PROJECT ACRONYM

**CUPIDO**

PROJECT TITLE

**Cardio Ultraefficient nanoParticles for Inhalation of Drug prOducts**

# Deliverable 3.5

# Validation of nanoparticle biodistribution in large animals

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# Table of Revisions

| REVISION NO. | DATE | WORK PERFORMED | CONTRIBUTOR(S) |
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| 1 | 02/01/2020 | Draft | Alessio Alogna |
| 2 | 09/01/2020 | Revision | Daniele Catalucci |
| 3 | 10/01/2020 | Revision | Ethics Board |
| 4 | 16/01/2020 | Revision | CCG |
| 5 | 23/01/2020 | Revision | IPR Team |
| 6 | 29/01/2020 | Formatting | Paulina Piotrowicz |

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# 1. Executive summary

This deliverable focuses on the biodistribution of the final product developed within CUPIDO. This product owns a certain technological complexity, consisting of i) a calcium phosphate (CaP) nanocarrier, encapsulating ii) a cardiac-specific biological active compound, a recently developed peptide ("Mimetic Peptide", MP). We, therefore, had to break down the biodistribution tests into two separate experiments, while investigating the two components of the CUPIDO technology (CaP and MP) separately. The activities performed during the deliverable concerned:

(1) *The biodistribution of the CaPs carrier*. In these in-vivo experiments, CaPs loaded with a marker-peptide, hemoagglutinin (HA), were acutely inhaled as a single-dose administration in anesthetized Landrace pigs. HA was detected in the myocardium as well as in the other tissues harvested at the end of the experiment, providing important information on the kinetic of the carrier.

(2) *The pharmacokinetic characteristics of the "free" MP and the self-internalizing MP (R7W-MP)*[1]. A preliminary pharmacokinetic analysis was performed in rodents as well as in vitro, assessing the persistency as well as the plasmatic stability of MP and *R7W-MP* when exposed to plasma from different species.

**Key deliverable achievements**:

The in-vivo experiments on the biodistribution of the CaP nanocarrier in large animals have been performed up to 10 hours after a single-dose inhalation of CaP-HA. The in-vitro stability of the biological active compound (MP) has been investigated, increasing our knowledge of the compound for the future in-vivo translation.

## 2. Abbreviations

CHA: CHARITE - Universitaetsmedizin Berlin

CNR: National Research Council

HA: Hemoagluttinin

MP: Mimetic peptide

NP: Nanoparticles

CaP: Calcium Phosphate nanoparticles

SAN: SANOFI - Aventis Recherche & Developpement

## 3. Cooperation between participants

In collaboration with CNR and SAN, different strategies have been adopted in order to overpass the critical issues related to the pharmacokinetics and biodistribution and the best in-process controls were found to obtain CaP in compliance with the TPP.

CHA, CNR and SAN shared information about the protocol and procedures of the experiments regarding the data on dose/efficiency during the CUPIDO semesterly meeting in Berlin (March 25, 26 2019).

CHA, CNR and SAN shared information about the data on dose/efficiency results during a conference call (April 12, 2019).

CHA, CNR and SAN shared information about the data on dose/efficiency results during a conference call (May 15, 2019).

CHA, CNR and SAN shared information about the data on dose/efficiency results during a conference call (June 25, 2019).

# 4. Introduction

The main goals of the utilization of nanoparticles (NP) in cardiovascular medicine are to improve the bioavailability, stability and safety of already existing drugs. By providing a higher solubility, NP-loaded drugs are expected to be protected from systemic degradation, show reduced toxicity and immunogenicity, possess ameliorated pharmacokinetics and increased half-life and exhibit increased bioavailability and precise biodistribution. Moreover, with the possibility to be functionalized with different classes of targeting moieties, nanodrug formulations are expected to enhance selective delivery to the site of interest and benefit from a lower clearance from the body[2].

The selection of a proper route of drug administration (currently mainly at oral and intravenous levels) is fundamental for nano-based drugs to be successfully released. For this reason, the CUPIDO project has been envisioning an innovative approach combining the inhalation, as a new administration route utilized in cardiology, with a novel NP formulation based on calcium phosphate[2].

Moreover, to reduce off-target toxicity issues and further implement the therapeutic selectivity to the heart, surface functionalization with cell-specific targeting groups (e.g. peptide, aptamers) can allow for a more direct and selective tuning of NP biodistribution toward the diseased myocardium. With the establishment of a further point-of-interaction with the targeted cells, more efficient NP internalization to the cell and thus enhanced release of the payload to cardiomyocytes may be envisaged[2].

However, despite attempts by many research groups and pharmaceutical companies, no clinically approved drugs have so far been obtained for this purpose. In particular, the biodistribution of the NP carrier, as well as of the mimetic peptide (MP) is yet to be investigated. Aim of this deliverable is to report on the experimental activities performed within this work package on the biodistribution and the pharmacokinetics of unloaded nanoparticles as well as on MP.

In specific, two different technologies needed to be tested:

1) CaPs as a drug carrier. In order to test the biodistribution the CaPs were loaded with hemoagglutinin (HA). The myocardium and other organs were then analyzed for the presence of CaPs-HA in two different protocols (at 5 and at 10 hours).

2) MP and its *self-internalizing version (R7W-MP)*[1] as a novel inotropic therapy. The in-vivo investigation of the biodistribution of a small peptide is quite complex and at the current stage of knowledge is difficult to standardize. Therefore, in parallel to a preliminary pharmacokinetic study in mice, the colleagues at SAN investigated the plasmatic stability of MP/R7W-MP in vitro.

# 5. Ethics requirement: Animals (A)

For the implementation of T3.3, CHA obtained:

- Authorization for experimenting on large animals in their laboratories;
- Ethical approval for research project and protocol relevant for running CUPIDO-related experiments.

In particular, authorization and ethical approval concern the following experiments: “Biodistribution u. kardiovaskuläre Effekte b. d. Inhalation von funktionelle Nanopartikeln in Landschwein” (G 0063/16 Inhalation von Nanopartikeln Schwein); “Inhalation Herx-Kreislauf-wirksamer Nanopartikel (Inhalation funktioneller Nanopartikel)”.

# 6. Experimental results

## 6.1. Biodistribution of the CaPs carrier

The biodistribution of the inhalable nanocarrier was assessed by nebulization of CaP-HA in anaesthetized healthy Landrace pigs (**Figure 1**A-B). Two separate groups of animals (each of those consisting of n= 6 animals) were investigated, accounting for an earlier (5 hours) and later (10 hours) time-point after a single-dose inhalation of the CaP-HA. These data have already been published in the paper: *Inhalation of peptide-loaded nanoparticles improves heart failure*[3]. Myocardial kinetics of inhaled CaP-HA was examined by dot blot analysis. Myocardial tissue after 5 hours was further analyzed by Western blot and confocal microscopy (**Figure 2**A, 2B, 2C). HA levels were significantly increased, providing evidence for effective delivery of CaP-HA to the myocardium, with a higher intensity of signal within the left ventricle. Furthermore, lower intensity of signal was detected in the other harvested organs, such as liver, aorta and trachea. Altogether, these data confirm the efficiency of our approach for the intra-myocardial delivery of small compounds in large animals. The final blotting analysis at 10 hours is still undergoing and it has been delayed by technical issues related to the detection of the presumably relatively small amount of the peptide enriching the heart 10 hours after inhalation.

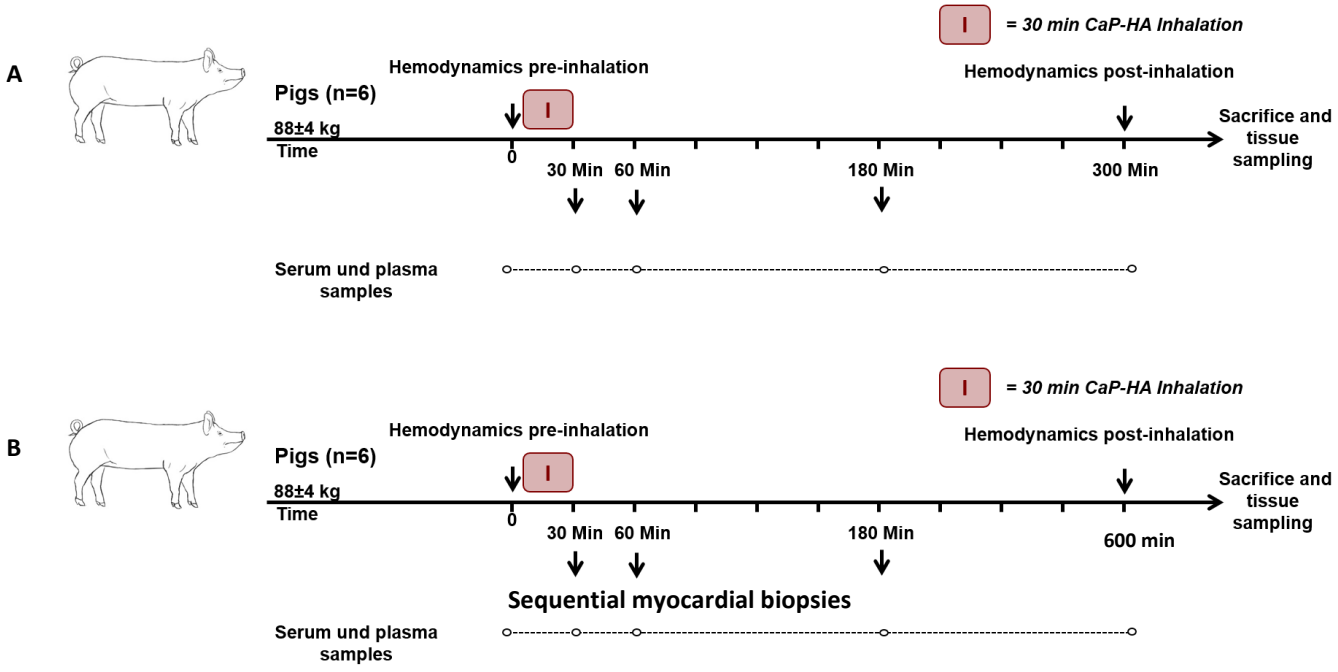


**Figure 1.** Design of the study. (A) n=6 animals inhaled nCaP-HA and tissues were sampled 5 hours after inhalation. (B) In a second group of pigs (n=6) sequential myocardial biopsies were performed and the tissues were sampled at 10 hours.

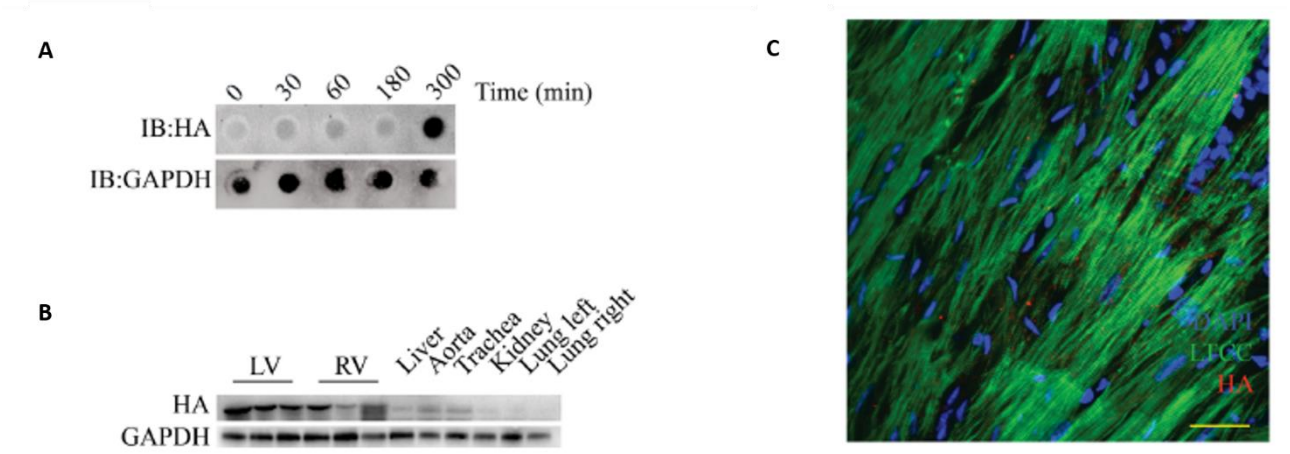


**Figure 2.** Kinetics of inhaled CaP-HA. (A) Kinetics of inhaled CaP-HA cardiac targeting as examined by dot blot analysis from biopsies obtained at the indicated times after inhalation. IB, immunoblotting. (B) Western blot analysis for HA on tissues of treated pigs. (C) Z-stack confocal laser scanning microscopy images showing HA peptide in myocardial tissue from pigs treated by CaP-HA inhalation. Immunofluorescence staining for HA (red), LTCC (green), and cell nuclei (DAPI; blue). Scale bar, 20 μm (n = 6).

## 6.2. Pharmacokinetic characteristics of the MP

In order to gain knowledge on the profile of the target product of CUPIDO, both MP as well as R7W-MP have been initially tested in-vitro, being exposed to plasma from different species, namely mouse, pig and human. The preliminary results show a higher stability and lower hydrolysis of the R7W-MP over time (**Figure 3**) when compared to MP.

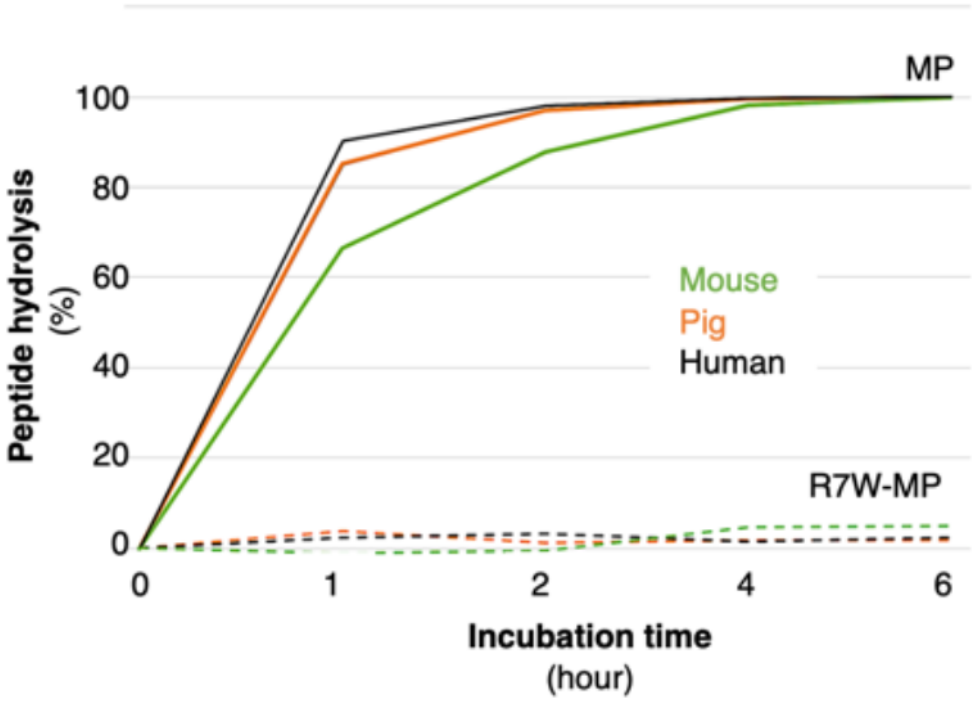


**Figure 3.** Hydrolysis time of MP versus R7W-MP. The peptide hydrolysis percentage after in vitro plasma incubation has been investigated in the mimetic peptide (MP) and in the R7W-MP (polyarginine tail substituted). A higher stability was observed in the R7W-MP compound.

Due to the high degree of MP instability, only the pharmacokinetic properties of R7W-MP were investigated by SAN in plasma after a single intravenous administration (3 mg/kg) to male C57BL6 mice. A rapid elimination of the R7W-MP was observed, with only a first sampling time point showing a quantifiable MP plasmatic concentration corresponding on average to 4-fold LLOQ (lower limit of quantification, i.e 250 ng/mL).

## 7. Conclusions

The in-vivo experiments on the biodistribution of the developed nanocarrier CaP in large animals have been performed, showing a favorable profile of myocardial enrichment up to 5 hours. Furthermore, the in-vitro stability of the biological active compound MP, together with its cell-penetrating form R7W-MP, has been investigated, increasing our knowledge of the compound for the future in-vivo translation. Ongoing studies are assessing the MP stability when encapsulated in CaPs.

## 8. Bibliography


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