Evaluation of the Potential for Genotoxicity and Heritable Changes with Acuitas Lipid Nanoparticle Platform Technology

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ACUITAS THERAPEUTICS

Abstract

With the increasing use of lipid nanoparticle (LNP) technology as a promising delivery platform particularly in the areas of gene editing and RNA-based therapeutics, determining the genotoxic potential of Acuitas' LNP platform technology is essential to support continued development. To address this, we evaluated the *in vitro* genotoxicity potential of three LNP formulations, in two standard *in vitro* genotoxicity assays to investigate their potential to cause chromosome damage or mutations in mammalian cells. Furthermore, given the safety concerns associated with genome editing and the potential transmission of genetic alterations to future off-spring, we evaluated the *in vivo* safety of LNP-mediated gene editing in a germline transmission study.

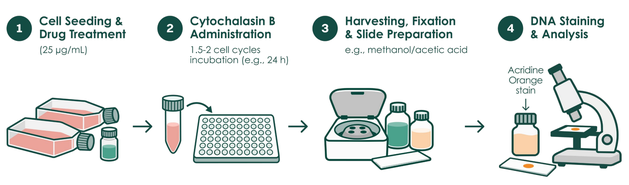
Two *in vitro* GLP genotoxicity assays were performed, including an *in vitro* mammalian cell micronucleus assay in TK6 cells to evaluate our LNP's potential to induce micronuclei (MN) in a human lymphoblastoid cell line and an *in vitro* mammalian cell forward gene mutation (CHO/HPRT) assay to evaluate our LNP's ability to induce forward mutations at the hypoxanthine-guanine phosphoribosyl transferase (HPRT) locus of Chinese hamster ovary (CHO) cells. LNP concentrations were selected based on preliminary toxicity assays and ranged from 0.05 to 3% (v/v) and from 0.001 to 10% (v/v) in the definitive micronucleus and definitive mutagenicity assay, respectively. Empty LNP07, Empty LNP09 and Empty LNP13, and/or metabolites of their lipid components, were concluded to be negative for the induction of micronuclei in TK6 cells and forward mutations in the HPRT locus in CHO cells, in both the presence and absence of exogenous metabolic activation.

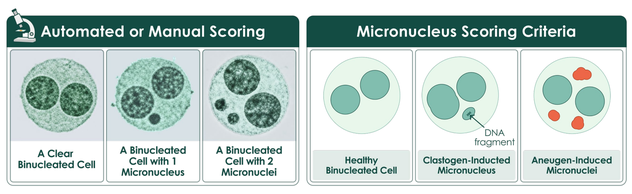
To examine the potential for germline transmission, a study in C57BL/6 female mice was conducted to quantify on-target PCSK9 liver editing in F0 generation females and in their F1 generation progeny using next-generation sequencing (NGS)-based methods. Two weeks before mating, F0 generation female mice were administered a single IV dose of an LNP formulation containing an PCSK9 genome editing payload (Cas9 mRNA + PSCK9 gRNA, 1:1) at 1 mg/kg RNA. The presence of on-target PCSK9 editing (48% Indel) was observed in the liver of treated mice, whereas no PCSK9 on-target gene editing (< LOD, 1% Indel) was found in the F1 generation off-spring, confirming that no germline transmission occurred. These results indicate that gene editing was limited to somatic cells and did not occur in the germline cells of the ovary.

These results provide compelling evidence that Acuitas' LNP delivery platform technology does not cause chromosomal damage and/or mutations in mammalian cells when evaluated in *in vitro* genotoxicity assays. In addition, transmission of heritable genetic alterations does not occur following IV administration of a gene editing LNP formulation, validating the favorable safety profile of Acuitas' LNP delivery platform technology.

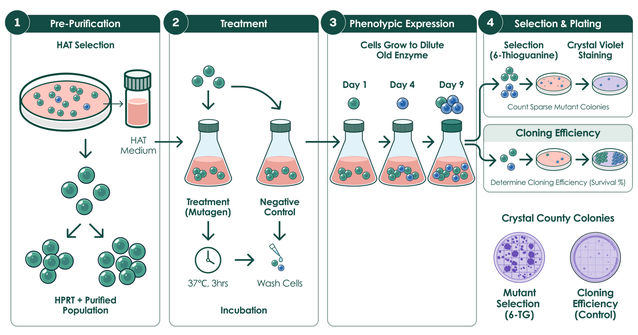
Methods

In Vitro Mammalian Cell Micronucleus Test (MNvit)

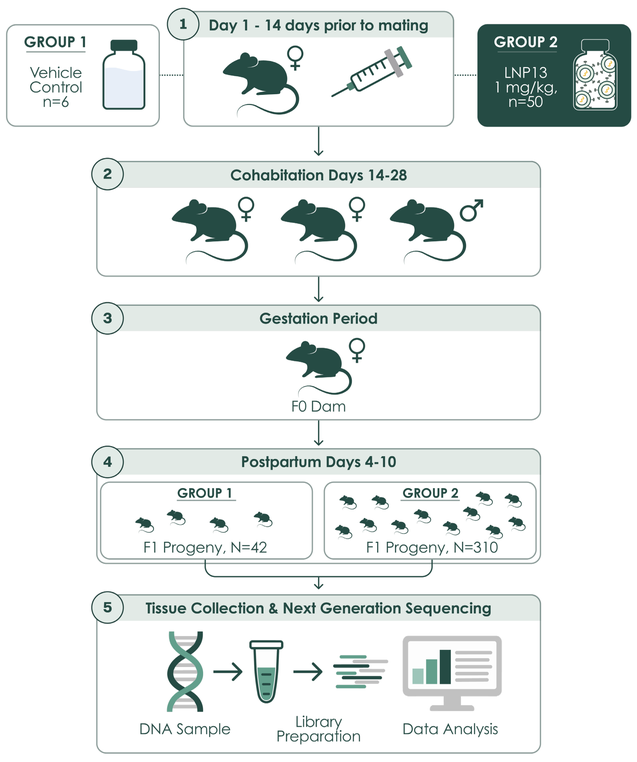




In Vitro HPRT/CHO Mutagenicity Assay



F1 Progeny Study Design



F1 progeny study. PCSK9 editing rates in livers of female (F0) and offspring (F1) mice following IV treatment of F0 females with either saline (n=6) or 1 mg/kg LNP13 carrying Cas9 mRNA and PSCK9 gRNA (n=50); 14 days later, all female animals were bred. Livers from F0 female dams, 42 pups from saline treated dams and 310 pups from LNP treated dams (the F1 generation) were collected for PCSK9 editing analysis. DNA was extracted and PCR was used to amplify the PCSK9 locus containing the editing site followed by library preparation and next-generation sequencing.

1 No Micronucleus Induction with LNP07, LNP09 or LNP13 Lipids (± S9 exogenous metabolic activation)

LNP07

Micronucleus Analysis of Empty LNP07 in the Absence of Exogenous Metabolic Activation: **4-Hour Treatment, 27-hour Harvest**

Treatment % (v/v)	Total Lipid (µg/mL)	Cytotoxicity (%)	Micronucleus Evaluation		
			Total Cells	MN Cells	MN%
Untreated	-		2000	14	0.70
Empty LNP07					
0.25	144	10	2000	14	0.70
0.5	287	10	2000	13	0.65
0.75 P	431	9	2000	16	0.80
MMC (µg/mL)					
0.12	-	52	2000	67	3.35 **

MN Cells=micronucleated mononucleated cells, P=precipitate, MMC=Mitomycin C

**p≤0.01 as compared to untreated control (Fisher Exact Test)

p>0.05, Cochran–Armitage Trend Test

Micronucleus Analysis of Empty LNP07 in the Presence of Exogenous Metabolic Activation: **4-Hour Treatment, 27-hour Harvest**

Treatment % (v/v)	Total Lipid (µg/mL)	Cytotoxicity (%)	Micronucleus Evaluation		
			Total Cells	MN Cells	MN%
Untreated	-		2000	14	0.70
Empty LNP07					
0.25	144	21	2000	16	0.80
0.5	287	22	2000	13	0.65
0.75 P	431	17	2000	18	0.90
CP (µg/mL)					
4	-	51	2000	63	3.15 **

MN Cells=micronucleated mononucleated cells, P=precipitate, CP=Cyclophosphamide

**p≤0.01 as compared to untreated control (Fisher Exact Test)

p>0.05, Cochran–Armitage Trend Test

Micronucleus Analysis of Empty LNP07 in the Absence of Exogenous Metabolic Activation: **27-Hour Treatment, 27-hour Harvest**

Treatment % (v/v)	Total Lipid (µg/mL)	Cytotoxicity (%)	Micronucleus Evaluation		
			Total Cells	MN Cells	MN%
Untreated	-		2000	15	0.75
Empty LNP07					
0.25	144	5	2000	12	0.60
0.5	287	5	2000	11	0.55
0.75 P	431	0	2000	17	0.85
VB (ng/mL)					
12	-	43	2000	217	10.85 **

MN Cells=micronucleated mononucleated cells, P=precipitate, VB=Vinblastine

**p≤0.01 as compared to untreated control (Fisher Exact Test)

p>0.05, Cochran–Armitage Trend Test

LNP09

Micronucleus Analysis of Empty LNP09 in the Absence of Exogenous Metabolic Activation: **4-Hour Treatment, 27-hour Harvest**

Treatment % (v/v)	Total Lipid (µg/mL)	Cytotoxicity (%)	Micronucleus Evaluation		
			Total Cells	MN Cells	MN%
Untreated	-		2000	7	0.35
Empty LNP09					
0.25	137	10	2000	10	0.50
0.5	273	13	2000	10	0.50
1.0 P	546	11	2000	9	0.45
MMC (µg/mL)					
0.12	-	48	2000	57	2.85 **

MN Cells=micronucleated mononucleated cells, P=precipitate, MMC=Mitomycin C

**p≤0.01 as compared to untreated control (Fisher Exact Test)

p>0.05, Cochran–Armitage Trend Test

Micronucleus Analysis of Empty LNP09 in the Presence of Exogenous Metabolic Activation: **4-Hour Treatment, 27-hour Harvest**

Treatment % (v/v)	Total Lipid (µg/mL)	Cytotoxicity (%)	Micronucleus Evaluation		
			Total Cells	MN Cells	MN%
Untreated	-		2000	9	0.45
Empty LNP09					
0.25	137	12	2000	7	0.35
0.5	273	17	2000	9	0.45
1.0 P	546	12	2000	10	0.50
CP (µg/mL)					
3	-	48	2000	38	1.90 **

MN Cells=micronucleated mononucleated cells, P=precipitate, CP=Cyclophosphamide

**p≤0.01 as compared to untreated control (Fisher Exact Test)

p>0.05, Cochran–Armitage Trend Test

Micronucleus Analysis of Empty LNP09 in the Absence of Exogenous Metabolic Activation: **27-Hour Treatment, 27-hour Harvest**

Treatment % (v/v)	Total Lipid (µg/mL)	Cytotoxicity (%)	Micronucleus Evaluation		
			Total Cells	MN Cells	MN%
Untreated	-		2000	7	0.35
Empty LNP09					
0.25	137	13	2000	7	0.35
0.5	273	17	2000	9	0.45
1.0 P	546	17	2000	8	0.40
VB (ng/mL)					
12	-	33	2000	128	6.40 **

MN Cells=micronucleated mononucleated cells, P=precipitate, VB=Vinblastine

**p≤0.01 as compared to untreated control (Fisher Exact Test)

p>0.05, Cochran–Armitage Trend Test

LNP13

Micronucleus Analysis of Empty LNP13 in the Absence of Exogenous Metabolic Activation: **4-Hour Treatment, 27-hour Harvest**

Treatment % (v/v)	Total Lipid (µg/mL)	Cytotoxicity (%)	Micronucleus Evaluation		
			Total Cells	MN Cells	MN%
Untreated	-		2000	14	0.70
Empty LNP13					
0.25	152	0	2000	13	0.65
0.5	304	0	2000	15	0.75
0.75 P	456	0	2000	13	0.65
MMC (µg/mL)					
0.12	-	38	2000	77	3.85 **

MN Cells=micronucleated mononucleated cells, P=precipitate, MMC=Mitomycin C

**p≤0.01 as compared to untreated control (Fisher Exact Test)

p>0.05, Cochran–Armitage Trend Test

Micronucleus Analysis of Empty LNP13 in the Presence of Exogenous Metabolic Activation: **4-Hour Treatment, 27-hour Harvest**

Treatment % (v/v)	Total Lipid (µg/mL)	Cytotoxicity (%)	Micronucleus Evaluation		
			Total Cells	MN Cells	MN%
Untreated	-		2000	16	0.80
Empty LNP13					
0.25	152	7	2000	16	0.80
0.5	304	9	2000	16	0.80
0.75 P	456	6	2000	21	1.05
CP (µg/mL)					
4	-	39	2000	45	2.25 **

MN Cells=micronucleated mononucleated cells, P=precipitate, CP=Cyclophosphamide

**p≤0.01 as compared to untreated control (Fisher Exact Test)

p>0.05, Cochran–Armitage Trend Test

Micronucleus Analysis of Empty LNP13 in the Absence of Exogenous Metabolic Activation: **27-Hour Treatment, 27-hour Harvest**

Treatment % (v/v)	Total Lipid (µg/mL)	Cytotoxicity (%)	Micronucleus Evaluation		
			Total Cells	MN Cells	MN%
Untreated	-		2000	18	0.90
Empty LNP13					
0.25	152	1	2000	16	0.80
0.5	304	3	2000	14	0.70
0.75 P	456	3	2000	16	0.80
VB (ng/mL)					
12	-	55	2000	225	11.25 **

MN Cells=micronucleated mononucleated cells, P=precipitate, VB=Vinblastine

**p≤0.01 as compared to untreated control (Fisher Exact Test)

p>0.05, Cochran–Armitage Trend Test

***In vitro* Mammalian Cell Micronucleus Assay in TK6 Cells treated with Empty LNP07, LNP09, or LNP13**. Three GLP-compliant *in vitro* MN Assays were performed to evaluate the potential of Empty LNP07, Empty LNP09 or Empty LNP13 to induce micronuclei in TK6 cells, a human lymphoblastoid cell line, in the presence and absence of an exogenous metabolic activation system (i.e., S9). The total lipid concentrations for Empty LNP07, LNP09 and LNP13 were 57.42, 54.59 and 60.77 mg/mL, respectively, and were approximately equivalent to the total lipid content associated with an mRNA-LNP formulation having an mRNA concentration of ~2.0 mg/mL. In the preliminary toxicity assays, the concentrations tested ranged from 1 to 10% (v/v) with and without S9. Cytotoxicity was not observed at any concentration in any of the three treatment groups, but visible precipitate (i.e., cloudiness) was observed at concentrations ≥ 1% (v/v). Based on these results, the concentrations chosen for the MN assay ranged from 0.05 to 3% (v/v) for all three exposure groups. In the definitive micronucleus assay, cytotoxicity was not observed at any concentration in any of the three treatment groups, but visible precipitate was observed at concentrations ≥ 0.75% (v/v) thus, the concentrations selected for evaluation of micronuclei for Empty LNP07 and Empty LNP13 were 0.25, 0.5, and 0.75% (v/v) for all three exposure groups, and 0.25, 0.5, and 1% (v/v) for Empty LNP09 in all three exposure groups. Empty LNP07, LNP09 and LNP13, and/or metabolites of lipid components, were concluded to be negative for the induction of micronuclei in TK6 cells, as neither statistically significant nor dose dependent increases in micronuclei induction were observed at any concentration in treatment groups with or without S9 ($p > 0.05$; Fisher's Exact and Cochran-Armitage tests).

2 No CHO/HPRT Mutations with LNP07, LNP09 and LNP13 Lipids (± S9 exogenous metabolic activation)

Summary of Representative Results for Definitive Mutagenicity Assay with Empty LNP07, LNP09 or LNP13

Treatment	Dose Level % (v/v)	S9	Initial Survival: Cells/mL x 10^6	Initial Survival: Cloning Efficiency Colonies/Plate			Initial Survival: Cloning Efficiency %	Initial Survival: Relative Survival (Adj.,%)	Selection: TG Mutants/Plate				Selection: Total Mutant Colonies	Selection: Cloning Efficiency Colonies/Plate			Selection: Cloning Efficiency %	Mutant Frequency (x10 ^{6}): Individual	Mutant Frequency (x10 ^{6}): Average
Untreated Control *	0	-	0.854	168	150	152	78.33	102.37	4	5	4	9	22	152	157	123	72.00	12.73	8.80
	0	-	0.948	140	135	125	66.67	96.79	2	6	5	3	16	276	266	281	137.17	4.86	
Empty LNP07	1.0 P	-	0.311	29	31	37	16.17	7.70	11	10	7	7	35	285	286	245	136.00	10.72	10.19
	1.0 P	-	0.308	94	111	125	55.00	25.94	3	4	6	10	23	198	203	194	99.17	9.66	
Empty LNP09	5.0 P	-	0.518	47	41	37	20.83	15.16	4	4	8	8	24	99	94	97	48.33	20.69	14.89
	5.0 P	-	0.529	20	39	38	16.17	12.37	3	5	1	2	11	91	104	108	50.50	9.08	
Empty LNP13	10.0 P	-	0.421	52	49	48	24.83	13.56	1	2	1	2	6	107	101	100	51.33	4.87	9.42
	10.0 P	-	0.468	51	54	68	28.83	17.51	6	1	3	10	20	116	112	130	59.67	13.97	
EMS (pos control) *	0.2 [a]	-	0.768	141	165	135	73.50	86.44	104	91	106	99	400	153	164	165	80.33	207.47	205.88**
	0.2 [a]	-	0.789	166	133	168	77.83	93.99	113	121	109	95	438	176	202	158	89.33	204.29	
Untreated Control *	0	+	0.768	120	128	123	61.83	97.22	4	3	4	4	15	151	123	171	74.17	8.43	10.61
	0	+	0.764	135	144	115	65.67	102.76	6	5	4	8	23	136	139	175	75.00	12.78	
Empty LNP07	10.0 P	+	0.526	84	76	63	37.17	40.04	3	6	5	2	16	149	141	157	74.50	8.95	7.42
	10.0 P	+	0.533	64	85	50	33.17	36.22	8	1	2	1	12	153	193	164	85.00	5.88	
Empty LNP09	10 P	+	0.544	47	40	53	23.33	21.36	2	3	1	1	7	163	198	170	88.50	3.30	2.96
	10 P	+	0.522	38	46	38	20.33	17.84	1	1	4	0	6	181	201	192	95.67	2.61	
Empty LNP13	5.0 P	+	0.398	117	70	88	45.83	24.83	2	2	0	4	8	189	179	152	86.67	3.85	4.30
	5.0 P	+	0.336	79	71	62	35.33	16.15	1	2	2	4	9	156	165	154	79.17	4.74	
B(a)P (pos control) *	4.0 [b]	+	0.740	72	97	91	43.33	65.66	44	74	57	47	222	201	189	188	96.33	96.02	117.90 **
	4.0 [b]	+	0.723	98	113	94	50.83	75.27	48	55	54	51	208	114	123	135	62.00	139.78	

[a] µL/mL,[b] µg/mL. P=Test article precipitate; EMS = Ethyl methane Sulfonate; B(a)P = Benzo(a)pyrene; - = not determined due to low relative survival (%)

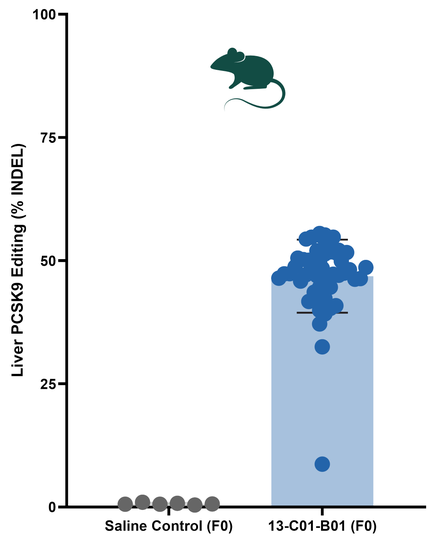
* Untreated and positive control data listed is representative data from the CHO/HPRT assay with Empty LNP07, similar results were produced in the CHO/HPRT assay with Empty LNP09 and Empty LNP13.

-S9 Without Dose-Related Trend (Lack of Fit Test), +S9 Without Dose-Related Trend (Lack of Fit Test); **p<0.01 compared to vehicle control (T-Test).

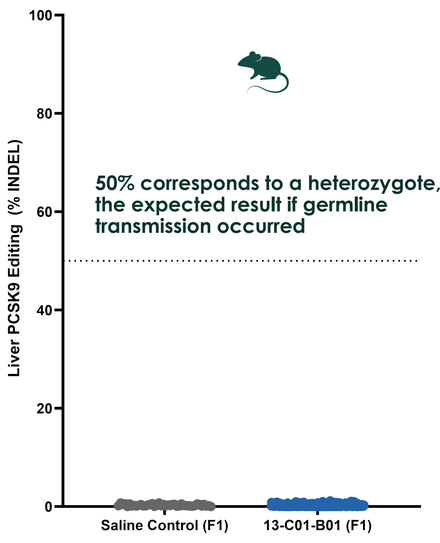
***In vitro* Mammalian Cell Forward Gene Mutation (CHO/HPRT) assays with Empty LNP07, LNP09 and LNP13.** Three GLP-compliant *in vitro* Mammalian Cell Forward Gene Mutation (CHO/HPRT) assays were performed to evaluate the potential of Empty LNP07, Empty LNP09 or Empty LNP13 to induce forward mutations at the hypoxanthine guanine phosphoribosyl transferase (HPRT) locus (hprt) of Chinese hamster ovary (CHO) cells, in the presence and absence of an exogenous metabolic activation system (i.e., S9). The total lipid concentrations for Empty LNP07, LNP09 and LNP13 were 57.42, 54.59 and 60.77 mg/mL, respectively, and were approximately equivalent to the total lipid content associated with an mRNA-LNP formulation having an mRNA concentration of ~2.0 mg/mL. In the preliminary toxicity assay, the concentrations tested were 0.05 to 10% v/v with and without S9. For Empty LNP07 and LNP13, visible precipitates (i.e., cloudiness) were observed at concentrations ≥1.0% v/v at the beginning of the treatment and at concentrations ≥5% v/v by the end of treatment. For Empty LNP09, visible precipitate (i.e., cloudiness) was observed at concentrations ≥5% v/v at beginning and the end of treatment. Based upon cytotoxicity and/or solubility limitations of the test article, the concentrations chosen for the definitive mutagenicity assay ranged from 0.001 to 10% (v/v), with and without S9. Empty LNP07, LNP09 and LNP13, and/or metabolites of lipid components, were concluded to be negative for the induction of forward mutations, as no significant increases in mutant frequency, relative to the concurrent untreated was observed at any concentration evaluated with or without S9 ($p > 0.05$). The positive controls induced significant increases in mutant frequency ($p < 0.01$) in all three assays.

No Germline Transmission to F1 Progeny from mRNA-LNP Edited Female Mice

3 mRNA-LNP Treated F0 Females



4 Offspring of mRNA-LNP treated Females (F1 Generation Pups)



5 Assay Cloudiness Does Not Affect MN Induction

Summary of Representative Results for Micronucleus Analysis of Empty LNP07, Empty LNP09 and Empty LNP13 in the Presence of Visible Precipitate, with or without Exogenous Metabolic Activation (Rat S9)

Treatment	Treatment / Harvest Condition (h)	Dose Level (%, v/v)	S9	Cytotoxicity (%)	Micronucleus Evaluation: Total Cells	MN Cells	MN%
Untreated	4 h/ 27 h	NA	-		2000	9	0.45
			+		2000	10	0.50
	27 h	NA	-		2000	8	0.40
Empty LNP07	4 h/ 27 h	10 P	-	35	2000	10	0.50
			+	33	2000	13	0.65
	27 h	10 P	-	9	2000	9	0.45
Empty LNP09	4 h/ 27 h	10 P	-	23	2000	6	0.30
			+	24	2000	12	0.60
	27 h	10 P	-	5	2000	7	0.35
Empty LNP13	4 h/ 27 h	10 P	-	24	2000	9	0.45
			+	18	2000	17	0.85
	27 h	10 P	-	0	2000	12	0.60
MMC	4 h/ 27 h	0.12 µg/mL	-	44	2000	81	4.05 **
CP		4 µg/mL	+	51	2000	46	2.30 **
VB	27 h	12 ng/mL	-	45	2000	185	9.25 **

MN Cells=micronucleated mononucleated cells, P=precipitate; MMC=Mitomycin C; CP=Cyclophosphamide; VB=Vinblastine; NA=Not applicable

**p≤0.01 as compared to untreated control (Fisher Exact Test)

p>0.05, Cochran–Armitage Trend Test

Evaluation of cloudiness (i.e., 'turbidity') in MN assay. LNP are inherently cloudy in appearance. This study was designed to investigate the effects of cloudiness in the *in vitro* MN assay. Cloudiness is synonymous to turbidity as per SOPs, limiting the upper concentrations used in the assay (OECD 487). Results demonstrate that cloudiness did not affect the results of the *in vitro* micronucleus assay under the conditions of the assay. Empty LNP07, LNP09 and LNP13 were concluded to be negative, at all three concentrations evaluated (2.5, 5.0 and 10% v/v) for all three exposure groups for the induction of micronuclei in both the non-activated and S9-activated test systems in the *in vitro* mammalian micronucleus assay using TK6 cells.

Summary

1. Empty LNP07, Empty LNP09 and Empty LNP13, and/or metabolites of their lipid components, were negative for the induction of micronuclei in TK6 cells, with and without exogenous metabolic activation.
2. Empty LNP07, Empty LNP09 and Empty LNP13, and/or metabolites of their lipid components, were negative for the induction of forward mutations in the HPRT locus in CHO cells, with and without exogenous metabolic activation.
3. Acuitas' platform LNP technology effectively delivered a gene editing system to mouse liver, resulting in substantial on-target liver editing quantified by next-generation sequencing methods.
4. No germline transmission of genetic alterations was observed in offspring (F1 generation pups), following IV administration of LNP encapsulating a mRNA-encoded gene editor to F0 female mice.
5. Cloudiness due to LNP, synonymous to 'turbidity' in the *in vitro* micronucleus assay SOPs, has no effect on MN induction up to the 10% (v/v) limit of the assay, suggesting that the visible appearance of the test article should be taken into consideration when selecting dose.
6. Taken together, these results indicate that Acuitas' LNP delivery platform technology is low risk for genotoxicity or transmission of heritable changes to offspring.

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Acknowledgement: We acknowledge Inotiv Genetic Toxicology, 9601 Medical Center Dr. Rockville, MD 20850 for performing the *in vitro* genotoxicity assays.