).

miR-221, a member of the miR-221/222 cluster gene, is located on the X chromosome and acts as 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 LNA-i-miR-221, which is a second-generation phosphorothioate (PS) antisense oligonucleotide (ASO), and takes advantages from locked nucleic acid (LNA) technology and PS backbone chemistry in terms of increased affinity to the target and resistance to nucleases. In the PS oligonucleotide, the sulphur 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\(^15,16\) by specific inhibition of miR-221 and consequent modulation of its canonical targets, including p27Kip1, PUMA, PTEN, and p57Kip2, regulators of cell cycle and apoptosis\(^17\). In vitro and
in vivo studies demonstrated that LNA-i-miR-221 exerts strong antitumor activity, providing the first evidence of its efficacy against multiple myeloma (MM) \(^{15}\) and other tumors \(^{18}\). Moreover, detectability of LNA-i-miR-221 in animals and tumor tissues as well as in plasma and urine specimens was demonstrated \(^{19}\) together with favorable pilot pharmacokinetics profile and rapid wide tissue distribution in mice and non-naïve monkeys \(^{20}\).

In the translational aim towards a first-in-human study, we here 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 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 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 \(^{20}\), where however, no toxicity was observed. The IV route of injection was selected since it is the intended as 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 are mentioned from 8% onwards. In particular increased weight in males kidney, spleen and liver ranged from 9 to 20% absolute values, while decreased weight in females spleen, adrenals and ovaries ranged from -11 to -21% absolute values. In spite of 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 2/3 females. This finding may correlate with the increased organ weights and has been related to the 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 NOAEL has not been established. In addition, in this study an animal group were allocated only for PK investigations to evaluate the systemic exposure for sex differences and time course of the LNA-i-miR-221, following IV bolus administration at a dose-level of 125 mg/kg/day over four consecutive days, for two cycles separated by a ten-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 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 Supplementary Fig S1. was similar in males and females, suggesting the absence of a sex effect. The plasma exposure of LNA-i-miR-221, based on C\(_0\) and AUC values, after multiple administrations at the highest dose (125 mg/kg/day), in males there was 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 in 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 intravenous bolus injection at dose-levels of 5, 12.5 and 125 mg/kg/day. The rational of two cycles is within our project to combine miR-221/222 inhibition with melphalan which results in restoring of cell sensitivity to alkylant agents, accordingly to our in vitro and in vivo findings in preclinical models of multiple myeloma. 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. In the aim to provide 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 on the clinical setting for a minimum chance of explore not only safety but also induction of clinical response. The dose-levels were selected based on the results of previous studies. Based on the MM mouse xenograft models, we reported a tumor growth inhibition at 25 mg/kg. This dose has been selected as therapeutic dose and used to identify the relative equivalent species doses for our formal toxicity studies, according to Preclinical Safety Evaluation of Biotechnology-derived Pharmaceuticals S6 (R1), Parent Guideline, 1997. In the light to provide, 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 genders, at all treatment doses. Clinical signs limited to hematomata on the tail chromodacryorrhea or alopecia as well as soft pale feces were considered due to the administration procedure, since it was observed in control group and in both genders not related to LNA-i-miR-221 treatment. At 5 and 12.5 mg/kg/day, in both genders, 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 vs controls on day 28, reaching statistical significance, p<0.5), while 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 of a minor toxicological significance, because it occurred in the absence of decrease in the food consumption and feed efficiency, it not occurred in female group, and it is not associated to signs of poor clinical condition.
(Supplementary data and Fig S2). Moreover, no changes were observed on breath or on CNS activity neither ocular findings were noted at all doses. Detailed information about evaluations of clinical examinations are available on Supplemental data.

**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 at 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 reticulocytes counts was observed in females. At the end of the recovery period (day 43), in males were observed 64-78% decreases of neutrophils, large unstained cells, and monocytes counts, and a slightly lower reticulocytes count was still observed in females, associated with a 64% decrease of large unstained cells (LUC). In a single animal occurred the decrease in eosinophils counts. It was considered as incidental. These changes were considered related to the LNA-i-miR-221 but of minor significance and of 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 genders. Detailed data are reported in Table 2 and Supplementary data.

**Biochemistry**

Regarding biochemistry on day 19, no relevant changes were observed in both genders, at 5 and 12.5 mg/kg/day, while at 125 mg/kg/day, increase of creatinine and urea concentrations, correlating with microscopic findings in the kidney (see below), were noted in both genders with statistical significance. Specifically, changes in biochemistry included 1.2- to 1.6-fold higher creatinine in both genders and 1.6-fold higher urea concentration, a 1.4-fold higher glucose concentration, 0.4-fold lower triglyceride concentration and a 1.5-fold higher 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 genders, 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 level (Table 2). These changes, were considered LNA-i-miR-221-related but no adverse, taking into the slight magnitude of changes. The urinary parameters did not change neither at the end of the treatment (day 19) nor at the end of the recovery period (day 43), at all doses and in both animal genders.

**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 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 highest dose were recorded even if not statistically significant. These increases correlated with microscopic tubular changes (basophilic granules, regeneration). Similarly, the slightly increase of the absolute mean and weight of spleen in males (+23; +25) and decrease in female (-9; -4) at highest dose correlated with the increased development of germinal centers observed microscopically. Finally, there were minimal increase in the mean absolute and relative weights testes (+10; +12) in males treated at 125 mg/kg/day that correlated with presence of interstitial granular macrophages observed at microscopic examination. Other organ weight changes were not considered to be related to the 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 (Supplementary Table S1). The pathology examinations performed at the end of the recovery period evidenced in males treated at 125 mg/kg/day a minimal decrease of the terminal body weight (-11% for the mean weight) as compared to controls, that could be considered related to the LNA-i-miR-221. In females a moderate increase of the absolute and relative weights liver (+46%) and kidneys (+35%) were detected (Supplementary Table S2). Only one male animal had a highest kidney weights (2,940 gr) 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 showed 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, and enlargement of lymph nodes in one animal of the highest dose group. Tan color of the kidneys was observed in 3/5 males and 2/4 females at 12.5 mg/kg/day, and in 4/5 males and all three females at 125 mg/kg/day dose treatment. This finding correlated microscopically, as below reported, with 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 lower dose of 5 mg/kg/day, tan color of the kidneys was also noted in 2/5 males and 1/4 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 1/5 males at highest dose and correlated with microscopic examination which evidenced an 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 1/2 females at highest dose treatment. The meaning of this finding remained unclear because no microscopic examination 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, evidenced 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. These effects involved degeneration/regeneration and basophilic granules in the kidneys, and foamy/granular macrophages in most organs/tissues. Specifically, in the kidneys at doses of 12.5 and 125 mg/kg/day were evidenced basophilic granules, vacuolization, degeneration/necrosis and regeneration of proximal tubular cells. Basophilic granules were characterized by variable size of dark blue/grey cytoplasmic granules located in proximal tubular cells, and 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 Supplementary Table S3. Infiltrates of foamy and/or granular macrophages (i.e. with vacuolar and/or basophilic granular cytoplasm) were noted at highest dose 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 (Supplementary Table S4). Basophilic granular cytoplasm was characterized by small dark grey 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, the 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 2/3 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, joints etc. Finally, the spleen showed an increased development (i.e. increased numbers and size) of germinal centers (i.e. center of secondary lymphoid follicles, Supplementary 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 the related to LNA-i-miR-221. To note, at 125 mg/kg/day, 1/3 females showed minimal diffuse hypertrophy of the thyroid follicular cells. This isolated finding was considered to be most probably incidental and unrelated to the 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 evidences, the NOAEL was established at 5 mg/kg/day which is the safety dose that ensure no adverse event.

**Pharmacokinetics**

In the pivotal rat GLP study, the systemic exposure was evaluated for sex difference and time course of LNA-i-miR-221 at low (5 mg/kg/day), mid (12.5 mg/kg/day) and high (125 mg/kg/day) dose following bolus injection for 4 consecutive days for 2 cycles with a 10-days washout (Fig.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, while in treated groups LNA-i-miR-221 was quantifiable from 0.08 hours (h) to 24h post-administration in all treated groups, except for the group treated with lower dose, specifically 3 males on day 1 and 2 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 C₀, 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 increase especially between 12.5 and 125 mg/kg/day in both sexes (Table 3). No significant time effect pattern was observed, but a trend towards 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. Definitely, the slight accumulation was observed in animals between cycles one and two, with no clear accumulation during the four consecutive administrations (between the 1st day and the 4th day of administration of the first cycle or between the 1st day (day 15) and the 4th day (day 18) of the 2nd cycle of administration). Specifically, on day 1 the AUC₀-24h (h*ng/mL) in male was 177070 and in female 190000, while on day 18 the AUC₀-24h (h*ng/mL) in male was 436000 and in female 344000. Logarithmic scale representation of LNA-i-miR-221 plasma concentration versus time profiles following single intravenous administration to male and female Sprague-Dawley rats at different days and dose are showed in Fig. 2-3. LNA-i-miR-221 TK parameters in plasma following intravenous (bolus) administration at a nominal dose-level of 5 mg/kg/day, 12.5 mg/kg/day and 125 mg/kg/day to male and female Sprague-Dawley rats, are reported (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 folds) established the AUC₀-24h increase of ~ 2.5-folds, 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-folds) and from 12.5 to 125 mg/kg (10-folds) established an increase in AUC₀-24h of 39.5- and 15.9-folds, respectively. On day 18 (cycle 2), the AUC₀-24h increase, from 5 to 12.5 mg/kg of dose, was ~ 3.4-folds, while from 5 to 125 and 12.5 to 125 mg/kg was 68.9- and 20.5-folds, respectively. In conclusion, the AUC₀-24h increment from day 1 to 18 was similar in the different dose range evaluated: 1.4
folds from 5 to 12.5 mg/kg, 1.7 folds from 5 to 125 mg/kg and 1.3 folds from 12.5 to 125 (10-folds) mg/kg. Similar results have been obtained by sex, as reported in Supplementary Table S6, was the male: female ratio was not significant. Taken together 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 the dose enhancement and time (day 18 vs 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 showed that in prostate carcinoma p27kip1 and miR-221 expression are inversely correlated. 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 G1 to S cell cycle phase transition. These results were also confirmed in neurological tumors, breast cancer, hepatocellular carcinoma (HCC), lung cancer, and myeloma. In liver, CDKN1C/p57 was also described as direct target of miR-221, suggesting that miR-221 has oncogenic function in HCC through negative regulation of cell cycle progression inhibitors. 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. Finally, downregulation of pro-apoptotic PUMA/BBC3, has been associated with drug-resistance, while its upregulation induced by miR-221/222 inhibition translates in sensitizing activity to temozolamide and melphalan.

So far, while in vitro and in vivo findings demonstrated therapeutic activity of miRNAs 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 and have been successfully used to efficiently inhibit endogenous non-coding RNAs. Recently, systemic injection of LNA-miR-122 inhibitor (Miravirsen, Santharis/Roche) has been investigated in patients carrying HCV infection up to phase II clinical trial, demonstrating 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 have been reported.

Using an original 13-mer LNA inhibitor, named LNA-i-miR-221, we previously demonstrated that a direct miR-221 inhibition upregulates canonical miR-221 targets, including p27kip1, in vitro and in vivo, translating in significant anti-tumor activity. This new agent was also associated with favorable PKs profile in pilot studies in mice and monkeys, making it a promising molecule for clinical translation. To this aim, we presented here 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 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 the LNA-i-miR-221 with three different doses (low, mid, high). A recovery group of animals was also included to determine the exposure over 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
the 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 condition. The
increase of creatinine in both genders and urea concentration in male were correlated with
microscopic findings in kidneys and, consistently with other reports\textsuperscript{44}, 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 enzymes concentration. 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\textsuperscript{44}. 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 ≥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
gender-related, taking into account that other ASOs do not show gender-related effects\textsuperscript{45}.
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.

Definitely, the PK profile was similar in all animal species tested\textsuperscript{20} and to other
oligonucleotides with a PS backbone\textsuperscript{46}. The bioavailability of LNA-i-miR-221 likely relies
upon the systemic distribution and retention by tissues after a process of internalization
and excretion favored by surface protein interactions and endocytosis similarly to other PS
ASOs class of molecules\textsuperscript{24,47}. We can speculate that, like 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\textsuperscript{20}. To predict an
acceptable human plasma clearance applying appropriate multiple allometric interspecies
scaling approaches\textsuperscript{48}, we developed a PK model by a 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 rat and monkey. 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 human by the use of HED\textsuperscript{49}. 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 calculation
recommended in the FDA Guidance\textsuperscript{50}. 
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 mid dose and high dose by tissue examination (Table S3). All these effects are chargeable to ASOs class and considered reversible based on available studies\textsuperscript{24}. 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 (EMEA/CHMP/SWP/28367/07). In detail, after the conversion of NOAEL to HED, on the basis of body surface area and correction factor (km), we considered that the use of the conventional safety factor (10 fold de-escalation) would led to a starting dose far from the presumable therapeutic dose, that does not fulfill our ethical standard. In fact, taking in 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 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 rat and monkey. In fact, the measured exposure AUC in rat at the NOAEL (Female 10,398 and Male 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\textsuperscript{49}. Moreover, as previously demonstrated\textsuperscript{20}, 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 multiple myeloma. 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.

**Methods and materials**

**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 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 Supplementary 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 intravenous (IV) administration (bolus injection) with a wash-out period of 10 days (total 8 injections) (Fig.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 (Supplementary Table S7). The study included also a group intended as satellite group animals treated with the same dose (125 mg/kg/day) and treatment schedule of principal group, for pharmacokinetic investigation only (see also Supplementary methods).

**Pivotal GLP rat toxicity study**

The pivotal study was performed according to 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 (Fig.1). In total 3 groups of 5 principal and 3 satellite animals per sex received LNA-i-miR-221 at 5, 12.5 and 125 mg/kg/day (Supplementary Table S8). Each animal was checked for clinical signs, body weight, food consumption, ophthalmology status, breath, and Central Nervous System (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 Supplementary methods.

**Toxicokinetic evaluation**

LNA-i-miR-221 quantification were performed using the rat plasma by LC-MS/MS analysis as already described (19,20). The toxicokinetics (TK) evaluation was performed using non-compartmental analysis on Phoenix WinNonlin software, version 6.4 (Pharsight Corporation, Mountain View, California 94040/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 Coefficient of Variation (CV) were calculated to assess inter-individual variability. The absence of quantifiable levels of the LNA-i-miR-221 at pre-dose (before the first administration) and in control animals was evaluated (see also Supplementary 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
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°C and protected from light. The analytical method as well as stability data were validated in Aptuit prior to dose formulation analysis. Acceptance criteria were fixed on the measured concentration equal to nominal concentration ± 10%. Prior to the first administration, the pH, density and osmolality were determined for the vehicle and the LNA-i-miR-221 formulations.

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Author contributions: MTDM, PST and PFT designed the experimental work, and write the manuscript; MTDM and MA, evaluated results FS, and DC reviewed results; PFT Funding Acquisition.

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**Figure legends**

Figure 1: Rat treatment timeline. LNA-i-miR-221 was administered for 4 consecutive days in two cycles: 4 administrations on Days 1, 2, 3, 4 for the first cycle and 4 administrations on Days 15, 16, 17, 18 for the second cycle (8 administrations in total). A period of ten (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.

Figure 2: LNA-i-miR-221 plasma concentration (mg/mL)-time (h) profiles following single intravenous bolus administration at 0, 5, 12.5 and 125 mg/kg/day to male and female on day 1 (A) on day 4 (B) plotted in semi logarithmic scale.

Figure 3: LNA-i-miR-221 plasma concentration (mg/mL)-time (h) profiles following single intravenous bolus administration at 0, 5, 12.5 and 125 mg/kg/day to male and female on day 15 (A) and on day 18 (B) plotted in semi logarithmic scale.

Figure 4: Citox software: to perform the statistical analyses of body weight, food consumption, hematology, blood biochemistry and urinalysis data, different software was applied according to the sequence here depicted.

**Tables**

Table 1: Main organ weights. The differences in % between treated and control animals were mentioned from 8% onwards. 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, thus the significance of the organ weight changes was considered to be not relevant. na: not applicable.

| Sex               | Male | Female |
|-------------------|------|--------|
| 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
Table 2: Blood parameters changes 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) measured in the animal group treated with 125 mg/kg/day of LNA-i-miR-221 and control (Vehicle) group.

| HEMATOLOGIC PARAMETER | day 19 | day 43 |
|------------------------|--------|--------|
|                        | Vehicle ± SD | 125 mg/kg/day ± SD | Vehicle ± SD | 125 mg/kg/day ± SD |
| Neutrophil             | 0.95 ± 0.443 * | 0.37 ± 0.08 F | 1.81 ± 0.567 M | 0.76 ± 0.057 F |
| Reticulocytes          | 2.88 ± 0.997** | 1.46 ± 0.219 F | 2.39 ± 0.656 F | 1.39 ± 0.354 M |
| Monocytes              | 0.55 ± 0.186 M | 0.14 ± 0.028 F | 0.19 ± 0.07 M | 0.08 ± 0.00 F |
| LUC                    | 0.11 ± 0.05 F | 0.04 ± 0.00 F | 0.19 ± 0.07 M | 0.08 ± 0.00 F |
| Eosinophils            | 0.08 ± 0.032 | 0.14 ± 0.045 |

| BIOCHEMISTRY PARAMETER | day 19 | day 43 |
|------------------------|--------|--------|
|                        | Vehicle ± SD | 125 mg/kg/day ± SD | Vehicle ± SD | 125 mg/kg/day ± SD |
| Creatinine             | 22.32 ± 0.980 M | 35.58 ± 5.109* F | 28.40 ± 1.563 M | 35.58 ± 1.450 F |
| Urea                   | 4.5 ± 0.68 M | 7.2 ± 1.77* F | 4.5 ± 0.52 M | 6.9 ± 0.71 F |
| Glucose                | 5.76 ± 0.534 M | 7.75 ± 1.041** F | 6.16 ± 0.855 M | 8.74 ± 0.184 F |
| Triglyceride           | 0.51 ± 0.151 M | 0.21 ± 0.037* F | 0.82 ± 0.339 M | 0.18 ± 0.071 F |
| ALAT                   | 39 ± 4.3 M | 59 ± 6.4* M | 47 ± 10.2 M | 34 ± 3.5 M |

*P<0.01; ** P<0.05
M: male F: female
Table 3. LNA-i-miR-221 toxicokinetic parameters in plasma following intravenous (bolus) administration at a nominal dose-level of 5 (A), 12.5 (B) and 125 (C) mg/kg/day to male and female Sprague-Dawley rats. SD was not calculated (NA) for group with reduced animals (<2).

(A) Period Sex | C₀ (ng/mL) | AUC₀→t (h*ng/mL) | AUC₀→24h* (h*ng/mL) | Vd (mL/kg) | CL (mL/h/kg) | t1/2 ±SD (h)
---|---|---|---|---|---|---
Day 1 | F | 21485 | 11221 | 12787 | 1879±1393 | 457±112 | 3.05±2.14
| M | 18150 | 6325 | 9382 | 658±132 | 782±166 | 0.585±0.014
Day 4 | F | 18112 | 7856 | 9801 | 1612±1815 | 662±220 | 1.91±2.35
| M | 20541 | 9862 | 9862 | 3553±861 | 514±104 | 4.76±0.217
Day 15 | F | 18707 | 9234 | 9234 | 3296±NA | 537±NA | 4.26±NA
| M | 14246 | 8083 | 8083 | 4396±NA | 612±NA | 4.97±NA
Day 18 | F | 19490 | 10398 | 10398 | 3545±NA | 469±NA | 5.25±NA
| M | 24469 | 12157 | 12157 | 3193±NA | 417±NA | 5.25±NA

(B) Period Sex | C₀ (ng/mL) | AUC₀→t (h*ng/mL) | AUC₀→24h* (h*ng/mL) | Vd (mL/kg) | CL (mL/h/kg) | t1/2 ±SD (h)
---|---|---|---|---|---|---
Day 1 | F | 51467 | 26620 | 26620 | 2597±445 | 473±66.4 | 3.80±0.168
| M | 60092 | 27016 | 27016 | 2654±583 | 473±93.5 | 3.187±0.099
Day 4 | F | 57188 | 31388 | 31388 | 2456±531 | 402±70.8 | 4.22±0.168
| M | 67055 | 31571 | 31571 | 2495±362 | 395±47.0 | 4.37±0.206
Day 15 | F | 45333 | 27599 | 27599 | 2606±316 | 454±63.4 | 3.98±0.142
| M | 65562 | 32812 | 32812 | 2539±NA | 379±NA | 4.64±NA
Day 18 | F | 55025 | 29363 | 29363 | 2998±NA | 423±NA | 4.94±NA
| M | 95080 | 45242 | 45242 | 2053±NA | 271±NA | 5.21±NA

(C) Period Sex | C₀ (ng/mL) | AUC₀→t (h*ng/mL) | AUC₀→24h* (h*ng/mL) | Vd (mL/kg) | CL (mL/h/kg) | t1/2 ±SD (h)
---|---|---|---|---|---|---
Day 1 | F | 799291 | 420966 | 420966 | 1510±169 | 298±36.2 | 3.51±0.035
| M | 633578 | 432082 | 432082 | 1565±307 | 290±37.8 | 3.71±0.241
Day 4 | F | 779878 | 450242 | 450242 | 1916±356 | 274±29.2 | 4.82±0.529
| M | 740797 | 470791 | 470791 | 1613±676 | 272±63.0 | 4.15±1.41
Day 15 | F | 690764 | 462730 | 462730 | 1571±NA | 269±NA | 4.04±NA
| M | 974027 | 694482 | 694482 | 1210±NA | 181±NA | 4.57±NA
Day 18 | F | 802382 | 620686 | 620686 | 1474±NA | 200±NA | 5.07±NA
| M | 1150506 | 900137 | 900137 | 1180±NA | 132±NA | 6.20±NA

*: is assumed to be equal to AUC₀→∞.
Figure 1

Day
1  2  3  4  15  16  17  18  28  29  42

Cycle I
Washout (10 days)

Cycle II

recovery

Sacrifice
Figure 4

- Number of animals/sex in each group ≥3?  
  - Yes → Test for data distribution normality
    - Kolmogorov-Lilliefors test
  - No → No statistical analysis
  - No → No statistical analysis

- Number of animals/sex in each group ≥5?  
  - Yes → Retest for data distribution normality
    - Kolmogorov-Lilliefors test
  - No → Logarithmic transformation of the values

- Normal distribution?  
  - Yes → Normal distribution?
    - Yes → ≥3 groups?  
      - Yes → Assessment of variance homogeneity between groups
        - Bartlett test
      - No → Homogenous?  
        - No → Dunn test
        - Yes → Dunn test
    - No → Assessment of variance homogeneity between groups
      - Fisher test
  - No → ≥3 groups?  
    - Yes → Comparison of treated and control groups using untransformed values: Dunn test
    - No → Normal distribution?
      - No → Mann-Whitney/Wilcoxon test
      - Yes → Dunnett test

- ≥3 groups?  
  - Yes → ≥3 groups?
    - Yes → Dunn test
    - No → Homogenous?  
      - No → Dunn test
      - Yes → Dunnett test
