

# PROTOCOL OF REGULATORY PRE-CLINICAL STUDY

## OBJECTIVES OF THE REGULATORY PRE-CLINICAL STUDY FOR HEPHA-440

The goal of these investigations (a preliminary study followed by a GLP study) is to investigate in rabbits the potential toxicity of HEPHA-440, a chemically detoxified lipopolysaccharide derivative from *Bordetella Pertussis* (BP) in liposomal formulation, which acts as a Toll-Like Receptor 4 (TLR4) agonist. The objectives are therefore to determine its toxicokinetics, acute toxicity, and toxicity after 3 administrations at 2-week intervals (*i.e.* covering a period of 1 month), including after a one-month recovery period, as well as the kinetics of cytokine production following repeated administration. This schedule has been selected to align with the planned schedule for human use: HEPHA-440 as monotherapy is envisaged to be administered intravenously (I.V.) every 2 weeks to patients. The nonclinical GLP study is essential to safely launch the first human trial with HEPHA-440, which will then be evaluated as monotherapy in the treatment of osteosarcoma and in combination with immune checkpoint inhibitors in the treatment of various types of solid cancers.

## REGULATORY PRE-CLINICAL TOXICOLOGY STUDY PROTOCOL

Vials of HEPHA-440 in PBS will be kept at 4°C. They will be diluted in NaCl 0.9% at the appropriate concentration on site prior administration.

### 1- Analytical method transfers and validation

A previously established method for quantifying HEPHA-440 will be transferred to the contract research organization (CRO) overseeing the study, and it will be validated according to GLP standards. This bioanalytical method must adhere to ICH-M10 guidelines, ensuring validation criteria such as selectivity, matrix effect, accuracy, precision, stability, etc., are met prior to commencing the GLP study.

Three quantification methods are currently under development.

- Suitable for dosage in formulation and blood:
  - o ELISA specific for lipooligosaccharide A (LOS-A) of *Bordetella pertussis*
  - o Liquid chromatography-mass spectrometry/high resolution mass spectrometry (LC-MS/HRMS) of the lipid A domain. The lipid A is cleaved from the drug substance using mild acidic hydrolysis, extracted from the blood matrix by liquid/liquid extraction or solid phase extraction and analyzed by LC-MS/HRMS. Separation is performed in a reversed-phase mode using a C8 stationary phase, ionization using Electrospray Ionization in negative mode (ESI-), and mass analysis with a resolution of  $R_s = 70,000$  for accurate mass determination. The spectrometer used for the development of the method is a hybrid quadrupole-Orbitrap Q Exactive Plus mass spectrometer, coupled to an UltiMate 3000 HPLC system.
- Suitable for dosage in formulation only:
  - o HPLC-CAD analysis of HEPHA-440.

The more sensitive and robust method(s) will be chosen for transfer and used to dose HEPHA-440 throughout the pharmacokinetics studies. Plasma samples have to be kept at -20°C before analytical study.

### 2- Dose Range Finding (DRF)

Parameters	Specifications
Objective	Administration of escalating doses to determine the « Highest Non-Severely Toxic Dose » (HNSTD)
Status	Non-regulatory
Animals	2 to 4 months old New Zealand rabbits 3 males/dose <b>Total:</b> 12 to 23 rabbits (depending on the maximal dose; it is highly unlikely that more than 5 dose levels will be necessary)

Groups	<ul style="list-style-type: none"> <li>- One group per dose range. Escalating doses until the highest non-severely toxic dose (HNSTD) is reached*.</li> </ul> <p>NB: The groups treated at different dose levels will be started with a two-week delay between each dose level, <i>i.e.</i> group dose 1 receives 1<sup>st</sup> infusion at week 1, group dose 2 receives 1<sup>st</sup> infusion at week 3, etc.</p> <ul style="list-style-type: none"> <li>- One control group (NaCl) will be started concomitantly to the group dose 1 (with sample collection at the same timepoints as all the groups).</li> <li>- One group with HEPHA-440 at HNSTD + anti-inflammatory compound** (started as soon as HNSTD has been identified)</li> <li>- Two animals may be added at HNSTD to confirm it</li> </ul>
Posology	3x30' IV infusions with a 2-weeks interval
Timing of samples collection during the experiment	<p><b>Blood draws:</b></p> <ul style="list-style-type: none"> <li>- For pharmacokinetics: T<sub>0</sub> T<sub>30'</sub> T<sub>1h</sub> T<sub>2h</sub> T<sub>4h</sub> T<sub>6h</sub> T<sub>12h</sub> T<sub>24h</sub> for all the administrations + T<sub>48h</sub> T<sub>72h</sub> T<sub>96h</sub> T<sub>144h</sub> for administrations 1 and 2</li> <li>- For biochemical blood parameters: T<sub>0</sub> T<sub>6h</sub> T<sub>24h</sub> for all the administrations + T<sub>48h</sub> for administrations 1 and 2</li> <li>- For cytokines: T<sub>0</sub> T<sub>2h</sub> T<sub>6h</sub> for each administration</li> </ul> <p><b>Total:</b> 2 x 8 + 12 = 28 blood draws/rabbit</p>
Sacrifice	24h after the 3 <sup>rd</sup> (and last) infusion
Measured parameters	<p><b>Animal observation:</b></p> <ul style="list-style-type: none"> <li>- Clinical signs of distress, mortality, prostration, diarrhea, vomiting + observation of animal behavior to detect potential cardiotoxicity, neurotoxicity or respiratory toxicity - after each infusion and 3 times a week</li> <li>- Measurement of weight and food consumption - 3 times a week</li> <li>- Observation of the injection site (local tolerance) - 4h and 24h after each infusion</li> </ul> <p><b>Temperature measurement</b> - before and 1h, 2h, 4h, 8h, 24h after each infusion</p> <p><b>Pharmacokinetics:</b> dosage (ELISA or LC-MS/HRMS) on blood samples</p> <ul style="list-style-type: none"> <li>- As a proof of exposure for all the groups - before and after the end of each infusion (<i>i.e.</i> T<sub>0</sub> and T<sub>30'</sub>)</li> <li>- For a detailed PK profile (<i>see appendix 1.1</i>) at HNSTD - before and 1h, 2h, 4h, 6h, 12h, 24h after all the infusions + 48h, 72h, 96h, 144h after infusions 1 and 2</li> </ul> <p><b>Blood analysis:</b> Hematology, coagulation parameters, clinical chemistry (<i>appendices 1.2 à 1.4</i>) and C-Reactive Protein (CRP) - 6h, 24h after each infusion + 48h after infusions 1 and 2</p> <p><b>Anatomopathological analysis:</b> macroscopic autopsy, collection, weighing, and paraffin embedding of organ of primary interest (<i>i.e.</i> liver, kidney, heart, thymus, spleen, draining lymph nodes around the injection site and bone marrow)</p> <p><b>Pharmacodynamics (PSE):</b> quantification of cytokines of the TLR4 pathway (IL-1<math>\beta</math> and/or TNF<math>\alpha</math> for the Myd88 pathway, CCL5 and/or CXCL10 for the TRIF pathway) by ELISA or qPCR - 2h and 6h after each infusion</p>

T<sub>0</sub> corresponds to the beginning of the infusion, meaning that T<sub>30'</sub> corresponds to the end of the infusion.

\* Each new dose level should be discussed with the sponsor.

Each new dose levels will be calculated as follows:

- If no adverse effect (AE) at dose<sub>n</sub>: increase of 100%, *i.e.* dose<sub>n+1</sub> = dose<sub>n</sub> × 2
- If moderate AE in 1/3 animals at dose<sub>n</sub>: increase of 33%, *i.e.* dose<sub>n+1</sub> = dose<sub>n</sub> × 1.33
- If moderate AE in 2/3 animals at dose<sub>n</sub>: HNSTD identified or include more animals (depending on the AE)
- If moderate AE in 3/3 animals at dose<sub>n</sub>: HNSTD identified
- If severe AE in 1/3 animals but no AE in the other animals at dose<sub>n</sub>: include more animals
- If severe AE in ≥1/3 animals and moderate AE in the others at dose<sub>n</sub>: STD identified → decrease to a dose between dose<sub>n</sub> and dose<sub>n-1</sub> to try to reach the HNSTD.

The NOAEL will also be determined during this escalating dose, as the highest dose with no observed AE.

NB: As explained above, transient, moderate and manageable hypothermia should not be considered as an adverse effect in this context and is likely to be observed at the dose which will be defined as the NOAEL.

\*\* The anti-inflammatory medication may be a steroid or a Non-Steroidal Anti-Inflammatory drug based on what is usually used to alleviate rabbits' inflammation/pain.

### 3- Comprehensive toxicity and toxicokinetics study according to Good Laboratory Practice (GLP)

Parameters	Specifications
Objective	Comprehensive characterization of the toxicity and the toxicokinetics with the final (GMP) formulation of HEPHA-440
Status	GLP
Animals	3.5 months old (+/- 1 week) New Zealand rabbits (age at 1 <sup>st</sup> administration) 3 males + 3 females for the « terminal sacrifice groups » (for all doses) 2 males + 2 females for the « recovery groups » (only for HNSTD and control) <b>Total:</b> 6x4 + 4x2 = 32 rabbits
Groups	Control (NaCl) High dose (HNSTD) Intermediate dose (geometric mean between HNSTD and NOAEL) Low dose (NOAEL)
Posology	3x30' IV infusions with 2-week intervals
Timing of samples collection during the experiment	<b>Blood draws:</b> <ul style="list-style-type: none"> <li>- For pharmacokinetics: T<sub>0</sub> T<sub>30'</sub> T<sub>24h</sub> + 3 other timepoints based on the PK profiles observed during the DRF</li> <li>- For biochemical blood parameters: T<sub>0</sub> T<sub>6h</sub> T<sub>24h</sub> for all the administrations + T<sub>48h</sub> for administrations 1 and 2 + before sacrifice of the rabbits of the « recovery group » (J<sub>57</sub>)</li> <li>- For cytokines: T<sub>0</sub> T<sub>2h</sub> T<sub>6h</sub> for each administration</li> <li>- For desensitization assessment: before the 1<sup>st</sup> infusion (T<sub>0</sub>), after the 3<sup>rd</sup> infusion (J<sub>29</sub>), and before sacrificing the rabbits of the « recovery groups » (J<sub>57</sub>)</li> </ul> <b>Total:</b> <ul style="list-style-type: none"> <li>- For the « terminal sacrifice groups »: 2 x 8 + 4 = 22 blood draws/rabbit</li> <li>- For the « recovery groups »: 2 x 8 + 4 + 1 = 23 blood draws/rabbit</li> </ul>
Sacrifice	24 hours after the 3 <sup>rd</sup> (and last) infusion for the "terminal sacrifice groups" 4 weeks after the 3 <sup>rd</sup> (and last) infusion for the "recovery groups", i.e. J <sub>57</sub>
Measured parameters	<b>Animal observation:</b> <ul style="list-style-type: none"> <li>- Clinical signs of distress, mortality, prostration, diarrhea, vomiting - after each infusion and 3 times a week</li> <li>- Measurement of weight and food consumption - 3 times a week</li> <li>- Observation of the injection site (local tolerance) - 4h and 24h after each infusion</li> </ul> <b>Safety pharmacology:</b> <ul style="list-style-type: none"> <li>- Respiratory study: quantification of inspirations/expiration</li> <li>- Neurological study: functional observational battery (FOB)</li> <li>- Careful observation of animal behavior and mobility to detect potential cardiological effects</li> </ul> <b>Temperature measurement</b> - before and 1h, 2h, 4h, 8h, 24h after each infusion <b>Ophthalmoscopic study</b> - after the 1 <sup>st</sup> infusion of each group + after the 2 <sup>nd</sup> and 3 <sup>rd</sup> infusion of the HNSTD group if something abnormal was observed after the 1 <sup>st</sup> infusion + before sacrificing the mice of the 'terminal sacrifice' group (i.e. J <sub>57</sub> ). <b>Pharmacokinetics:</b> dosage (ELISA or LC-MS/HRMS) on blood samples to determine PK (see appendix 1.1) – before and after 30', 24h + 3 other timepoints based on the

results of the PK profiles observed during the DRF - for the « terminal sacrifice groups » only

**Blood analysis:** Hematology, coagulation parameters, clinical chemistry (*appendices 1.2 à 1.4*) and C-Reactive Protein (CRP) - 6h, 24h, 48h (when possible) after each infusion

**Anatomopathologic analysis:** macroscopic autopsy, organs weighing and standard complete histopathology (*see the list of organs in appendix 2*)

**Pharmacodynamics (PSE):** quantification of cytokines of the TLR4 pathway (IL-1 $\beta$  and TNF $\alpha$  for the Myd88 pathway, CCL5 for the TRIF pathway) by ELISa or qPCR - 2h and 6h after each infusion

**Desensitization (TO DO ONLY IF THE PARAMETERS MEASURED AFTER EACH ADMINISTRATION DURING THE DRF SUGGEST A DESENSITIZATION):** ELISa on blood samples to detect the presence of « anti-drug antibodies » (ADAs) – before infusion, after the 3<sup>rd</sup> infusion and before sacrificing the rabbits of the « recovery groups »

T<sub>0</sub> corresponds to the beginning of the infusion, meaning that T<sub>30'</sub> corresponds to the end of the infusion.

If feasible, an additional 1 mL of blood will be collected at T<sub>0</sub>, T<sub>2h</sub>, T<sub>6h</sub>, T<sub>24h</sub> for each rabbit. Plasma and white blood cells will be separated and collected, then shipped to the sponsor (UCBL, Lyon). The organs embedded in paraffin will also be provided to the sponsor.

## Appendix 1: Measurement parameters (data to be measured or calculated and included in the report)

### 1.1. Pharmacokinetics

Theoretical concentration at time 0 (C<sub>0</sub>)

Maximum observed concentration (C<sub>max</sub>)

Time at which C<sub>max</sub> has been observed (t<sub>max</sub>)

Last timepoint at which the last measurable concentration of HEPHA-440 is observed (t<sub>last</sub>)

Area under the concentration/time curve from t<sub>0</sub> to theoretical t<sub>inf</sub> (AUC<sub>0-inf</sub>)

Area under the concentration/time curve from t<sub>0</sub> to t<sub>last</sub> (AUC<sub>tlast</sub>)

Half-life (t<sub>1/2</sub>)

Total clearance of the body (Cl)

Volume of distribution (V<sub>d</sub>)

Ratio between AUC<sub>tlast</sub> after administrations 2/3 and AUC<sub>tlast</sub> after administration 1 (R<sub>AUC-inj2</sub>/R<sub>AUC-inj3</sub>)

C<sub>0</sub>/Dose

C<sub>max</sub>/Dose

AUC<sub>tlast</sub>/Dose

### 1.2. Hematology

Red blood cell count

Hemoglobin concentration

Hematocrit

Mean corpuscular volume

Mean corpuscular hemoglobin concentration

Mean corpuscular hemoglobin

Reticulocyte count

Platelet count

White blood cell count

Neutrophil count

Lymphocyte count

Monocyte count

Eosinophil count

Basophil count

Large unstained cells count

### 1.3. Coagulation parameters

Activated partial thromboplastin time

Fibrinogen  
Prothrombin time  
Sample quality

#### 1.4. Clinical chemistry

Alanine aminotransferase (ALAT)  
Aspartate aminotransferase (ASAT)  
Alkaline phosphatase (PA)  
Gamma-glutamyltransferase (GGT)  
Total bilirubin  
Unconjugated bilirubin  
Urea nitrogen  
Creatinine  
Calcium  
Phosphorus  
Total protein  
Albumin  
Globulin (calculated)  
Albumin/globulin ratio  
Glucose  
Cholesterol  
Triglycerides  
Sodium  
Potassium  
Chloride  
Magnesium  
C reactive protein  
Sample quality

#### Appendix 2: Organs to be collected (guidelines EMA on repeated dose toxicity)

Adrenal gland  
Aorta  
Bone  
Bone marrow  
Brain  
Cecum  
Colon  
Duodenum  
Epididymis  
Esophagus  
Eye  
Gallbladder  
Harderian gland  
Heart<sup>#</sup>  
Ileum  
Jejunum  
Kidney<sup>#</sup>  
Liver<sup>#</sup>  
Lung  
Lymph node(s)  
Mammary gland (females)  
Ovary (Females)  
Pancreas  
Parathyroid gland  
Peripheral nerve  
Pituitary

Prostate (males)  
Salivary gland  
Seminal vesicle (males)  
Skeletal muscle  
Skin  
Spinal cord  
Spleen#  
Stomach  
Testis (males)  
Thymus#  
Thyroid gland  
Trachea  
Urinary bladder  
Uterus  
Vagina (Females)  
Other organs or tissues with gross lesions  
Tissue masses  
Ear  
Vein  
Draining lymph nodes around the injection site#

#Organs of primary interest: to be collected during the DRF