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Google Translate was used to produce a rough translation. It was compared with the original Japanese. Grammar and syntax were corrected as necessary. Some statements were creatively reinterpreted for clarity while some idiosycratic terms were left as-is in order to preserve the flavor of the Japanese language.

Emphases and comments were added at discretion.

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SARS-CoV-2 mRNA Vaccine (BNT162, PF-07302048)

2.6.4 Summary of pharmacokinetic study PFIZER CONFIDENTIAL

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Terms and abbreviations used in this section

Term / Abbreviation - Full Expression: Definition

ALC-0159 - PEG lipid added to this drug

ALC-0315 - Amino lipid added to this drug

[3H]-CHE - Radiolabeled [Cholesteryl-1,2- 3H(N)] - Cholesteryl Hexadecyl Ether

DSPC - 1,2-Distearoyl-SN-Glycero-3-Phosphocholine

GLP - Good Laboratory Practice: Criteria for conducting non-clinical studies on drug safety

LNP - Lipid-nanoparticle

modRNA - Nucleoside-modified mRNA

mRNA - Messenger RNA

m/z - (m over z): A dimensionless quantity obtained by dividing the mass of an ion by the unified atomic mass unit (= Dalton) and further dividing it by the absolute value of the number of charges of the ion.

PEG - Polyethylene glycol

PK - Pharmacokinetics

RNA - Ribonucleic acid

S9 - Supernatant fraction obtained from liver homogenate by centrifuging at 9000G

WHO - World Health Organization

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1. Summary

BNT162b2 (BioNTech code number: BNT162, Pfizer code number: PF-07302048) is a modified nucleoside mRNA (modRNA) that encodes the ***full-length spike glycoprotein**** (S protein) of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which is being developed [*as of this study*] as the essence of an mRNA vaccine against SARS-CoV-2 infections.

[* *BNT162b1(one) is the mRNA version that encodes only the RBD whereas BNT162b2(two) encodes the whole S protein.*]

In formulating the BNT162b2 encapusalting nano-particle vaccine, two functional lipids, ALC-0315 (aminolipid) and ALC-0159 (PEG lipid), and two structural lipids DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine) and cholesterol are mixed together. Lipid nanoparticles (LNP) that encapsulate BNT162b2 are thus formed (hereinafter, "BNT162b2-encapsulating LNP").

Nonclinical pharmacokinetics (PK) of ALC-0315 and ALC-0159 components of BNT162b2 encapsulating LNP were evaluated in vivo and in vitro in studies assessing absorption, metabolism, and excretion of said components. In addition, biodistribution studies using luciferase encoding RNA as an alternative marker, and radiolabeled lipids were carried out. Based on the fact that the development of vaccines aimed at preventing infectious diseases does not require evaluation of systemic exposure (WHO, 2005; Non-clinical study guidelines for infectious disease preventive vaccines)^1,^2, no muscle injection internal PK study of BNT162b2 encapsulating LNP was performed. In addition, two other types of lipids cholesterol and DSPC are naturally occurring lipids that are thought to be metabolized and excreted in the same way as endogenous lipids. *Also, BNT162b2 is degraded by ribonucleases in the cells that have taken it up, resulting in nucleic acid charges. The S protein from BNT162b2 is expected to undergo proteolysis. Hence, it was considered unnecessary to evaluate the metabolism and excretion of these components.*

Luciferase encoding RNA was used as an alternative reporter for BNT162b2. LNP having the same lipid composition as the BNT162b2 encapsulating LNP was used. In a PK study in which Luciferase RNA-encapsulating LNP was *intravenously administered* to Wistar Han rats, plasma, urine, feces and liver samples were collected over time and the concentrations of ALC-0315 and ALC-0159 in each sample were measured. ALC-0315 and ALC-0159 were shown to be rapidly distributed from the blood to the liver. About 1% and 50% of the doses of ALC-0315 and ALC-0159 respectively were excreted in feces as unchanged drug. Both were below the detection limit in urine.

In the biodistribution test, luciferase RNA encapsulating LNP was *intramuscularly administered* to BALB/c mice. The expression of luciferase was observed in the liver as well as at the administration site, albeit at a substantially lower level. Expression at the administration site of luciferase was observed from 6 hours after administration, and was not observable after 9 days. Expression in the liver was also observed 6 hours after administration and was not observable by 48 hours after administration. In the intramuscular administration of radiolabeled LNP containing luciferase RNA to rats to quantify biodistribution, the radioactivity concentration was the highest at the administration site. Liver was prominent and the next highest (up to 18% of the dose).

Metabolism of ALC-0315 and ALC-0159 was evaluated in vitro using CD-1/ICR mouse, Wistar Han or Sprague Dawley rat, cynomolgus monkey or human blood, liver microsomes, liver S9 fraction and hepatocytes. In vivo metabolism was also examined using plasma, urine, feces and liver samples collected in the above rat intravenous PK test. These in vitro and in vivo studies showed that ALC-0315 and ALC-0159 were slowly metabolized by hydrolysis of ester and amide bonds, respectively, in any of the animal species tested.

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The above nonclinical pharmacokinetic evaluation showed that the primary destination of the LNP that entered the circulation was the liver. In addition, metabolism and fecal excretion may be involved in the clearance of ALC-0315 and ALC-0159, respectively.

2. Analytical Method

Report number: PF-07302048_06xxx_072424

An appropriate LC/MS [*liquid chromatography / mass spectrometry*] pharmacokinetic test for quantifying the concentration ALC-0315 and ALC-0159 was developed [without GLP] (M2.6.4.3):

* 20 µL plasma;

* Liver homogenate prepared using sections collected from three parts of the liver, the "pool" thereof diluted with an appropriate blank matrix;

* Urine and fecal homogenate diluted with an appropriate blank matrix;

-- Each apportioned sample containing an internal standard substance (PEG-2000), after protein denaturing wth acetonitrile, was centrifuged, and the supernatant was subjected to LC-MS/MS measurement.

[*Above may not an acceptable style for a research paper. Is there a better way to disentangle?*]

3. Absorption

Report number: PF-07302048_06xxx_072424, Summary table: 2.6.5.3

To investigate the pharmacokinetics of ALC-0315 and ALC-0159, luciferase RNA encapsulating LNP containing *single intravenous dose* of 1 mg RNA/kg was administered to male Wistar Han rats. Over-time sampling (pre-dose, then post-dose at hours 0.1, 0.25, 0.5, 1, 3, 6, and 24, as well as on days 2, 4, 8, and 14) was performed (3 animals / time point). ALC-0315 and ALC-0159 concentrations were measured in plasma and liver, and the PK parameters were calculated (Table 1). It is noteworthy that the plasma concentration 24 hours after administration was less than 1% of the maximum plasma concentration (Figure 1). The apparent terminal phase elimination half-life (t½) was similar in plasma and liver, with ALC-0315 at 6-8 days and ALC-0159 at 2-3 days. The results suggest that the liver is one of the major tissues that take up ALC-0315 and ALC-0159 from the plasma.

Results of examination of urinary and fecal concentrations of ALC-0315 and ALC-0159 conducted in this study are described. M2.6.4.6.

Table 1 - Pharmacokinetics of ALC-0315 and ALC-0159 when luciferase RNA-encapsulating LNP was intravenously administered to Wistar Han rats at a dose of 1 mg RNA/kg.

Analytical material	ALC-0315	ALC-0159
Dosage of analyte (mg/kg)	15.3	1.96
Gender/N	Male/3^b	Male/3^b
t½ (h)	139	72.7
AUC inf(µg • h/mL)	1030	99.2
AUC last(Mg • h/mL)	1020	98.6

To the liver Distribution ratio (%)^a 60 20

a. Calculated as [maximum liver distribution (µg)] / [dose (µg)].

b. 3 animals at each time point. Sparse sampling.

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Figure 1- Plasma and hepatic concentrations of ALC-0315 and ALC-0159 when luciferase RNA-encapsulated LNP was intravenously administered to Wistar Han rats at a dose of 1 mg RNA/kg.

// Fig. 1 Left: Plasma; Right: Liver

4. Distribution

Report number: R-x -0072, 185350, Summary table: **2.6.5.5A, 2.6.5.5B**

Female BALB/c mice (3 mice) were administered luciferase RNA-encapsulating LNP, and the biodistribution of BNT163b2 was examined usuing luciferase luminescence as an alternative marker. Luciferase RNA encapsulating LNP was *intramuscularly administered* to the left and right hind limbs of mice at a dose of 1 µg RNA (2 µg RNA in total).

5 minutes prior to each luminescence detection, luciferin substrate was adminsitered. Then, under isoflurane anesthesia, luminescence was emitted in vivo using the Xenogen IVIS Spectrum at hours 6 and 24, and on days 2, 3, 6, and 9. The expression of luciferase protein in the same individual was evaluated over time. Expression of luciferase at the administration site was observable from hour 6 to day 9. *Expression in the liver was also observable from hour 6*, but disappeared by 48 hours after administration. The distribution to the liver was considered to indicate that a part of the locally administered luciferase RNA-encapsulating LNP entered the circulation and was taken up by the liver. As detailed in Section M2.6.4.3, when rats were intravenously administered Luciferase RNA-encapsulating LNP, the liver was the major destination for ALC-0315 and ALC-0159. It has been suggested that intramuscular administration distribution will match the intravenous route distribution. The results of this study confirm this. No toxic findings indicating liver damage were found in the rat repeated-dose toxicity test (M2.6.6.3).

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Figure 2 - In vivo luminescence in BALB/c mice intramuscularly administered with luciferase RNA-encapsulating LNP

/* Figure 2 goes here */

[3H]-Cholesteryl hexadecyl ether-labeled LNP (Luciferase RNA-encapsulating LNP with [3H] -CHE) was intramuscularly administered at a dose of 50 µg RNA to male and female Wistar Han rats. At 15 minutes and 1, 2, 4, 8, 24 and 48 hours after administration, blood, plasma and tissue were collected from each of the three animals, and the in vivo distribution of LNP was evaluated by measuring the radioactivity concentration by the liquid scintillation counting method. In both males and females, the radioactivity concentration was highest at the administration site at all measurement points. The radioactivity concentration in plasma was the highest 1 to 4 hours after administration. Biodistribution was mainly in the liver, spleen, adrenal gland and ovaries, and the highest radioactivity concentration in these tissues was 8 to 48 hours after administration. The total radioactivity recovery rate other than the administration site is highest in the liver (up to 18%), compared with the spleen (1.0% or less), adrenal gland (0.11% or less) and ovaries (0.095% or less), which was significantly lower. The average concentration of radioactivity and the tissue distribution pattern were generally similar between males and females.

The in vivo expression distribution of the antigen encoded by BNT162b2 is considered to depend on the LNP distribution. Since the lipid composition of the luciferase RNA-encapsulated LNP used in this study is the same as that of the application product of BNT162b2, it is considered that the results of this study show the distribution of BNT162b2-encapsulated LNP.

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5. Metabolism

Report number: 01049-xx008 , 01049-xx009 , 01049-xx010 , 01049-xx020 , 01049-xx021 , 01049-

xx022 ,PF-07302048_05xxxx_043725,

Summary table: 2.6.5.10A , 2.6.5.10B , 2.6.5.10C , 2.6.5.10D

In vitro metabolic stability of ALC-0315 and ALC-0159 was evaluated using CD-1/ICR mice, Wistar Han or Sprague Dawley rats, cynomolgus monkeys and human liver microsomes, liver S9 fractions and hepatocytes. ALC-0315 or ALC-0159 was added to the liver microsomes or liver S9 fraction (120 minutes incubation) or hepatocytes (240 minutes incubation) of each animal species, and the proportion of unchanged drug after incubation was measured. ALC-0315 and ALC-0159 were metabolically stable in all animal species and test systems, and the final proportion of unchanged drug was over 82%.

Furthermore, the metabolic pathways of ALC-0315 and ALC-0159 were evaluated in vitro and in vivo. These studies evaluated in vitro metabolism using blood, hepatic S9 fractions and hepatocytes from CD-1 mice, Wistar Han rats, cynomolgus monkeys and humans. In vivo metabolism was evaluated using plasma, urine, feces and liver samples collected in the rat PK test (M2.6.4.3). The test results clarified that the metabolisms of ALC-0315 and ALC-0159 are slow, and that they are metabolized by hydrolysis of ester bond and amide bond, respectively. The hydrolysis-induced metabolism shown in Figure 3 and Figure 4 was observed in all the animal species evaluated.

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Figure 3 - Estimated in vivo metabolic pathway of ALC-0315 in various animal species.

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In blood (Mo, R) In hepatocytes (Mo, R, Mk, H) In liver S9 (Mo, R, H) Plasma (R)In blood (Mo, R)

In hepatocytes (Mo, R, Mk, H) In liver S9 (Mo, R, H) Plasma (R) In blood (Mo, R) Liver S9 (Mk)

Plasma (R) Liver (R) In blood (Mo, R) Liver S9 (Mk) Plasma (R)

Urinary (R) Feces (R) Liver (R) Urinary (R)Glucuronide

*/

/* end metabolite shown is glucuronide */

H: human, Mk: monkey, Mo: mouse, R: rat

ALC-0315 is metabolized by undergoing ester hydrolysis twice in a row. These two hydrolyses first

produce a monoester metabolite (m/z 528), and then a double deesterified metabolite (m/z 290). Only the rat urine PK test detected a conjugate of the latter (m/z 466). It was also confirmed that the acidic product of the two hydrolyses was 6-hexyldecanoic acid (m/z 255).

Figure 4 - Estimated in vivo metabolic pathway of ALC-0159 in various animal species

H: human, Mk: monkey, Mo: mouse, R: rat

The main metabolic pathway of ALC-0159 was the production of N, N-ditetradecylamine (m/z 410) by hydrolysis of the amide bond. This metabolite was detected in mouse and rat blood and in mouse, rat, monkey and human hepatocytes and liver S9 fractions. No metabolites of ALC-0159 were identified in the in vivo samples.

6. Excretion

Urine and feces concentrations of ALC-0315 and ALC-0159 were measured at specified times after administration of intravenous luciferase RNA-encapsulating LNP in rats at a dose of 1 mg RNA/kg (M2.6.4.3). Neither ALC-0315 nor ALC-0159 in unchanged form was detected in urine. On the other hand, unchanged forms of ALC-0315 and ALC-0159 were detected in feces, and the ratios per dose were about 1% and about 50%, respectively. As shown in Figure 3, a metabolite of ALC-0315 was detected in urine.

7. Pharmacokinetic drug interactions

No pharmacokinetic drug interaction studies have been conducted with this vaccine.

8. Other pharmacokinetic studies

No other pharmacokinetic studies of this vaccine have been conducted.

9. Discussion and conclusion

By two weeks post dose in the rat PK study, plasma and liver ALC-0315 levels were reduced to approximately 1/7000 and 1/4 of the maximum levels, and ALC-0159 levels were reduced to approximately 1/8000 and 1/250. T½ is comparable in plasma and liver: ALC-0315 is 6-8 days, and ALC-0159 is 2-3 days. The plasma t½ value is considered to indicate that each lipid was distributed in tissues

as LNP and then redistributed in plasma during the elimination process.

Very little of the unchanged form of ALC-0315 was detected in either urine or feces, but monoester metabolites, double deesterified metabolites and 6-Hexyldecanoic acid were detected from feces and plasma samples taken in the rat PK test, and in the rat urine were double deesterified metabolite and glucuronic acid conjugate. This metabolic process is thought to be the major clearance mechanism of ALC-0315, but no quantitative data have been obtained to test this hypothesis.

On the other hand, about 50% of the dose of ALC-0159 was excreted in feces as unchanged drug. In in vitro metabolism experiments, it was slowly metabolized by hydrolysis of amide bonds.

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In blood (Mo, R) In hepatocytes (Mo, R, Mk, H) In liver S9 (Mo, R, Mk, H)

N, N-ditetradecylamine m/z 410

Since the in vivo expression distribution of the antigen encoded by BNT162b2 is considered to depend on the LNP distribution, luciferase RNA-encapsulating LNP was intramuscularly administered to BALB/c mice, and the in vivo distribution of the alternative reporter protein was investigated. While luciferase was observed prominently at the administration site, it was also observed in the liver albeit to much a leser degree. Expression at the administration site of luciferase was observed from 6 hours after administration, and disappeared 9 days after administration. Expression in the liver was observed from 6 hours after administration and disappeared by 48 hours after administration. The distribution to the liver was considered to indicate that the locally administered luciferase RNA-encapsulated LNP reached the circulating blood and was taken up by the liver. Intramuscular administration of a radioactivity-labeled luciferase RNA-encapsulating LNP to rats showed the highest radioactivity concentration at the administration site. Other than the site of administration, it was highest in the liver, followed by spleen, adrenal gland, and ovaries, but the total radioactivity recovery rate for the dose in the latter tissues was significantly lower than that in the liver. This result was consistent with the expression of luciferase in the liver in the mouse biodistribution test. No toxic findings indicating liver damage were found in the rat repeated-dose toxicity test (M2.6.6.3).

From the above nonclinical pharmacokinetic evaluation, it was shown that LNP that reached the circulating blood was taken up by the liver. It was also suggested that metabolism and fecal excretion are involved in the clearance of ALC-0315 and ALC-0159, respectively.

10. Chart

Charts are shown in the text and in the summary table.

References

1 - World Health Organization. Annex 1. Guidelines on the nonclinical evaluation ofvaccines. In: WHO Technical Report Series No. 927, Geneva, Switzerland. WorldHealth Organization; 2005: 31-63.

2 - Non-clinical study guidelines for infectious disease preventive vaccines (No. 0527 from Yaksik Examination No. 1, May 27, 2010)

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