Centrale Commissie Dierproeven

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information on the project proposal, see the Guidelines to the project licence application form for animal procedures on our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0800-7890789).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	50100
1.2	Provide the name of the licenced establishment.	TNO
1.3	List the serial number and type of animal procedure *Use the numbers provided at 3.4.3 of the project proposal.*	Serial number: 1 Type of animal procedure: Rat and Guinea Pig models to study the toxicology of chemical threat agents and countermeasure evaluation

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

General aims of the procedures

The main objective of the proposed project is to determine the efficacy of medical interventions in qualified models of toxic chemical exposure. This objective is divided into three subgoals/components, that are aimed at assessing and characterizing the threat posed by selected toxic chemicals depending on dose and exposure route, investigating the therapeutic efficacy of available medical countermeasures, and assessing the safety of these countermeasures at the required dosages. For this, we will use rats or guinea pigs which will be exposed to the chemical threat and treated with selected medical counter measures (MCM). First the experimental approaches for the three subgoals are outlined in the different components of this research proposal, followed by a description of the primary outcome parameters.

Animal model selection

With respect to the animal model development, the range of models that could be used is quite broad, depending on the requirements, e.g. challenge agent, route of exposure, time frame of exposure, human target and the desired benefit of prospective countermeasures on read out parameters. A general methodology for selection is as follows:

a. Preselect a list of relevant animal models based on historic evidence.
b. On the basis of the challenge agent of the particular Task: consider the pathophysiology, the target organ(s) / disease patterns; take into account possible differences in the target population (such as gender, age, predisposition).

c. Create a match between animal models considered appropriate for those target organs and target end points to be mitigated; the result is a re-ranked short-list.
d. On the basis of the drugs to be studied as potential countermeasures, for example based on translational PK/PD: match these with short-list (point c); the result is an updated short-list.
e. Starting off with list (d): take into consideration the route of exposure of the challenge agent (most likely inhalational or dermal) and that of the drugs (most likely parenteral, oral or dermal) check for best fit with (operational) requirement.
f. Integrate all previous points to select appropriate rat or guinea pig models

This methodology is in agreement with points addressed in the "Guidance for Industry: Product Development under the Animal Rule" providing an excellent starting point for selection of animal models for separate challenge agents and efficacy requirements for treatment guidelines. As the models in this appendix are used for evaluation of therapeutic safety and efficacy, this has to be taken into account here already.

An overview of rationales with regard to read-out parameters or etiology are described below. As small animal models, (hairless) rats, and (hairless) guinea pigs are appropriate models to address different research questions.

1. Rats are animals suitable for addressing research questions with regard to animal behavior or issues related to the central nervous system (CNS), including brain drug or agent levels and seizure development and culmination. This can be relevant for follow up questions with regard to improvements in the context of intermediate term recovery.
2. (Hairless) guinea pigs are generally used to assess the effects of interventions (e.g. decontamination or treatment) on skin penetration and kinetics. To this end, the species with skin properties most closely approximating the human skin will be used. In addition to hairless guinea pigs, hairless rats have become available recently.
3. For organophosphate research, certain enzymes (carboxylesterase among others) act as endogenous scavengers which can lower the sensitivity for organophosphate poisoning. Guinea pigs have carboxylesterase levels more comparable to humans than do rats, making them the preferred model for studies involving scavengers, for example. Generally, they are considered to hold the most predictive value in terms of therapeutic efficacy observed in primates. However, as long as the impact of relevant biological differences is known and taken into account during interpretation of the data, rats are very suitable for organophosphate research as well.
4. For opioid research, the respiratory system of rats is slightly more similar to humans than guinea pigs, but the opioid receptor system of guinea pig resembles the human situation better than rats. The choice of species will therefore highly depend on the specific biological systems to be targeted by the interventions.
5. For regulatory purposes, efficacy of treatments might have to be confirmed in at least two different small animal species, which might drive the choice for either rat or guinea pig.

In spite of a different airway architecture from humans, these small animal species can also be valid to elucidate mechanisms of toxicity of inhaled agents, as in general the organ toxicity depends on the amount of agent reaching the target site, that should be similar to that in human. This can be titrated depending on the species.

Selection of readout parameters

Depending on the pathophysiology, a range of readout parameters will be selected, that should allow for defining possible intervention times (trigger to treat), and consequently allow for determination of therapeutic efficacy of such interventions in other stages of the project. The following list is a representation of possible readout parameters, that could be assessed over time, ranging from several hours to 14 days. The readout parameters apply to studies performed for all subgoals; though the specific subgoal per study might differ, the experimental approach is the same.

a) Clinical signs: Observation of the animal for specific signs of toxicity associated with the type of toxic compound, such as chewing, twitching, grimacing, salivation, involuntary movements, labored breathing, or skin discoloration.

b) *Physiological signs*:

- brain activity, measured by means of electroencephalography (EEG), using implanted telemetry devices

- cardiovascular parameters (such as electrocardiography (ECG) and blood pressure), using implanted telemetry devices
- Skeletal muscle activity, measured by means of electromyography (EMG), using implanted telemetry devices.
- Core or subcutaneous temperature, assessed using telemetry
- respiratory function (such as respiratory rate, apnea, or markers indicative of bronchoconstriction), assessed non-invasively using (whole body) plethysmography

c) ***Blood parameters*** – assessed in blood samples, obtained from unrestrained animals (pre-implanted indwelling catheter) or brief restraint (e.g. tail vein) in anesthetized or unanesthetized animals

a. Toxicokinetics: levels of chemical threat agent and any active metabolites in the blood
b. Biochemistry: specific enzyme activity, representative for a specific chemical, specific chemical adducts (biomarkers)
c. Clinical chemistry: Oxygen levels, ionic balance, enzyme/ protein levels

Primary indicators for progression of toxicity will be based on real-time monitoring of physiology and clinical signs. For substances primarily acting on the central nervous system, EEG will be included, whereas for irritants, respiratory parameters may be more indicative for progression of toxicity. ECG-derived parameters, such as heart rate and heart rate variability (HRV) are often used as indicators for systemic burden of a poisoning, but can also be used as important parameters to assess the experimental endpoint. Additionally, blood samples could be obtained to assess toxicokinetics when considered of additional value, and/ or to obtain biomarkers or general clinical chemistry. A designated combination of parameters should lead to a dose – time to effect range following exposure to a specific threat agent.
In selected cases, for example in the animal model development component, ultimately death, or time to death, or humane endpoint (in case a point of no return can be established) could be a readout. To establish a dose and time to effect relation, dose-range finding studies, based on the literature, will be performed.

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

Component 1. **Development of translational animal models**.
This will include characterization of the toxic effects (toxidrome) induced by the threat agent being studied, the underlying mechanism of toxicity, as well as the toxicokinetics and metabolic pathways. Model validation is a difficult issue, especially as human data is extremely scarce for the threat agents of interest. However, validity of the model used will be investigated by comparing the toxidrome (face validity) and the molecular pathways affected (construct validity) to what is known in humans. All relevant exposure routes may be studied by which incidents are expected to happen, such as inhalational exposure to gases, vapors and aerosols or percutaneous exposures to liquids, vapors and aerosols. Invasive routes, either as bolus administration or prolonged infusion, may also be employed as model routes for one of the aforementioned routes, if the toxicokinetic or toxicodynamic profiles are similar. These routes may be preferred for some of the studies as they are associated with smaller degrees of experimental variability and experimental risk. The aim of this subgoal/component is to design or use animal models to characterize response at (sub)lethal doses of chemicals, with specified triggers to treat. These triggers may be based on the toxidrome (occurrence of a (combination of) specific clinical sign(s) or physiological change(s)) or on the timing that is relevant for the expected casualty scenario. These studies will also be suitable to aid in the development of forensic tools and identification of biomarkers of exposure, or determine the biological fate of the chemical to which the animal has been exposed.

Ultimately, this paradigm will lead to an animal model which exhibits a well-defined, translationally valid, and consistent response to a specific toxic chemical, which can be used for the evaluation of medical countermeasures.

Component 2. Pharmacokinetics (PK) and pharmacodynamics (PD) of therapeutics and adverse effects
Studies in this project will investigate the pharmacokinetics of existing or prospective therapeutics when used as countermeasures for intoxications with highly toxic agents for various relevant routes of administration, such as intramuscular, intranasal, subcutaneous, intraosseous, topical/percutaneous. In particular, these studies will be aimed at providing information useful for addressing bioavailability, changes in pharmacokinetics and pharmacodynamics depending on the route of administration. This will allow the determination of optimal timing of dosing in relation to the clinical signs of toxicity as determined in phase I, to optimize the design of studies

for the determination of therapeutic efficacy. Some of the research performed will be focused on therapeutics that are either already FDA-approved (repurposing) or in clinical development. Therefore, in most cases pharmacokinetic data will be available in the literature. However, the studies performed here focus on acute chemical intoxications that often require substantially higher doses or different administration routes, for which the pharmacokinetic data is not available and existing pharmacokinetic models are not suitable.
Importantly, studies performed could be used to establish PK-PD models that aid in the translation from animal models to the human situation. For many chemical threat agents, due to the absence of clinical data, therapeutic doses for medical countermeasures in humans have not been established. Translation of efficacious doses from animals to humans could be assisted by means of allometric scaling, incorporating animal and human PK data, or by translation of PK-PD studies, in which efficacious doses in humans can be established by comparing physiological effects observed both in animals and humans at therapeutic doses confirmed in animals.
Potential adverse effects of therapeutics in the dose-range required for efficacy against toxicants will be determined in the animal models. An example of such an adverse effect is incapacitation due to excessive sedation, which could occur in case of a misdiagnosis or false alarm. Incapacitation could have major implications when occurring in a military setting and therefore these risks need to be known.

Component 3. Efficacy of medical countermeasures against intoxication by chemical threat agents
The efficacy of interventions to counter the adverse effects of chemical threat agents will be studied in already available or newly developed animal models. This includes the evaluation of the efficacy of therapeutics, but also other types of interventions may be studied, such as procedures to decontaminate the skin. The studies will be aimed at establishing both short term efficacy, such as survival for up to 96 h, reduction of life-threatening effects induced by the threat agent, as well as intermediate term efficacy, such as recovery from the effects of the intoxication after treatment, which may be studied for a period of up to 14 days post-intoxication. Treatment may be focused on generic, supportive care, or on curative treatment, directly countering the effects of the investigated substance. In this respect, the public setting for which the models are to be developed requires an adequate identification of intervention points that would be feasible for example in a mass casualty situation or in a follow-up situation. In a military setting, other operational circumstances may exist, such as adequate training of personnel, and rapidly available countermeasures based on threat evaluations. Among others, the intervention point determines which animal model is feasible to address the efficacy of a certain intervention.
This means that each clinical phase following exposure should be separately considered for intervention, based on the hypothesis to be tested. Initial treatments could be aimed at improving survival, or lowering the burden on follow-up intensive care, whereas intermediate treatments are most likely aimed at preventing debilitating impairments affecting the quality of life.

Animal procedures
The animal procedures for experiments covered in this appendix can be divided into three phases, (1) a preparation phase in which animals may be equipped with catheters, telemetry devices according to the strategy described above. This is followed by (2) the exposure phase, in which the same animals are exposed to a certain dose of a chemical agent and/or therapeutic intervention and (3) monitoring of the animals response to the toxic challenge and/or intervention.

Preparation
All surgical procedures will be carried out under species- and procedure appropriate anesthesia. These will be specified in the individual research plan which will submitted to the Animal Welfare Body (Dutch: IvD) for review before start of each separate experiment. Appropriate perioperative care will be provided (analgesia and antibiotic treatment if necessary). Animals will be surgically equipped with one or more of the following, dependent on compatibility of techniques and required read-outs:

Jugular vein and/ or carotid/femoral artery cannula
For permanent venous or arterial access (to draw blood or to inject intravenously), it is necessary to implant animals with an indwelling catheter in accessible blood vessels, such as the jugular vein or the carotid/femoral artery. The catheters will be exteriorized to allow for stress-free repeated sampling. An example is subcutaneous tunneling, and fixation to the skull bone with dental cement in small animal models. Other techniques, such as the use of vascular access buttons, may also be used to provide an access point for repeated blood sampling during an experiment.

EEG electrode (telemetry)
Electrodes to obtain brain electrical activity will be placed through the skull bone to access the dura mater. The head of the anesthetized animal is fixed, small holes are drilled, and screw electrodes or flexible leads are fixed to the skull, making light contact with the dura. The electrodes/leads are either attached to a connector or directly to a telemetry device. In case a connector is used, this small connector is fixed to the skull. In case they are attached to a telemetry device, the leads are tunneled subcutaneously and the device is placed in a subcutaneous pouch or in the abdominal cavity. Dental cement is used to cover and fix the electrodes/leads and connector. Screws and instrumentation will be appropriately dimensioned for the animal species under study.

ECG electrode (telemetry)
Leads to obtain cardiac functioning information are placed in an appropriate configuration, such as Lead II configuration, e.g. one lead at the right collar bone and one lead at the lower left chest. Leads are fixed in superficial muscular layers. The leads are led subcutaneously to a connector fixed on the skull (see EEG electrode), or to a telemetric body. In some cases the wireless transmitters are implanted in the animals instead of exteriorized as described above.

EMG electrode (telemetry)
Leads are placed in an appropriate configuration, in order to allow for monitoring of specific muscle activity. Leads may, for example, be fixed to diaphragm intercostal or trapezius muscles for monitoring the main or accessory respiratory activity respectively.

Skin microdialysis probe
A skin microdialysis probe (flexible) is inserted in the skin of an animal, via a guide that is inserted over a fixed distance in the skin, e.g. 1 cm. The probe is fixed to the skin with glue or a suture (concentric probe).

Brain microdialysis guide
Animals are fixed in a stereotaxic frame under anesthesia. Via the stereotact, the guide will be placed into a specified brain area by stereotaxic coordinates. Holes for screw fixation and probe guide access are prepared using small electrical drills. The probe is fixed to the skull and fixation screw with dental cement. Our lab has experience with all of the aforementioned techniques, for examples see[1][2].

Exposure / Treatment
Preparation is followed by exposure/treatment as described below. The preparation phase is followed by an appropriate post-surgery recovery period of minimally 3 days up to 2 weeks, depending on the instrumentation and surgery. After this recover the rats or guinea pigs will be exposed to the chemical threat, treatment or their combination. Wherever feasible, we will administer the substance while the animals are under anesthesia. Although a toxic challenge under anesthesia would be preferred from an animal welfare point of view, interference of the anesthetic with the toxicological mechanism of action often significantly hampers the translational value of the experiment, and is as such considered unethical to perform. In such cases, the challenge is performed in awake animals .

Depending on the research question, animals may be exposed either under anesthesia or manual restraint. The following administration routes may be employed. The dose, dosing regimen, and route of administration depend on the nature of the research question and will be such that they are in line with current guidance such as described by the IQ 3Rs Leadership group (2016)[3].

[1] [redacted]

[2] [redacted]

[3] IQ 3Rs Leadership Group - Contract Research Organization Working Group. Recommended Dose Volumes for Common Laboratory Animals, URL https://iqconsortium.org/images/LG-3Rs/IQ-CRO_Recommended_Dose_Volumes_for_Common_Laboratory_Animals_June_2016_%282%29.pdf, accessed 17-Sep2021

*__subcutaneous/intraperitoneal__ injection/infusion of a solution or other formulation (i.e. gel). Alternatively, implanted devices (such as osmotic pumps) may be used to administer treatment (all species)

*__intravenous__ injection/infusion

Under restraint via the tail vein (rat), under anesthesia in the penile vein (guinea pig) or via indwelling permanent catheter (all species)

*__intramuscular__ injection

***intraosseous** injection/infusion of a solution under anesthesia

*__percutaneous__ administration by dropping a known amount of solution on the skin; generally in the microliter range. Alternatively, patches (microneedle technology) may be used.

*__inhalational__ exposure

Animals will be restrained in a nose-only exposure restraint device (size dependent on the species) or a mask may be applied. The device will be attached to the generation system and the animal will be exposed nose-only for a predefined time

***Intranasal/buccal** administration by spraying using an atomizer/nebulizer or dropping a known amount of solution or other formulation (i.e. gel)

Monitoring

Blood sampling

Generally from the tail under restraint (rat, in case of sparse sampling) or via an indwelling cannula for guinea pigs for when larger volumes and short intervals are needed. Maximum volumes are determined as described by the IQ 3Rs Leadership Group[3].

Microdialysis sampling

A probe will be inserted under gentle fixation in the brain guide ~24 hours before the experiment. Flexible tubing will be attached to the fixed probes and dialysates will be collected.

Metabolism

Animals are individually housed in metabolism cages, with access to water and food *ad libitum* to allow for collection of faeces and/ or urine.

EEG, ECG, EMG monitoring (Telemetry)

The electrical signals (ECG/EEG/EMG) are transmitted wirelessly, from the telemetry device attached to the head stage or implanted subcutaneously/intraperitoneally. The animals are housed individually during assessment to prevent interference of the signals of the different animals.

Respiration monitoring

Animals are placed individually in a whole body plethysmograph. In this way, breathing patterns can be assessed non-invasively. The animals will be in the plethysmograph for 8 hours, depending on the sampling time required.

Behavioral observations

Animals will be monitored live or via video recording to observe behavioral changes relevant for the induced toxidrome, as well as recovery.

At the end of the monitoring phase, tissues are collected after appropriate euthanasia.

The procedures described above are summarized in the following table. The animals will undergo a single surgical procedure, during which they are equipped with (a combination of) electrodes and/or catheters. Though different combinations of telemetry and catheters could be made, recovery of the animals is fast and the cumulative discomfort is similar for all animals due to the discomfort of anesthesia (moderate). Following an appropriate recovery period, animals will be exposed to a chemical threat agent and/or receive treatment, after which animals are monitored. In case of an exposure (or exposure+treatment), monitoring will be limited to a single occurrence. In case only treatment is administered, this may be repeated up to a maximum of two times, only if each occurrence is associated with 'mild' discomfort. In these cases, the monitoring period will not exceed 14 days and the cumulative discomfort will not exceed 'moderate', as described in section F.

Phase	Procedure	Frequency	Duration
Preparatory surgery	Jugular vein and/or carotid/femoral artery cannula placement	Once	< 1.5h total surgery time
	EEG electrode placement	Once	
	ECG electrode placement	Once	
	EMG electrode placement	Once	
	Skin microdialysis guide placement	Once	
	Brain microdialysis guide placement	Once	
Exposure/ treatment	Subcutaneous injection/infusion	Once for exposure to chemical threat agent, variable (up to 3 injections per day) for interventions	Variable
	Intravenous injection/infusion		20 sec – 24 h
	Intraperitoneal injection		<30 sec
	Intramuscular injection		<30 sec
	Dermal application (percutaneous)		<30 sec
	Intraosseous injection		<5 min (under light anesthesia)
	Inhalation		Variable
	Intranasal/buccal		<30 sec
Monitoring	Blood sampling	Variable	n/a
	Microdialysis sampling	1-3	Max 8h per occurrence
	Metabolism	Once	Max 14 days
	EEG, ECG, EMG monitoring (telemetry)	1-3	Max 14 days in total
	Respiration monitoring	1-3	Max 8h per occurrence
	Behavioral observations	1-3	Max 14 days in total

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

Where possible, different modalities of data are gathered in the same animal: pharmacokinetic and pharmacodynamic data are measured simultaneously. This enables us to collect a large amount of biological data per animal, improving scientific quality and reducing the number of animals needed. Further methods that are employed to minimize animal use include testing of multiple treatments per animal instead of testing one treatment per animal. In case multiple therapeutic treatment groups are included in a study (for example: vehicle, treatment A, treatment B, and combi treatment A+B) which do not all require a toxic challenge exposure, multiple treatments can be tested in one animal, provided the total burden of the treatment allows for it, such as performed in previous studies[4]. Adequate washout of substances as well as recovery needs to be determined first. Designs in this case will be chosen such that confounding effects due to treatment combinations are equal for all treatments and are thus controlled for. While this approach introduces a layer of complexity, the total number of animals can be substantially reduced. This approach applies only to treatments with therapeutics that allow full recovery in between, animals are never exposed to chemical threat agents more than once.

All studies performed will include a power analysis, for which the critical output parameter and appropriate statistical approach for data analysis will be defined depending on the research question. Whereas for the full analysis of the experiment, multiple statistical methods could be applied to ensure proper analysis for each parameter, the major output (i.e. maximum effect achieved of a treatment) is generally used in a group-wise fashion to determine the total number of animals to be used.

[redacted]

A typical study could consist of a threat agent dose-range finding phase, a pharmacokinetic/pharmacodynamic evaluation of the experimental treatment in healthy animals and an efficacy experiment in which a treatment is provided after exposure to the toxic compound. For each phase, different approaches ensure sufficient power, while preventing overpowered studies.
Pilot studies with a small number of animals may be performed first, if information regarding variability of a specific parameter is unknown. If, for example, a new administration route such as buccal is evaluated, a pilot study may be performed to assess the general variability associated with its absorption and pharmacokinetics. Mechanistic insight obtained from these studies is then used to design studies in which toxicological/pharmacological processes can be fully characterized and accurately quantified. The number of animals selected for pilot studies are based on previous experience, advice from colleagues from other institutes and the Animal Welfare Body.
Follow-up experiments, such as pharmacokinetic/pharmacodynamic analysis of treatments in healthy animals or efficacy experiments can employ well-defined effect sizes and necessary group sizes from a priori sample size calculations based on available pilot data or previous studies. Such experiments may incorporate more complex analyses, such as dose-response studies in which drug-drug interactions are investigated by pharmacokinetic analysis or synergy by isobolographic analysis.

In a typical experiment the efficacy of a treatment may be assessed by its effect on a physiological parameter (such as respiratory minute ventilation), for which the one-hour average value is taken. A treatment may be compared to the control group at 4 doses. To demonstrate a (physiologically relevant) 30% improvement between the control and one of the treatment groups, considering a (conservative) Bonferroni correction for multiple tests, with an overall variability (SD) of 15%, a group size of 8 animals (power 0.8, alpha =0.0125, 2-sided) is needed, resulting in a total number of 40 animals. A dropout rate of 15% can be expected and as a result, 7 reserve animals are needed.

In the context of life-long learning, innovative statistical techniques and study designs will be explored in order to maximize statistical power and minimize animal use, building on experience with similar, previously executed, studies and advanced knowledge of the scientific community. An example of this is improving designs of dose-response studies such as described by Bornkamp, Bretz, Pinheiro 2009[5], in which multiple comparison and regression techniques are combined.

The response to the toxic effects of certain compounds are known to exhibit seasonal variability (notably the organophosphate class). As the dose-response may be extremely steep for these compounds, this means that unexplained variability could result in suboptimal challenge doses in the efficacy studies. In these cases, a pilot dose-response study will be executed to find the optimal challenge dose by using an Up-and-Down Procedure (UDP). The UDP is an efficient method for (re-)establishing the dose-response relationship for compounds with steep dose-response relationships with a smaller number of animals, compared to conventional LD_{50} approaches[6]. Various methods exist for implementing an UDP, such as described by Abal et al., 2017[7]. A similar approach may be used in our lab, where an experiment of 24 animals is used to accurately establish an ED_{50} of a given toxic response, which can then be used to design efficacy experiments.

[5]Bornkamp B, Pinheiro J, Bretz F. MCPMod: An R package for the design and analysis of dose finding studies. J Stat Software 2009; 29: 1-23

[6] Lichtman AH. The up-and-down method substantially reduces the number of animals required to determine antinociceptive ED50 values. J Pharmacol Toxicol Methods. 1998;40(2):81–5.

[7]Abal P, Louzao MC, Antelo A, Alvarez M, Cagide E, Vilariño N, et al. Acute Oral Tox c ty of Tetrodotoxin in Mice: Determination of Lethal Dose 50 (LD50) and No Observed Adverse Effect Level (NOAEL). Toxins (Basel). 2017 Mar;9(3):75. doi:10.3390/toxins9030075.

Estimated numbers in the 5-year project period:

Annex I	Rats	Guinea pigs
Total number of animals	1000	1500
Nr of studies	12-20	25-30
1 Non-recovery	5%	5%
2 Mild	3%	3%
3 Moderate	85%	85%
4 Severe	7%	7%

The estimated number of guinea pigs in this project proposal is higher as compared to the previous project to account for the diversification of the research scope. The estimated percentages of discomfort are updated and based on the realized percentages of discomfort of the previous project.

B. The animals

Specify the species, origin, life stages, estimated numbers, gender, genetic alterations and, if important for achieving the immediate goal, the strain.

Serial number	Species	Origin	Life stages	Number	Gender	Genetically altered	Strain
1	Rat	commercial supplier	Multiple	1000	Male / Female	No	Multiple
2	Guinea Pig	commercial supplier	Multiple	1500	Male/ Female	No	Multiple

Provide justifications for these choices

Species	The methodology that is used for species selection is described in section A.
Origin	All animals will be purchased from commercial suppliers.
Life stages	Depending on the target population for registration of new medical treatments, juvenile, adult or aged animals will be used
Number	The number of animals is based on the estimated number of studies performed under the current proposal, the number of research questions identified, staff availability, and the number of animals typically needed per study, as listed in the table above. As an example; a typical study could include a dose-finding phase, incorporating a up-and-down procedure of 24 animals, in order to find one or more suitable challenge doses. The next phase could be where the efficacy of a therapeutic is tested at 4 levels, which would (including a control group) and reserve animals result in 47 animals.
Gender	The choice of gender will be based on literature, and depending on the target population for drug use. Initial phases will most likely be performed in one gender only. From the animal model selection, it will be derived whether the response to toxic chemicals is gender specific or not. For the anticipated levels of exposure, to be investigated in the project proposal, the usually rather subtle gender related differences following exposure are not expected to be a pivotal factor, that could affect the outcome of medical countermeasures. However, to minimize possible variations, and as the discomfort induced by the experiments can be classified up to severe, initial studies will preferably be performed in one gender at a time, and in follow-up stages critical challenges and doses could be on request of regulation or scientific validation transferred to the opposite gender. Historically, males have been used more frequently. The broadening of the scope of threat agents requires more frequent development of new models. This will allow us to select females as a species of choice more often, thereby balancing the ratio of males/females in the *total* use of animals.
Genetic alterations	As our studies investigate chemical exposures, they do not need genetic alterations to recreate a clinical condition.

Strain	The choice of strain is included in the methodology for species selection, generally dependent upon past experience.

C. Accommodation and care

Is the housing and care of the animals used in experimental procedures in accordance with Annex III of the Directive 2010/63/EU?

☐ Yes

☒ No > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices

Whenever possible, housing conditions used will be in concordance with Annex III of the Directive 2010/63/EU. However, in some cases, short-term solitary housing will be used for the post-operative recovery period, which is generally 7-14 days. This is only done in cases where certain surgical areas (wounds or catheter access ports) could potentially be damaged by other animals. Housing will also be solitary during the monitoring phase of the experiment, without full cage enrichment, as physiology/video data acquisition is not possible with more than one animal per cage/chamber. The maximum duration of this phase is 2 weeks. Animals may be placed in special whole-body plethysmography chambers for a maximum of 24 hours.

D. Pain and compromised animal welfare

Will the animals experience pain during or after the procedures?

☐ No

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☐ No > Justify why pain relieving methods will not be used.

☒ Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

Surgical procedures will be carried out under species appropriate anesthesia, such as isoflurane anesthesia (4-5% induction, 1-2% maintenance), depending on surgical procedure and animal species. The anesthesia and analgesia procedures will be specified in the individual research plan.
Appropriate perioperative care will be provided, with analgesics and/ or antibiotics administered before surgery and up to 96 hours following surgery to minimize pain and improve recovery. Animals will be monitored frequently immediately after surgery until full recovery, based on voluntary movement and consciousness, then lowered to twice daily, or once at later stage (after ~1 week). See humane endpoints for observation criteria. Anesthesia/ Pain relief can generally not be applied during the toxicological/pharmacological part of the study, as this would substantially affect the responses elicited by the chemical compounds administered and cloud the experimental outcomes.

Describe which other adverse effects on the animals' welfare may be expected?

The expected adverse effects could be laboured breathing, loss of consciousness, tremor, epileptic seizures, apnoea, cardiovascular compromise, all direct results from the toxic challenge.
In the event a surgery results in major complications, this could lead to compromised recovery. Measures to be taken in such a case are described in paragraph E (humane endpoints).

Explain why these effects may emerge.

The effects described are the direct result from a toxic challenge. The observation of these effects is the aim of the study.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

If scientifically valid, the exposure to toxic chemicals will be performed under anaesthesia. Surgery will be performed using appropriate analgesia and anaesthesia, and animals will be monitored closely for adverse signs during recovery from a procedure. In general, chemical challenges will be as low as possible, without compromising the scientific aim. Furthermore, animals will be observed closely after a chemical challenge, and humane endpoints are defined. Depending on the severity, and aim, the experiments will be as short as possible to minimize the duration of discomfort (initially 4-6 hours, up to a maximum of 24 hours in this phase, depending on the aim of the model use).

E. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

☐ No > Continue with question F

☒ Yes > Describe the criteria that will be used to identify the humane endpoints.

At high doses, the challenge will be lethal within a very short time (minutes to hours), in most cases initiated by rapid loss of consciousness. Animals might be challenged to a lower but toxic dose, leading to a longer period of less severe clinical signs. In particular in those cases, a defined humane endpoint will be chosen, to minimize suffering, and additionally to obtain scientifically comparable outputs.
A humane endpoint could be a certain duration of a decline in cardiovascular parameters, or breathing. In case spontaneous death resulting from the chemical challenge can be prevented (reaching humane endpoint), the animals will be sacrificed. Furthermore a drop in heart rate under ~35% of baseline level is an indication for early termination of the experiment. Specific endpoints will be specified per individual study plan.

In earlier phases, a failure from the animals to recover from surgery (i.e. when the animal has not returned to its' initial body weight at the day of exposure after surgery) is an indication for exclusion from the experiment. A short body weight dip of around 10% after surgery is expected, but after 1-2 day(s) they typically start to gain weight and return to their pre-operative weight within a 1-5 day recovery period. Adequate recovery is assessed by means of the following scores.

Scoring for overall condition after surgery and prior to a chemical challenge:
0: No clinical signs, normal weight gain, smooth fur
1: Normal effects of surgery, normal feeding and drinking, stable weight, no weight gain
2: Slightly decreased eating and drinking, bad appearance of fur, slight weight decrease (<20%), recovering weight)
3: Bad appearance of fur, weight decrease >20%, lethargy, poor coordination, no eating and drinking
4: No eating, drinking, weight decrease >20%, progressive weight loss >2 days, Painful, hunched posture, or worse

Conditions 0-2 are expected to recover, progression to 3 could occur for a maximum of 1 day, but should return to 2 the next day. During the entire recovery process, a single occurrence of score 4, or more than two occurrences of score 3 indicates a poor recovery and a cumulative discomfort that is considered to be severe; in these cases, a humane endpoint is reached and animals will be euthanized. This list of criteria is not exhaustive: In the event that specific signs are observed that do not fall in any of the categories, this may be considered a humane endpoint, in consultation with the AWB.

Indicate the likely incidence.

In the majority of cases, at lethal and sublethal challenges, the animals will reach the humane endpoint, or lose consciousness rapidly (within minutes to hours after exposure to the toxic agent), as an endpoint of the study.

F. Classification of severity of procedures

Provide information on the experimental factors contributing to the discomfort of the animals and indicate to which category these factors are assigned ('non-recovery', 'mild', 'moderate', 'severe'). In addition, provide for each species and treatment group information on the expected levels of cumulative discomfort (in percentages).

- Non-recovery: for challenges under anaesthesia immediately after instrumentation.
- Mild: For subclinical dose exposures in which no/very limited clinical signs of exposure are seen.
- Moderate: Though instrumentation of the animals with one of the modalities as displayed in the table might not be classified as 'moderate', the combination of anaesthesia, instrumentation and recovery leads to every surgical instrumentation being classified as 'moderate' discomfort. Different combinations of instrumentation are possible, but the total discomfort of the surgery with recovery will not reach 'severe' discomfort in order to guarantee that animals can sufficiently recover from the surgery within 1-2 weeks before the start of the experiment. For studies in which the effects of therapeutics are studied in healthy animals, administration may occur on a maximum of three days, with three injections per day. Each of the administrations separately will not be associated with more than 'mild' discomfort, in such a manner that the cumulative discomfort does not exceed 'moderate'. For short-term, acute challenges, during which loss of consciousness occurs within ~60 minutes after onset of moderate clinical signs. Within the scientific boundaries of the project, rapid death might be an outcome parameter. It is recognized that this intuitively could be classified as 'severe' discomfort. However, due to the anticipated speed of the progression of toxicity and in most cases rapid loss of consciousness in case of challenging awake animals, the resulting discomfort is rated as "moderate".
- Severe: In case of lower dose challenges, the effects arise more slowly and will be of longer duration. Such effects would be severe bronchoconstriction, or for example paralysis. This can lead to a maximum of 'severe' discomfort, when a longer duration of observation is required for the model (> 8 hours, up to 24 hours). Whenever possible, the humane endpoints will be taken into account and the duration of effects will be kept as short as possible (~4-6 hours). If humane endpoints can be applied in an early stage, the discomfort of the animals could also be classified as "moderate".

Description	Animal species/strain and sex	Number of animals	Discomfort (expected cumulative discomfort)	Discomfort is a sum of the following listed procedures:
All three components	Rat	1000	1 Non-recovery 5% 2 Mild 3% 3 Moderate 85% 4 Severe 7%	**Preparation surgery (moderate)** Jugular vein and/or carotid/femoral artery cannula placement EEG electrode placement ECG electrode placement EMG electrode placement Skin microdialysis probe placement Brain microdialysis guide placement **Exposure / treatment** subcutaneous injection/infusion (mild) Intravenous injection/infusion (mild) Intraperitioneal injection (mild) intramuscular injection (mild) dermal application (percutaneous) (mild) Intraosseus injection (mild) Inhalation (mild) Intranasal/buccal (mild) Subclinical dose exposure (mild) Sublethal dose exposure (moderate / severe) Lethal dose exposure (moderate / severe) Anesthetized model (non-recovery) **Monitoring** Blood sampling (mild) Microdialysis sampling (mild) Metabolism (mild) EEG, ECG, EMG monitoring (telemetry) (mild)

				Respiration monitoring (mild) Behavioral observations (mild) Euthanasia
All three components	Guinea pig	1500	1 Non-recovery 5% Mild 3% 3 Moderate 85% 4 Severe 7%	**As above**
	Total:	2500	1 Non-recovery 5% Mild 3% 3 Moderate 85% 4 Severe 7%	**As Above**

G. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Replacement	In the context of replacement, strict selection criteria are used to determine if *in vivo* research is warranted, including prior *in vitro* research on the mechanism of toxicity, drug target and therapeutic mechanism (see section 3.4 and figure 1 of the project proposal). Knowledge on these topics could be derived either from scientific literature, studies performed by other labs or inhouse. The research described here will be follow-up research, from for example *in vitro* target identification. Signs of toxicity *in vivo*, are in most cases the result of cascade interactions in a body. For example, nerve agent poisoning highly selectively inhibits acetylcholinesterase, but the subsequent signs of toxicity arise from eventual recruitment of glutamatergic systems. Cyanide selectively interferes with intracellular oxygen metabolism, but this results in a number of changes in clinical chemistry, and cardiovascular compromise. As certain chemicals are highly reactive, the bonding to a key physiological target can be extremely strong (covalent binding), which requires an extremely targeted, highly reactive drug action. This is extremely challenging and in a number of cases chemically impossible (this requires *in vitro* research in critical organ cells for example, or just protein targets). It can be possible however, to intervene with the cascade effects, and let the body resolve the intoxication, by regeneration of targets for example. To achieve that, artificial respiration, or certain anesthetic regimens may be employed. Such cascade interactions cannot be replaced by *in vitro* approaches. In general, for drug approval, phase III (Clinical efficacy) trials are necessary to indicate a medical intervention for a specific condition. As patient populations are not available for exposure to highly toxic chemicals, this phase is replaced by the testing in a qualified animal model under quality standards, which are currently not available. ADME/PK/TK studies are obligatory for registration of a test substance or in support of clinical trials, or for use in Phase III or replacement. If the guideline suggests to use alternatives that do not involve animals, then these types of tests will be used for registration purposes. However, in recent guidelines, no authority approved-alternatives for ADME/PK/TK studies exist. For Drug approval for the general population in the USA, FDA approval is required. In Europe, EMA regulates approval of new drugs.
Reduction	The number of animals used is reduced by well-designed studies (e.g. choice of dose, choice of dosing route, choice of animal, etc.). With regard to development of challenge models of highly toxic compounds to test efficacy of medical countermeasures, initial research stages will have involved *in vitro*, *ex vivo* and *in vivo* studies. In the proposed project, animals could for example be equipped with telemetric devices, in order to obtain a range of precise and adequate readout parameters. Known baseline variations from experience or other studies of critical parameters and possibly the variation in response to a chemical challenge will allow statistical power analysis to calculate the number of animals for a dose range finding experiment. Furthermore, the research

	strategy, up- and down dosing, described under A(2) allows the reduction of the number of animals per group. This will be based on sequential analysis, meaning that in case exposure of additional animals in a group will not lead to significant improvement or decline (the difference already being too big), the group will be terminated.
Refinement	Monitoring and blood sampling techniques are chosen to be minimally invasive. Based on recent microsurgical insights, optimal perioperative care will be used, which will be explained in more detail in the next section. For each study, application of species appropriate anesthesia and sedatives as refinement for a chemical challenge will be considered if they do not affect the toxicology of the chemical threat agent under study.

Are adverse environmental effects expected? Explain what measures will be taken to minimise these effects.

☒ No

☐ Yes > Describe the environmental effects and explain what measures will be taken to minimise these effects.

H. Re-use

Will animals be used that have already been used in other animal procedures ?

☐ No > Continue with question I.

☒ Yes > Explain why re-use is considered acceptable for this animal procedure.

In case multiple therapeutic treatment groups are included in a study (for example: vehicle, treatment A, treatment B, and combi treatment A+B) which do not all require a toxic challenge exposure, a maximum of three treatments can be tested in one animal, provided the total burden of the treatments prior to the final experimental day is mild. In case the discomfort of a treatment is higher than mild, the animal will be excluded from re-use and will be euthanized at the end of the experimental day. This approach applies only to treatments with therapeutics that allow full recovery in between and animals are never exposed to chemical threat agents more than once. As full recovery between repeated experimental days within one animal is essential, extra discomfort due to re-use will be limited. For each study with planned re-use, cumulative discomfort as a consequence of re-use will be discussed with the animal welfare body.

Are the previous or proposed animal procedures classified as 'severe'?

☒ No

☐ Yes > Provide specific justifications for the re-use of these animals during the procedures.

I. Repetition

Explain for legally required animal procedures what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, describe why duplication is required.

Review of the available scientific literature will be part of the establishment of research questions. Our lab is also involved in various international collaborations, in order to align interests and avoid repetition. For some studies, repetition is specifically requested by regulatory authorities to further approval of new treatments (for example to proof efficacy in two small animal models).

J. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

☒ No > Continue with question K.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

End of experiment

K. Destination of the animals

Will the animals be killed during or after the procedures?

☐ No > Provide information on the destination of the animals.

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☐ No > Describe the method of killing that will be used and provide justifications for this choice.

Tissues are required for analysis of organ toxicity, or for example amounts of chemical agent in the tissues. This will be at a fixed time point following challenge, or after reaching the humane endpoints.

☒ Yes > Will a method of killing be used for which specific requirements apply?

☒ No > Describe the method of killing.

Euthanasia in all cases is preferably performed using an overdose of pentobarbital (>100 mg/kg), administered iv, if the animal is equipped with a venous/arterial catheter, or ip if not.

☐ Yes > Describe the method of killing that will be used and provide justifications for this choice.

If animals are killed for non-scientific reasons, justify why it is not feasible to rehome the animals.