CLINICAL STUDY PROTOCOL

The effects of intravenous heme arginate on HO-1 expression and oxidative stress in the human heart

[redacted]

1) Department of Cardiac Surgery, Medical University of Vienna
2) Department of Laboratory Medicine, Medical University of Vienna
3) Department of Clinical Pharmacology, Medical University of Vienna

EudraCT: 2013-000887-27 NCT number: NCT02314780
Version 2.0 – 8th May, 2013)

Clinical investigator: [redacted]

Sponsor:	Department of Cardiac Surgery, Medical University of Vienna
Monitor:	TBA (Clinical Studies Support Center)
Clinical Trial Centre:	Department of Cardiac Surgery Medical University of Vienna Währinger Gürtel 18-20, A-1090 Vienna
Test Drug (IMP):	Heme arginate
Study phase:	1

[redacted]

Introduction

Project background

Ischemia and ischemia-reperfusion injury play a crucial role in today's medicine[1]. Several approaches were developed to decrease tissue injury during or after ischemia in the animal model. However, despite major effort is put in new substances and techniques, the timely reperfusions remains the main approach to avoid sustained tissue damage[2].

Our working group focused on an already well-established concept based on data gathered in animal research, which was not further developed in the clinical setting until our first project. With the support of the Vienna Science and Technology Fund (Life Science Call 2007 – Therapy of ischema-reperfusion injury by heme oxygenase-1 induction in skeletal muscle and ischemic kidney), we evaluated the induction of heme oxygenase – 1 (HO-1) by heme arginate. HO-1 is a well-known cellular protein protecting the tissue from ischemia-reperfusion injury[3,4]. We could provide data regarding HO-1 induction in peripheral blood mononuclear cells (PBMCs) as well as the characterisation of ischemia and ischemia-reperfusion injury in the healthy skeletal muscle[5]. Further, we established a human in-vivo model supporting the hypothesis of the protection induced by heme arginate in healthy human skeletal muscle after ischemia-reperfusion injury[6].

Our next step regarding the introduction of this promising therapy into daily clinical practice is the application of heme arginate in a distinct disease model. The human heart seems to be ideal as a target organ for HO-1 mediated protection. First, ischemic heart disease represents still one of the leading causes of death in the industrialist world[1,7]. An improved protection from ischemia may be beneficial in any kind of acute coronary syndromes regardless of the planned revascularisation strategy. In addition, several surgical procedures involving the heart and the great vessel necessitate some degree of myocardial ischemia, which may lead to adverse events. Improved protection from ischemia-reperfusion injury may therefore optimize the outcome of aneurysm surgery, valvular heart procedures as well as heart transplantation.

In conclusion, we intend to evaluate and optimize the induction of HO-1 in the human heart by heme arginate in this clinical project. This shall provide sufficient data to enable large-scale clinical trials for the evaluation of this promising therapeutic approach.

Ischemia and Ischemia Reperfusion Injury

Ischemic injury to muscular tissue is a common problem in cardiovascular medicine. Myocardial infarction and peripheral artery disease account substantially for morbidity and mortality in the industrialized world[1,7]. The most effective treatment to avoid ischemic damage is the rapid re-establishment of reperfusion. However, reperfusion itself can result in additional damage to ischemic tissue[8]. This phenomenon is called ischemia – reperfusion (IR) injury and is caused by different pathologic mechanisms[8,9].
One of the major pathologic mechanisms is the increased production of free oxygen radicals, thereby overwhelming the cellular antioxidant protection and damaging cellular proteins and membranes[10]. In a second step, neutrophil activation and invasion is suggested to be part of the deleterious cascade of IR injury[11].

Several strategies have been demonstrated to prevent IR injury in various experimental settings, including mechanical preconditioning and postconditioning, administering of antioxidant substances like vitamin C and treatment with endothelin receptor antagonists[12-16]. These experimental findings support the hypothesis that the reduction of reactive oxygen species is mandatory in the treatment of reperfusion injury. As a new approach in humans, the enzyme induction of heme oxygenase 1 (HO – 1) may represent a potent strategy to diminish IR injury.

[redacted]

On-pump coronary artery bypass grafting

Coronary artery bypass grafting (CABG) is still the treatment of choice in patients with complex coronary artery disease or left main stenosis. The majority of cases are operated on-pump, leaving the heart without blood support for 1 to 2 hours. Cold blood cardioplegia is routinely used to protect the heart during this period. Although this method is very safe, regional and temporal ischemia still occurs. Therefore, we

suggest that an antioxidant treatment like HO-1 induction prior to CABG may additionally protect the heart and improve early postoperative performance[17].

The role of heme oxygenase and bilirubin

HO - 1 is the rate-limiting enzyme that catalyzes the degradation of heme b (Fe-protoporphyrin-IX) into biliverdin (which is rapidly converted to bilirubin), carbon monoxide (CO) and iron[18]. It is a heat shock protein (HSP 32) and serves as a protective enzyme due to its anti-inflammatory, antioxidant, anti-apoptotic and antiproliferative actions[19,20]. HO-1 is expressed in several organs, in endothelial, epithelial, smooth muscle cells and the heart[3,21-23].

One hypothesis to explain the beneficial effects of induced HO-1 focuses on the pleiotropic cytoprotective actions of HO-1 and the products of heme degradation. These are potently anti-inflammatory and anti-oxidant. In addition, they modulate apoptotic and cell proliferative responses (but always in the direction of what appears to be a homeostasis-inducing response). Thus, after vascular injury, CO enhances the proliferation of endothelial cells (EC), which benefit return to homeostasis as the endothelium is more rapidly repaired, while blocking vascular smooth muscle cells proliferation, which prevents obliteration of the lumen of the vessel. Thus, these diametrically opposed actions are both homeostasis supporting. The same is true for apoptosis. HO-1 also impacts the immunological response by enhancing T cell mediated suppression (relatively more T regulatory cells) and suppressing the rejection response (fewer allo-aggressive T cells).
Regarding the inflammatory cascades, IRI leads to increased leukocyte extravasation, which includes the production of the injurious chemokines and cytokines - such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and monocyte/macrophage chemoattractant protein (MCP)-1 - ultimately leading to early dysfunction or primary non-function of transplanted organs. In previous studies using animal models, induction of HO-1 resulted in diminished production of pro-inflammatory cytokines in various IRI associated situations. We hypothesize that induced HO-1 expression results in diminished pro-inflammatory cytokine expression following IRI. Lastly, HO-1 acts as a therapeutic funnel in that a large number of physiologically active molecules (e.g. IL-10) appear to function by up-regulating HO-1 with HO-1 then mediating, at least the anti-inflammatory, therapeutic actions of that

molecule. Without HO-1, the molecule loses its anti-inflammatory effects. The beneficial effects of HO-1 stimulation on IRI has been evaluated in multiple organs studied in rodents and in pig studies in liver IRI.

Pharmacologic concept

A promising concept for the reduction of IRI is the induction of heme oxygenase 1 (HO-1). Heme oxygenases are expressed in the cellular microsomes. HO-2 is the constitutively expressed form in humans. In contrast, HO-1 can be expressed in response to several stimuli, which are generally pro-inflammatory. As a new therapeutic approach in humans, the induction of the enzyme HO-1 may be employed to mitigate IRI[20].

Heat shock proteins' transcriptional regulation responds to several chemical and biological stimuli. Although it has been shown that heme is such an inducer of HO-1 [24], the molecular steps and signal transduction pathways underlying HO-1 up-regulation in general, and by heme, in particular, remain largely unclear [25,26].

IRI may be attenuated by pharmacological HO-1 induction with heme arginate as shown in a rodent hemorrhagic shock model[20,27-29]. Previous data have confirmed dose-dependent induction of HO-1 mRNA and protein by heme arginate in venous blood of healthy subjects [5,30].

There are two heme-containing drugs for intravenous human application available. Hemin is the ferriheme chloride. Heme arginate is the conjugation of hemin with L-arginine in a solution of propylene glycol, ethanol and water. HA is commercially available from Orphan Europe Ltd. as Normosang® which is the approved form of heme in Europe for episodes of acute intermittent porphyria (AIP), variegate porphyria (VP) and hereditary coproporphyria (HCP). The usual dose of HA is 3 to 4 mg/kg body weight given as intravenous infusion once daily over 4 to 7 days.

Safety and tolerability

Heme arginate has the same advantages as hemin with lesser side-effects. Most important, heme arginate is less harmful to the endothelium and thereby reduces vasculopathy and thrombotic side effects[31-33]. Further, no adverse effects on hemostasis are observed[34]. Acute toxicity occurs at doses of 48 to 70 mg/kg body weight (depending on animal species).

Table 1: Known adverse reactions to heme arginate[35]

frequency	adverse reaction
very common (> 10 %)	difficult venous access (with repeated administration)
common (1-10 %)	injection site pain, injection site swelling, phlebitis at injection site
uncommon (0.1-1 %)	elevated serum ferritin concentration
rare (0.01-0.1 %)	fever, anaphylactoid reaction, allergic reactions (dermatitis medicamentosa, tongue edema)

Pharmacokinetics

Intravenously administered HA is bound to hemopexin and albumin and as heme-hemopexin-complex transported to the liver. There it is degraded by HO-1 and biliverdin reductase to bilirubin and excreted into the bile.

Table 2: Relevant pharmacokinetic parameters of heme arginate[36]

Half-life (single dose)	10.8 hours
Volume of distribution	3.37 litres
Total plasma clearance	3.7 ml/min

Rationale of the study

Ischemia reperfusion injury may be attenuated by HO-1 induction. Our previous data confirmed strong HO-1 induction in peripheral blood cells following heme arginate infusion in healthy humans[5]. Furthermore, we could demonstrate the amelioration of experimental ischemia reperfusion injury in the calf musculature by heme arginate in healthy subjects as measured by functional MRI[6].

Therefore, we propose that HO-1 induction in the human heart may be a suitable target to mitigate cardiac ischemia-reperfusion injury.

The HO-1 induction will be assessed in a clinical trial by myocardial biopsy prior to and after aortic cross clamping in subjects with or without preceding heme arginate treatment in two different dosages. The HO-1 expression will also be measured in the clinical trials in peripheral blood mononuclear cells. As additional outcome, levels of myoglobin, creatine-kinase and troponin T and reactive oxygen species will be measured in plasma according to standard laboratory procedures.

Risk/Benefit assessment

The study drug heme arginate is approved in humans for acute episodes of hepatic porphyrias, used since 1985, and is well tolerated. The most commonly observed AEs of intravenous infusion of heme arginate are local reactions at the injection site. Known AEs are listed in detail in Table 1.

Biopsies will be taken under direct vision from a superficial part of the right ventricle and oversewn immediately. Therefore, the risk of bleeding is very low because the site of biopsy can be observed after the administration of protamin until the sternum is closed. Right ventricular biopsies are routinely performed in heart-transplanted patients and are safe regarding ventricular dysfunction.

[redacted]

There is a potential benefit for the participants although clinical data in humans are lacking until now. However, study related procedures do not lead to an increased length of stay for the patient. This study may provide new insights into the mechanisms of reperfusion injury and the protective capacity of HO-1 stimulation in cardiovascular pathologies. Therefore the risk / benefit assessment is acceptable.

Study objectives

1) To evaluate the effects of heme arginate administration in two different doses on myocardial HO – 1 induction
2) To evaluate postoperative plasma levels of myoglobin, creatin-kinase, troponin T and reactive oxygen species according to treatment period.

Investigational plan

Design

A placebo controlled, double blind study design with two treatment groups and one placebo group will be used for this purpose in 36 patients undergoing aortic valve replacement (AVR). Screening and drug administration will be performed on the day of hospitalisation. Subjects will be randomized to the study periods by a computer program ("Randomizer"). AVR will be performed 24 hours after heme arginate administration.

Dosage and treatment duration

The currently approved dose for heme arginate therapy in acute hepatic porphyrias is 3 mg/kg once daily over 4 to 7 days. A single intravenous dose of 1 mg/kg will be used in this study for the first treatment cohort and a dose of 3mg/kg will be used for the second treatment cohort.

Treatments

All subjects will be randomized to receive a predefined treatment 24 hours prior to AVR surgery:

- Placebo (12 patients): intravenous infusion of 110 ml 0.9 % NaCl solution.
- Treatment cohort A (12 patients): intravenous infusion of 1 mg/kg heme arginate diluteded to 110 mL with 0.9 % NaCl.
- Treatment cohort B (12 patients): intravenous infusion of 3 mg/kg heme arginate diluteded to 110 mL with 0.9 % NaCl.

Inclusion criteria

- Signed informed consent
- Men and women aged between 40 and 85 years (inclusive)
- Planed aortic valve replacement
- Body mass index <35 kg/m^2
- Ability to communicate well with the investigator in the local language and to understand and comply with the requirements of the study

Exclusion criteria

Any of the following will exclude a subject from the study:

- Known hypersensitivity to the study drug or any excipients of the drug formulation
- Treatment with another investigational drug within 3 weeks prior to screening
- History or clinical evidence of any disease and/or existence of any surgical or medical condition, which might interfere with the absorption, distribution, metabolism or excretion of the study drug
- Severe renal failure (glomerula filtration rate < 30ml/min)
- Moderately or severe impaired left ventricular function (ejection fraction < 40%)
- Moderately or severe impaired right ventricular function
- Systolic pulmonary pressure > 45mmHg
- Acute or recent (<7 days) myocardial infarction
- Child bearing potential

Description of study days

Informed consent will be obtained after admission for AVR at the Medical University of Vienna. All patients are admitted with an internal approval for AVR and all necessary echocardiographic and laboratory data. Therefore, only a physical examination will be performed and subjects are directly sent for randomization and heme arginate or placebo infusion to the Department of Clinical Pharmacology. Blood pressure and heart rate will be obtained before and after drug infusion. Subjects will be scheduled for AVR on the day after infusion.

[redacted]

During surgery, a small biopsy (1-2mm) will be taken from the right ventricle prior to cross clamping and 10 minutes after protamin administration prior to chest closure. Biopsies will be performed under direct vision and every bleeding can be controlled directly. A blood sample (15 ml) will be taken prior to AVR, after chest closure as well as 24, 48 and 72 hours post surgery. Routine postoperative controls of creatine kinase, myoglobin and troponin T will be performed at admission to the ICU as well as 24, 48 and 72 hours post surgery.

Study medication

Substance: heme arginate (25 mg/mL NORMOSANG® vials á 10 mL)
Manufacturer: Orphan Europe Ltd., Paris, France
Placebo: 0.9 % NaCl solution

Dosage and administration

Dose: 1 mg/kg body weight (treatment cohort A); 3 mg/kg body weight (treatment cohort B)
Preparation: the specified amount of NORMOSANG® is diluted to 100 mL with 0.9% NaCl solution (in glass bottles), preparation is done by the pharmacist / investigator or his / her designee under sterile conditions.
Route: within 1 hour after preparation the study drug is administered intravenously in a cubital vein or large forearm vein via a transfusion set.
Timing: the study drug will be administered between 9.00 and 11.00
Administration: infusion is administered within 15 minutes, which correspond to an infusion rate of 400 ml/min. To ensure a correct timing an infusomat (IP 85-2, Döring, Munic, Germany) is used. The post-treatment infusion of a 0.9 % NaCl solution is administered over the same infusion line to rinse out heme arginate solution (20 mL filling volume of infusion line) and prevent loss of heme arginate.
Post-treatment: infusion of 250 ml 0.9 % NaCl solution over 10-15 min.

[redacted]

Treatment after end of study

After end of the study further treatment will be performed at the Department of Cardiac Surgery until discharge from hospital. Thereafter, subjects will be sent to their cardiologist for routine control.

Treatment assignment

The assignment of number and code for the subject identification is based on the obligation for anonymity. Only their subject number, initials (first name and last name) and date of birth will identify subjects.

Outcome Variables

Main outcome variables

HO – 1 mRNA and protein induction in the heart due to heme arginate pretreatment

HO – 1 mRNA and protein induction in PMBC in patients undergoing cardiac surgery due to heme arginate pretreatment

Evaluate dose-response of HO-1 induction in the heart

Additional outcome

Reactive oxygen species levels (Biopsies and blood samples)

Postoperative plasma levels of creatine kinase, troponin T and myoglobin

Safety variables

Major cardiac adverse events

Methods of evaluation

HO-1 protein and mRNA levels in heart biopsies and PBMCs

HO-1 levels will be analyzed in cardiac biopsies and peripheral blood mononuclear cells (PBMC). PBMC are considered effectors of oxidative stress-mediated injury and

influenced by HO-1 expression. PBMCs will be isolated from EDTA-blood with Ficoll-Plaque® (Amersham Biosiences, United Kingdom) prefilled tubes (Leusosep®, Greiner bio-one, Austria). Cell pellets and biopsy samples for HO-1 mRNA analysis are treated with lysis buffer (Buffer RLT, Qiagen Sciences, Maryland, USA) and immediately frozen on liquid nitrogen. Lysates and cell pellets are stored at -80°C until analysis. First-strand cDNAs will be synthesized from approximately 1 µg of total RNA with the use of MLV reverse transcriptase and random hexamer primers according to the manufacturer's instructions (RT-PCR Core Kit; Takara Bio). For quantitative real-time polymerase chain reaction (RT-PCR), sense and antisense primers (Invitrogen, Paisley, Scotland) and fluorogenic probes (Eurogentec, Herstal, Belgium) for HO-1 and 18S will be used as described previously [37]: for HO-1, the primers and probe used were 5'-GACTGCGTTCCTGCTCAACAT-3' (sense), 5'-GCTCTGGTCCTTGGTGTCATG-3' (antisense) and 5'-TCAGCAGCTCCTGCAACTCCTCAAAGAG-3' (probe), generating a 75 base pair PCR product. For 18S, the primers and probe used were 5'-CGGCTACCACATCCAAGG-3' (sense), 5'-CCAATTACAGGGCCTCGAAA-3' (antisense) and 5'-CGCGCAAATTACCCACTCCCGA-3' (probe), generating a 109 base pair PCR fragment. The ABI PRISM 7700 (Applied Biosystems, Foster City, CA) will be used for PCR. Results are expressed as the target/reference ratio. The difference between the HO-1 mRNA levels of heme arginate and vehicle-treated cells are considered to reflect the capacity of cells to upregulate HO-1 mRNA, and were expressed as ΔHO-1 mRNA.

For Western blot analysis the dry cell pellets will be transferred from -80°C to dry ice and the cells will immediately lysed with a lysis buffer (5X extraction reagent diluted with water) containing protease inhibitors. After centrifugation (14000 rpm for 15 min at 4 °C) the resulting supernatant will be used for measurements using a bicinchominic acid protein assay kit using a BSA standard (Thermo Scientific). 30 µL of a solution containing SDS loading buffer and mercaptoethanol are combined with 50 µg of sample protein and lysis buffer up to 100 µL. Samples are heated at 93°C for 5 min and loaded onto a gel for protein separation. The gel will be run at 120 V for 1.5-2 hours and then transferred to a Western Blot membrane (20 V for 1.5 hrs) using the semi-dry method. The membranes will be blocked in 5 % milk for 1 hour, washed three times for 5 min with TBST (tris-buffered saline tween-20) and incubated with the primary HO-1 antibody over night at 4 °C, washed and incubated

with the second antibody for 1 hr. The membranes will be incubated for 5 min in substrate solution and imaged using clear X-ray films (Thermo Scientific). The antibody staining and development procedure are repeated again using an anit-β-actin antibody for normalization of the results.

Western Blot analyses

Western blotting will performed as previously described.[38] Equal amounts of protein will be loaded and Ponceau staining is performed to verify protein loading. Primary antibody used is anti-Heme-Oxygenase 1 (rabbit monoclonal antibody to heme-oxygenase 1; Abcam, Cat. No.: 52947). Secondary antibody used is goat anti-rabbit IgG (Thermo Scientific, Cat. No.: 32460). Quantification of bands is performed using the Quantity One Software (Quantity one, Biorad).

Reactive oxigen species

The Reactive Oxygen Species (ROS) ELISA Kit (ABIN511031, Antibodies Online, Atlanta, USA) will be used according to the manufactorers specifications for ROS measurement.

Statistical methodology and analysis

Sample size considerations

We could demonstrate a significantly improved reperfusion by 38% after 20 minutes ischemia in the skeletal muscle 24 hours after heme arginate administration in a sample size of 12 healthy subjects[6]. We used this data concerning the skeletal muscle for the sample size calculation of this trial. For a desired alpha of 0.05 and a power of 80%, the calculated sample size is 8. However, myocardial tissue may react in a different way to heme arginate administration. Therefore, the randomizer program will be programmed to distribute the first 24 patients evenly in all three groups. An intermediate evaluation of the outcome parameters will be performed by ANOVA (applying the Bonferroni correction). We hypothesize that the sample size of 12 patients per group for this trial may be appropriate. If the intermediate evaluation provides significant results to answer the study endpoints, no additional subjects will be included. If not, a sample size calculation will be performed based on the results of the first 24 patients. If more than 36 patients are required to answer the proposed questions, an amendment will be formulated to the ethical board and the competent authority.

Safety and tolerability endpoints

The treatment-emergent AEs will be tabulated (if possible, coded by using the MedDRA dictionary. Adverse events leading to premature discontinuation of study drug will be listed and summarized similarly to AEs. Treatment-emergent SAEs will be listed and summarized similarly to AEs, separately for treatment-emergent SAEs and SAEs occurring before study drug initiation and after study drug discontinuation. Reasons for premature discontinuation of study drug will be listed and summarized by frequency.
Vital signs (blood pressure and pulse rate) will be summarized for the absolute values and for the change from baseline to each scheduled time point of measurement. Individual subject listings will be provided.

Baseline parameters and concomitant medications

Continuous demographic variables (age, height, weight, BMI) will be summarized by the usual location and scale statistics (mean, median, standard deviation, minimum, maximum and number of available observations).
Qualitative demographic characteristics (race) will be summarized by counts and percentages. Other subject characteristics (medical history, physical examination findings, previous and concomitant medications) will only be listed.
Previous and concomitant medications will be coded according to the WHO drug code and the ATC code. They will be summarized by type (i.e., previous and concomitant) by tabulating the number and percentages of subjects having received each treatment.

Ethical and legal aspects

The study will be conducted according to the principles of Good Clinical Practice and the Declaration of Helsinki and in agreement with Austrian laws and regulation. Project management and monitoring will be done by the KKS or a comparable institution. The study protocol shall be registered in a public database (clinicaltrials.gov).

Informed consent of subjects

Following comprehensive instruction regarding the nature, significance, impact and risks of this clinical trial, the patient must give written consent to participation in the study.
During the instruction the patients are to be made aware of the fact that they can withdraw their consent – without giving reasons – at any time without their further medical care being influenced in any way.
In addition to the comprehensive instructions given to the patients by the investigator, the patients also receive a written patient information sheet in comprehensible language, explaining the nature and purpose of the study and its progress.
The patients must agree to the possibility of study-related data being passed on to relevant authorities.
The patients must be informed in detail of their obligations in relation to the study insurance in order not to jeopardize insurance cover.

Withdrawal and replacement of subjects

Criteria for withdrawal

[redacted]

Subjects may prematurely discontinue from the study at any time. Premature discontinuation from the study is to be understood when the subject did not undergo biopsy.

Subjects must be withdrawn under the following circumstances:

- at their own request
- if the investigator feels it would not be in the best interest of the subject to continue
- if the subject violates conditions laid out in the consent form / information sheet or disregards instructions by the study personal

In all cases, the reason why subjects are withdrawn must be recorded in detail in the CRF and in the subject's medical records. Should the study be discontinued prematurely, all study materials (complete, partially completed and empty CRFs) will be retained.
Subjects prematurely discontinued from the study for any reason will be replaced.

Follow-up of patients withdrawn from the study

In case of premature discontinuation after study drug intake, the subjects will be closely followed until dismissal. They may request that from the time point of withdrawal no more data will be recorded and that all biological samples collected in the course of the study will be destroyed.

Premature termination of the study

The sponsor has the right to close this study at any time. The IEC and the competent regulatory authority must be informed. The trial will be terminated prematurely in the following cases:
- If adverse events occur which are so serious that the risk-benefit ratio is not acceptable
- If the number of dropouts is so high that proper completion of the trial cannot realistically be expected

Safety definitions and reporting requirements

Averse events (AEs)

Summary of known and potential risks of the study drug

Heme arginate has been used in humans to treat acute episodes of hepatic porphyrias since 1985 and is well tolerated. The most commonly observed AEs of intravenous infusion of heme arginate are local reactions. Known AEs are listed in detail in Table 2.

Definition of adverse events

An AE is any adverse change from the subject's baseline condition, i.e., any unfavourable and unintended sign including an abnormal laboratory finding, symptom or disease that occurs during the course of the study, whether or not considered related to the study drug.
A treatment-emergent AE is any AE temporally associated with the use of a study drug, whether or not considered related to the study drug.

Adverse events include:
- Exacerbation of a pre-existing disease.
- Increase in frequency or intensity of a pre-existing episodic disease or medical condition.
- Disease or medical condition detected or diagnosed after study drug administration even though it may have been present prior to the start of the study.
- Continuous persistent disease or symptoms present at baseline that worsen following the start of the study.
- Lack of efficacy in the acute treatment of a life-threatening disease.
- Events considered by the investigator to be related to study-mandated procedures.
- Abnormal assessments, e.g., ECG and physical examination findings, must be reported as AEs if they represent a clinically significant finding that was not present at baseline or worsened during the course of the study.
- Laboratory test abnormalities must be reported as AEs if they represent a clinically significant finding, symptomatic or not, which was not present at baseline or worsened during the course of the study or led to dose reduction, interruption or permanent discontinuation of study drug.

Adverse events do not include:

- Medical or surgical procedure, e.g., surgery, endoscopy, tooth extraction, transfusion. However, the event leading to the procedure is an AE. If this event is serious, the procedure must be described in the SAE narrative.
- Pre-existing disease or medical condition that does not worsen.
- Situations in which an adverse change did not occur, e.g., hospitalizations for cosmetic elective surgery or for social and/or convenience reasons.
- Overdose of either study drug or concomitant medication without any signs or symptoms. However, overdose must be mentioned in the Study Drug Log.

Serious adverse events (SAEs)

A Serious Adverse Event (SAE) is defined by the International Conference on Harmonization (ICH) guidelines as any AE fulfilling at least one of the following criteria:
- Fatal (including fetal death).
- Life-threatening – defined as an event in which the subject was, in the judgment of the investigator, at risk of death at the time of the event; it does not refer to an event that hypothetically might have caused death had it been more severe.
- Requiring subject's hospitalization or prolongation of existing hospitalization – inpatient hospitalization refers to any inpatient admission, regardless of length of stay.
- Resulting in persistent or significant disability or incapacity (i.e., a substantial disruption of a person’s ability to conduct normal life functions).
- Congenital anomaly or birth defect.
- Is medically significant or requires intervention to prevent at least one of the outcomes listed above.

Life-threatening refers to an event in which the subject/subject was at risk of death at the time of the event. It does not refer to an event that hypothetically might have caused death if it were more severe.
Important medical events that may not immediately result in death, be life-threatening, or require hospitalization may be considered as SAEs when, based upon appropriate medical judgment, they may jeopardize the subject and may require medical or surgical intervention to prevent one of the outcomes listed in the definitions above.

Hospitalization – Prolongation of existing hospitalization

Hospitalization is defined as an overnight stay in a hospital unit and/or emergency room.
An additional overnight stay defines a prolongation of existing hospitalization.
The following is not considered an SAE and should be reported as an AE only:
- Treatment on an emergency or outsubject basis for an event not fulfilling the definition of seriousness given above and not resulting in hospitalization.

The following reasons for hospitalizations are not considered AEs, and therefore not SAEs:
- Hospitalizations for cosmetic elective surgery, social and/or convenience reasons.
- Standard monitoring of a pre-existing disease or medical condition that did not worsen, e.g., hospitalization for coronary angiography in a subject with stable angina pectoris.
- Elective treatment of a pre-existing disease or medical condition that did not worsen, e.g., hospitalization for chemotherapy for cancer, elective hip replacement for arthritis.

SAEs related to study-mandated procedures

Such SAEs are defined as SAEs that appear to have a reasonable possibility of causal relationship (i.e., a relationship cannot be ruled out) to study-mandated procedures (excluding administration of study drug) such as discontinuation of subject's previous treatment during a washout period, or complication of a mandated invasive procedure (e.g., blood sampling, heart catheterization), or car accident on the way to the hospital for a study visit, etc.

Suspected unexpected serious adverse reactions (SUSARs)

SUSARs are all suspected adverse reactions related to the study drug that are both unexpected (not previously described in the SmPC) and serious.

Severity of adverse events

The severity of clinical AEs is graded on a three-point scale: mild, moderate, severe, and reported on specific AE pages of the CRF.
If the severity of an AE worsens during study drug administration, only the worst intensity should be reported on the AE page. If the AE lessens in intensity, no change in the severity is required.
If an AE occurs during a washout or placebo run-in phase and afterwards worsens during the treatment phase, a new AE page must be filled in with the intensity observed during study drug administration.

Mild

Event may be noticeable to subject; does not influence daily activities; the AE resolves spontaneously or may require minimal therapeutic intervention;

Moderate

Event may make subject uncomfortable; performance of daily activities may be influenced; intervention may be needed; the AE produces no sequelae.

Severe

Event may cause noticeable discomfort; usually interferes with daily activities; subject may not be able to continue in the study; the AE produces sequelae, which require prolonged therapeutic intervention.

A mild, moderate or severe AE may or may not be serious. These terms are used to describe the intensity of a specific event (as in mild, moderate, or severe myocardial infarction). However, a severe event may be of relatively minor medical significance (such as severe headache) and is not necessarily serious. For example, nausea lasting several hours may be rated as severe, but may not be clinically serious. Fever of 39°C that is not considered severe may become serious if it prolongs hospital

discharge by a day. Seriousness rather than severity serves as a guide for defining regulatory reporting obligations.
These definitions do not apply to clinically significant and asymptomatic laboratory test abnormalities or abnormal assessments (e.g., ECG findings) considered as AEs. The investigator should tick non-applicable on the AE page of the CRF to qualify the intensity of the AE.

Relationship to study drug

For all AEs, the investigator will assess the causal relationship between the study drug and the AE using his/her clinical expertise and judgment according to the following algorithm that best fits the circumstances of the AE:
Unrelated:
- May or may not follow a reasonable temporal sequence from administration of the study product
- Is biologically implausible and does not follow known response pattern to the suspect study drug (if response pattern is previously known)
- Can be explained by the known characteristics of the subject's clinical state or other modes of therapy administered to the subject.

Possibly related:
- Follows a reasonable temporal sequence from administration of the study product.
- May follow a known response pattern to the study drug (if response pattern is previously known).
- May also be reasonably explained by the subject's clinical state or other modes of therapy administered to the subject.

Probably related:
- Follows a reasonable temporal sequence form administration of the study drug.
- May follow a known response pattern to the study drug (if response pattern is previously known).
- Could not be reasonable not be reasonably explained by the known characteristics of the subject's clinical state or other modes of therapy administered to the subject, if applicable.

Definitely related:
- Follows a reasonable temporal sequence form administration of the study drug.
- Follows a known response pattern to the study drug (if response pattern is previously known).
- No other reasonable cause is present.

Reporting procedures

A special section is designated to adverse events in the case report form. The following details must thereby be entered:
- Type of adverse event
- Start (date and time)
- End (date and time)
- Severity (mild, moderate, severe)
- Serious (no / yes)
- Unexpected (no / yes)
- Outcome (resolved, ongoing, ongoing – improved, ongoing – worsening)
- Relation to study drug (unrelated, possibly related, probably related, definitely related)

Adverse events are to be documented in the case report form in accordance with the above mentioned criteria.

Reporting procedures for SAEs

In the event of serious, the investigator has to use all supportive measures for best patient treatment. A written report is also to be prepared and made available to the clinical investigator within five days. The following details should at least be available:
- Patient initials and number
- Patient: date of birth, sex, ethical origin
- The suspected investigational medical product (IMP)
- The adverse event assessed as serious
- Short description of the event and outcome

Reporting procedures for SUSARs

It must be remembered that the regulatory authorities, and in case of SUSARs which could possibly concern the safety of the study participants, also the Institutional Review Board / Independent Ethics Committee (IRB / IEC) are to be informed. Such reports shall be made by the study management and the following details should be at least available:

- Patient initials and number
- Patient: date of birth, sex, ethical origin
- Name of investigator and investigating site
- Period of administration
- The suspected investigational medical product (IMP)
- The adverse event assessed as serious and unexpected, and for which there is a reasonable suspected causal relationship to the IMP
- Concomitant disease and medication
- Short description of the event (description, onset and (if applicable) end, therapeutic intervention, causal relationship, hospitalization of prolongation of hospitalization, death, life-threatening, persistent or significant disability or incapacity

Electronic reporting should be the expected method for reporting of SUSARs to the competent authority. In that case, the format and content as defined by Guidance {Module, #68} should be adhered to. The latest version of MedDRA should be applied. Lower level terms (LLT) should be used.

Data handling procedures

A case record form will be completed for each volunteer. Trained personnel will check the entries and any errors or inconsistencies will be resolved immediately. The results of the prestudy screening examination will be documented in the study masterfile.

[redacted]

Acknowledgement / approval of the study

The investigator will submit this protocol and any related document provided to the subject (such as subject information used to obtain informed consent) to the Ethics

Committee (EC) of the Medical University of Vienna. Approval from the committee will be obtained before starting the study.

Adverse events - whether serious and/or unexpected, and possibly endangering the safety of the study participants - are likewise to be reported to the EC.
The clinical trial shall be performed in full compliance with the valid legal regulations according to the Drug Law (AMG - Arzneimittelgesetz) of the Republic of Austria.
The study must be notified to the Austrian Agency for Health and Food Safety (AGES) and to the European Agency for the Evaluation of Medicinal Products (EMEA) and registered to the European Clinical Trial Database (EurdaCT) using the required forms.

Protocol amendments

Modifications made to the protocol after receipt of the EC/IRB approval must also be submitted as amendments by the investigator to the EC/IRB in accordance with local procedures and regulations.

Finance and insurance

During their participation in the clinical trial the patients will be insured as defined by legal requirements. The principal investigator of the clinical trial will receive a copy of the insurance conditions of the 'proband insurance'. The sponsor is providing insurance in order to indemnify (legal and financial coverage) the investigator/center against claims arising from the study, except for claims that arise from malpractice and/or negligence. The compensation of the subject in the event of study-related injuries will comply with the applicable regulations.
Details on the existing proband insurance are given in the patient information sheet.

[redacted]

Confidentiality

The information contained in this document, especially unpublished data, is the property of the sponsor of this study, the Department of Clinical Pharmacology, Medical University Vienna. It is therefore provided to you in confidence as an

investigator, potential investigator, or consultant, for review by you, your staff, and an Ethics Committee or Institutional Review Board. It is understood that this information will not be disclosed to others without written authorization from the Department of Clinical Pharmacology, Medical University Vienna, except to the extent necessary to obtain informed consent from those persons to whom the study drug may be administered.

Ethics and Good clinical Practice (GCP)

The investigator will ensure that this study is conducted in full conformance with the principles of the "Declaration of Helsinki" (as amended at the 56th WMA General Assembly, Tokyo, Japan, 2004) and with the laws and regulations of the country in which the clinical research is conducted.
The principal investigator of the clinical trial shall guarantee that only appropriately trained personnel will be involved in the study. All studies must follow the ICH GCP Guidelines (June 1996) and, if applicable, the Code of Federal Regulations (USA). In other countries in which GCP Guidelines exist, the investigators will strictly ensure adherence to the stated provisions.

Documentation of study results

A Subject Screening and Enrollment Log will be completed for all eligible or non-eligible subjects with the reasons for exclusion.
For each subject enrolled, regardless of study drug initiation, a CRF must be completed and signed by the principal investigator or co-investigator. This also applies to those subjects who fail to complete the study. If a subject withdraws from the study, the reason must be noted on the CRF. Case report forms are to be completed on an ongoing basis.
All forms should be completed using a black pen and must be legible. Trained personnel will check the entries and any errors or inconsistencies will be checked immediately. Errors should be crossed out but not obliterated, the correction inserted, and the change initialled and dated by the investigator, co-investigator or study nurse.
The monitor will collect original completed and signed CRFs at the end of the study. A copy of the completed and signed CRFs will remain on site.

Safekeeping

The investigator will maintain adequate and accurate records to enable the conduct of the study to be fully documented and the study data to be subsequently verified. These documents will be classified into two different categories: investigator's file, and subject clinical source documents.
The investigator's file will contain the protocol/amendments, EudraCT forms, CRFs and data clarification and query forms, EC/IRB and Health Authority approval with correspondence, informed consent, drug records, staff curriculum vitae and authorization forms, screening and enrollment logs, and other appropriate documents/correspondence as per ICH/Good Clinical Practice (GCP) and local regulations.
Subject clinical source documents include, but are not limited to subject hospital/clinic records, physician's and nurse's notes, appointment book, original laboratory reports, ECG, X-ray, pathology and special assessment reports, consultant letters, etc.
These two categories of documents must be kept on file by the investigator for as long as needed to comply with national and international regulations (in Austria 15 years after discontinuing clinical development or after the last marketing approval). No study document should be destroyed without prior written approval from the Department of Clinical Pharmacology.
When source documents are required for the continued care of the subject, appropriate copies should be made for storing outside of the site.

Monitoring

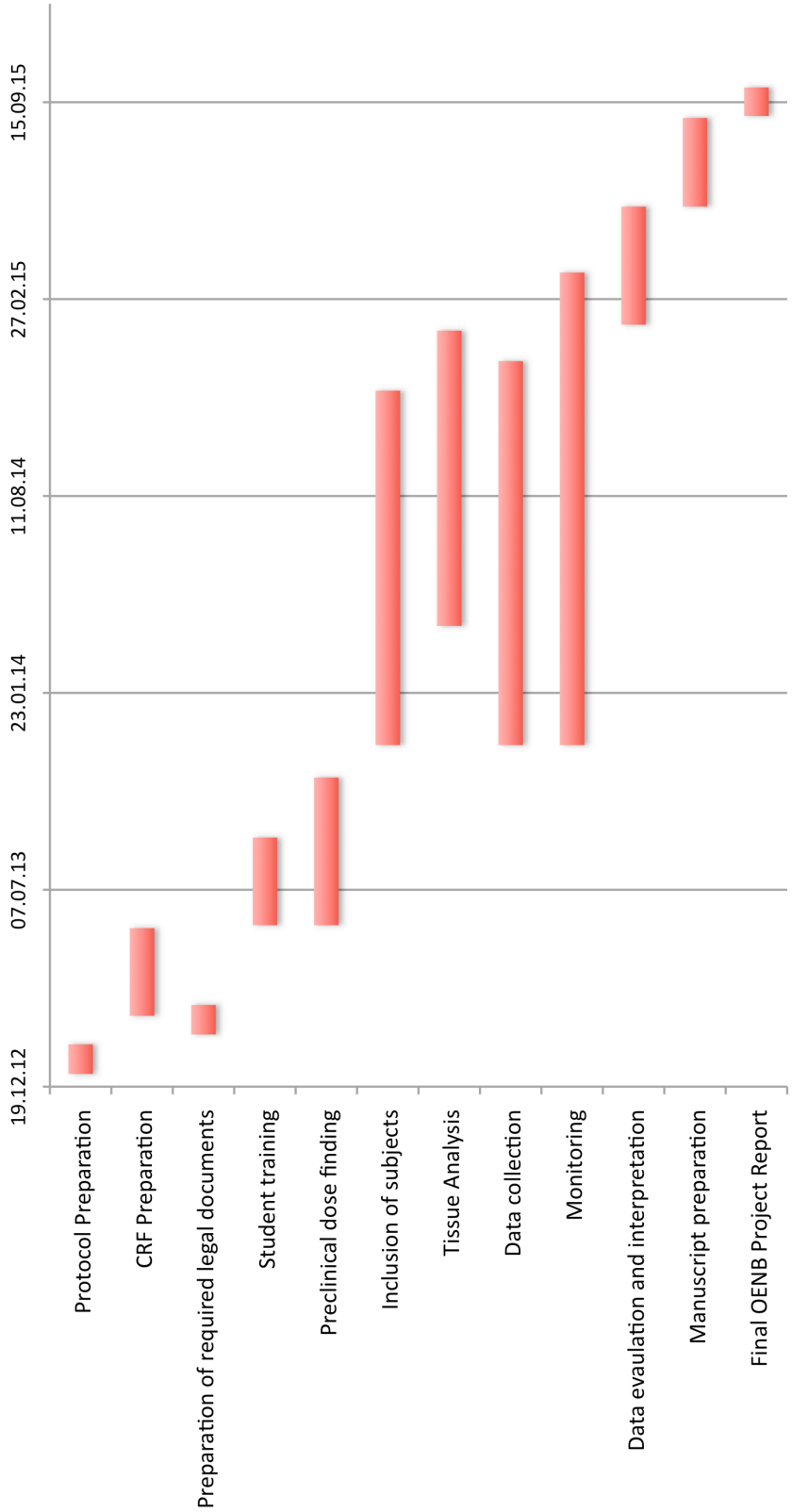
The monitor will contact and visit the investigator regularly and will be allowed, on request, to have access to all source documents needed to verify the entries on the CRF and other protocol-related documents provided that subject confidentiality is maintained in agreement with local regulations. It will be the monitor's responsibility to inspect the CRF at regular intervals throughout the study, to verify the adherence to the protocol and the completeness, consistency and accuracy of the data being entered on them. The monitoring standards require full verification for the presence

of informed consent, adherence to the inclusion/exclusion criteria, documentation of SAEs and the recording of the main efficacy, safety, and tolerability endpoints.

Publication of study results

The findings of this study will be published by the investigators in a scientific journal and presented at scientific meetings. The manuscript will be circulated to all co-investigators before submission.

Study time course



References

1. Cooper R, Cutler J, Desvigne-Nickens P, et al. Trends and disparities in coronary heart disease, stroke, and other cardiovascular diseases in the United States: findings of the national conference on cardiovascular disease prevention. Circulation 2000;102:3137-47.
2. Morel O, Perret T, Delarche N, et al. Pharmacological approaches to reperfusion therapy. Cardiovasc Res 2012;94:246-52.
3. Otterbein LE, Soares MP, Yamashita K, Bach FH. Heme oxygenase-1: unleashing the protective properties of heme. Trends Immunol 2003;24:449-55.
4. Tsuchihashi S, Fondevila C, Kupiec-Weglinski JW. Heme oxygenase system in ischemia and reperfusion injury. Ann Transplant 2004;9:84-7.
5. Doberer D, Haschemi A, Andreas M, et al. Haem arginate infusion stimulates haem oxygenase-1 expression in healthy subjects. Br J Pharmacol 2010;161:1751-62.
6. Andreas M, Schmid AI, Doberer D, et al. Heme arginate improves reperfusion patterns after ischemia: a randomized, placebo-controlled trial in healthy male subjects. J Cardiovasc Magn Reson 2012;14:55.
7. Lloyd-Jones DM, Larson MG, Beiser A, Levy D. Lifetime risk of developing coronary heart disease. Lancet 1999;353:89-92.
8. Matsumura K, Jeremy RW, Schaper J, Becker LC. Progression of myocardial necrosis during reperfusion of ischemic myocardium. Circulation 1998;97:795-804.
9. Eefting F, Rensing B, Wigman J, et al. Role of apoptosis in reperfusion injury. Cardiovasc Res 2004;61:414-26.
10. Zweier JL, Talukder MA. The role of oxidants and free radicals in reperfusion injury. Cardiovasc Res 2006;70:181-90.
11. Vinten-Johansen J. Involvement of neutrophils in the pathogenesis of lethal myocardial reperfusion injury. Cardiovasc Res 2004;61:481-97.
12. Zhao ZQ, Vinten-Johansen J. Postconditioning: reduction of reperfusion-induced injury. Cardiovasc Res 2006;70:200-11.
13. Pleiner J, Schaller G, Mittermayer F, Bayerle-Eder M, Roden M, Wolzt M. FFA-induced endothelial dysfunction can be corrected by vitamin C. J Clin Endocrinol Metab 2002;87:2913-7.
14. Bayerle-Eder M, Pleiner J, Mittermayer F, et al. Effect of systemic vitamin C on free fatty acid-induced lipid peroxidation. Diabetes Metab 2004;30:433-9.
15. Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation 1986;74:1124-36.
16. Kharbanda RK, Peters M, Walton B, et al. Ischemic preconditioning prevents endothelial injury and systemic neutrophil activation during ischemia-reperfusion in humans in vivo. Circulation 2001;103:1624-30.
17. De Backer D, Dubois MJ, Schmartz D, et al. Microcirculatory alterations in cardiac surgery: effects of cardiopulmonary bypass and anesthesia. Ann Thorac Surg 2009;88:1396-403.
18. Tenhunen R, Marver HS, Schmid R. The enzymatic conversion of heme to bilirubin by microsomal heme oxygenase. Proc Natl Acad Sci U S A 1968;61:748-55.
19. Vitek L, Schwertner HA. The heme catabolic pathway and its protective effects on oxidative stress-mediated diseases. Adv Clin Chem 2007;43:1-57.
20. Ryter SW, Alam J, Choi AM. Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications. Physiol Rev 2006;86:583-650.

21. Clark JE, Foresti R, Sarathchandra P, Kaur H, Green CJ, Motterlini R. Heme oxygenase-1-derived bilirubin ameliorates postischemic myocardial dysfunction. Am J Physiol Heart Circ Physiol 2000;278:H643-51.
22. Terry CM, Clikeman JA, Hoidal JR, Callahan KS. TNF-alpha and IL-1alpha induce heme oxygenase-1 via protein kinase C, Ca2+, and phospholipase A2 in endothelial cells. Am J Physiol 1999;276:H1493-501.
23. Kawamura K, Ishikawa K, Wada Y, et al. Bilirubin from heme oxygenase-1 attenuates vascular endothelial activation and dysfunction. Arterioscler Thromb Vasc Biol 2005;25:155-60.
24. Yoshida T, Biro P, Cohen T, Muller RM, Shibahara S. Human heme oxygenase cDNA and induction of its mRNA by hemin. Eur J Biochem 1988;171:457-61.
25. Alam J, Killeen E, Gong P, et al. Heme activates the heme oxygenase-1 gene in renal epithelial cells by stabilizing Nrf2. Am J Physiol Renal Physiol 2003;284:F743-52.
26. Alam J, Cook JL. Transcriptional regulation of the heme oxygenase-1 gene via the stress response element pathway. Curr Pharm Des 2003;9:2499-511.
27. Ferenbach DA, Nkejabega NC, McKay J, et al. The induction of macrophage hemeoxygenase-1 is protective during acute kidney injury in aging mice. Kidney Int 2011;79:966-76.
28. Maeshima K, Takahashi T, Uehara K, et al. Prevention of hemorrhagic shock-induced lung injury by heme arginate treatment in rats. Biochem Pharmacol 2005;69:1667-80.
29. Sasaki T, Takahashi T, Maeshima K, et al. Heme arginate pretreatment attenuates pulmonary NF-kappaB and AP-1 activation induced by hemorrhagic shock via heme oxygenase-1 induction. Med Chem 2006;2:271-4.
30. Bharucha AE, Kulkarni A, Choi KM, et al. First-in-human study demonstrating pharmacological activation of heme oxygenase-1 in humans. Clin Pharmacol Ther 2010;87:187-90.
31. Mustajoki P, Tenhunen R, Tokola O, Gothoni G. Haem arginate in the treatment of acute hepatic porphyrias. Br Med J (Clin Res Ed) 1986;293:538-9.
32. Herrick A, McColl KE, McLellan A, Moore MR, Brodie MJ, Goldberg A. Effect of haem arginate therapy on porphyrin metabolism and mixed function oxygenase activity in acute hepatic porphyria. Lancet 1987;2:1178-9.
33. Tenhunen R, Mustajoki P. Acute porphyria: treatment with heme. Semin Liver Dis 1998;18:53-5.
34. Volin L, Rasi V, Vahtera E, Tenhunen R. Heme arginate: effects on hemostasis. Blood 1988;71:625-8.
35. German full package insert for Normosang in Austria.
36. Tokola O, Tenhunen R, Volin L, Mustajoki P. Pharmacokinetics of intravenously administered haem arginate. Br J Clin Pharmacol 1986;22:331-5.
37. Geuken E, Buis CI, Visser DS, et al. Expression of heme oxygenase-1 in human livers before transplantation correlates with graft injury and function after transplantation. Am J Transplant 2005;5:1875-85.
38. Bernhard D, Skvortsov S, Tinhofer I, et al. Inhibition of histone deacetylase activity enhances Fas receptor-mediated apoptosis in leukemic lymphoblasts. Cell death and differentiation 2001;8:1014-21.