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, see our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

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 serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 3.4.4.3 Type of animal procedure: Determination of treatment efficacy of drugs against the effects of exposure to highly toxic chemicals

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.

The aim of this part of the project is to determine the efficacy of treatment (medical countermeasures) in qualified models for exposure to highly toxic chemicals (existing models or models as developed in appendix 3.4.1.1). In this phase, dose range finding and possible side effects of medical pharmacological interventions will be characterized in animals challenged with highly toxic chemicals. Critical physiological parameters and/ or pharmacokinetics will be determined to estimate best practices for intervention in a field situation.
Medical countermeasures to be tested could be well know from literature, or already in use for other applications. In other cases, basic pharmacokinetic and/ or pharmacodynamic parameters will have been determined within the framework of appendix 3.4.4.2. This information will be used to address issues such as dose setting, route of administration, bioavailability, tissue disposition, target organs and efficacy. Based on this information, the experimental studies described in this appendix will be prepared.
The animal model, will be based on the (expected) human pathophysiology following exposure to the chemical agent (route, dose, timing; depending on the expected exposure levels of the public in a certain setting) as described in appendix 3.4.4.1.
An example of an outcome of a study under appendix 3.4.4.1 is a trigger to treat (objective change in the read out parameters) at a certain time point after exposure. This means that the time point of subsequent intervention can be objectively determined, in line with feasibility for the modelled intervention, but also in line with the expected level of toxic challenge and thus intensity of and latency to certain signs.
A time frame to mimic the time for first responders to arrive and perform triage(looking for triggers to

treat) would be longer than 20 minutes after the challenge, but within 60 minutes (if no signs are present, immediate intervention is of less importance). In such a case, the aim is to find a sublethal dose leading to apparent clinical signs (heart rate or blood pressure change, breathing rate, epileptic seizures, etc.), within that time frame. In case of readily available countermeasures, such as during military operations (for nerve agent poisoning so-called autoinjectors are for example available, similar to self-administering epinephrin injections for anaphylactic shock), an appropriate time frame could be much shorter , and be within 1-2 minutes after challenge or occurrence of signs. In both cases, the aim is to have a model available that allows an increase in the time to death, or progression of poisoning by administration of medical countermeasures in follow-up phases. An alternate aim can be to further elucidate mechanisms of toxicity, to aid scientific development of new medical interventions, or to find biomarkers of exposure and/ or effect.
So, briefly: Initially the time to intervention will be determined from an available model from stage 3.4.4.1, following the same challenge procedure. The intervention, for example characterized in appendix 3.4.4.2, will be crossed with the outcome of 3.4.4.1 in this stage. Similar readout parameters will be established.

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

Depending on the outcome of studies under appendices 3.4.4.1 and 3.4.4.2 and/or based on literature, the animals will be challenged with a highly toxic chemical (a model based on literature or as described in appendix 3.4.4.1) and at a certain time point after challenge or after the onset of toxic effects (trigger to treat) the animals will be treated with a countermeasure. The pharmacokinetic profile of the treatment substance is known from experiments in healthy animals (based on literature and/or see appendix 3.4.4.2). For these studies animals will undergo the following three steps:

1. Preparation:

Similar to the preparation described in appendix 3.4.4.1. All surgical procedures will be carried out under species- and procedure appropriate anaesthesia. Details will be specified in the individual study plan. Appropriate peri-operative care will be provided (such as analgesia). Animals will be surgically equipped with one or more of the following:
Jugular vein and/ or carotid artery cannula; EEG electrode; ECG Electrode; Implantation of osmotic minipump or transmitter; Skin microdialysis probe; Brain microdialysis guide. For details, see appendices 3.4.4.1 and 3.4.4.2

2. Challenge Phase:

In this phase the animals, will be challenged with a toxic chemical inducing a specific range of clinical signs as determined in appendix 3.4.4.1. Generally, gentle fixation by hand is used to restrain the animals. Depending on the research question, animals could be exposed under anesthesia. Animals can be exposed according to the one of the following routes: **subcutaneous or** i**ntravenous** injection, **percutaneous** exposure or **inhalatory.**

3. Countermeasure:

In this phase the animals will be treated with a medical countermeasure and a range of readout parameters as established in the model will be measured. At a certain time point, a set of clinical or physiological signs, or a fixed time after challenge, a medical pharmacological intervention will be administered to address therapeutic efficacy, via the **intravenous, intramuscular**, intraperitoneal, **intraosseus** (see appendix 3.4.4.2 for extra information on intraosseus administration), **topical** (for example decontamination) or **inhalatory** route. Dosing and administration routes will be based on the anticipated route of exposure in the human situation.

4. Monitoring (up to ~48 hours)

Directly after challenge, the effect of the countermeasure on (a selection of) the following parameters will be established: **Blood sampling** *(toxicokinetics, pharmacokinetics);* **Microdialysis sampling; Metabolism; EEG, ECG monitoring (Telemetry); Respiration monitoring**

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

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

A typical study design is shown below, accounting for a dose –effect relationship. All animals will be subject to the same challenge and readouts, and 3 different doses are employed. The number of animals will be based on a statistical power analysis. The power analysis will be based on critical outcome parameters, which have to be significantly and biologically relevantly improved. This could be an onset time of clinical signs, effects on respiration, duration of seizures, or cardiovascular effects. The effect of a treatment should improve towards baseline values. The variation of the parameters will be derived from the challenge model (appendix 3.4.4.1), or historical data, allowing appropriate calculation of the required number of animals, corrected for multiple comparisons. Additionally, variability in the response to a treatment on top of the challenge may be larger, and is estimated based on expected efficacy. In general, the variability in the positive and negative control groups, is lower than in treatment groups, hence requiring less animals. In the table below, it would for example be the question to derive the lowest dose that is still maximally effective; or to derive a dose response effect. Depending on the mechanism of action of the medical countermeasure, a dose response effect could be expected, for example when receptor action is involved (e.g. an anticonvulsant). On the other hand, a countermeasure could causally reverse the toxic action and culminate the toxic cascade (for example a reactivator (oxime) reversing organophosphate-induced cholinesterase inhibition. This knowledge will be important to assess the appropriate experimental design and accordingly the required number of animals.

GROUP	Challenge (fixed dose)	Treatment	Number
1	Challenge agent	Vehicle	6
2	Challenge agent	Low	8
3	Challenge agent	Mid	8
4	Challenge agent	High	8
5	Saline/ Vehicle	Vehicle	6

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Based on the research question the animal model as described in appendix 3.4.4.1 will be used, ranging from small animals (mice, rats, guinea pigs), to larger animals (minipigs). For foundation: see appendix 3.4.4.1.
Depending on the target population for the treatment, juvenile, adult or aged animals will be used. The choice of gender will be based on literature, but also in line with the sex to be used in the efficacy phase. Initial phases will most likely be performed in one gender only, in line with results obtained in studies performed in the framework of appendices 3.4.4.1 and 3.4.4.2, taking into account whether the response to pharmaceutical is gender specific or not. However, to minimize possible variations, initial studies will preferably be performed in one gender at a time, and in follow up stages critical challenges and doses might be transferred to the opposite sex. (as described in appendix 1).
Estimated numbers in 5-year project period:

Annex III	Mice	Rats	Guinea pigs	Minipigs
Total estimated 5-year	90	400	300	120
Number of studies	2-3	8-10	6-8	3-4
1 Non recovery	60%	10%	10%	20%
2 Mild	0%	0%	0%	0%
3 Moderate	40%	40%	40%	30%
4 Severe	0%	50%	50%	50%

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

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

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.

D. 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.

<u>Replacement</u>

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 extremely targeted, highly reactive drug action. To achieve such high reactivity towards a specific complex 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 *in vitro*.
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 for drug approval. A range of animal models for mechanistic research is available.

<u>Reduction</u>

The number of animals used to establish if a compound is suited for the intended use can be 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 the variation in response to a chemical challenge will allow statistical power analysis to calculate the number of animals for an efficacy experiment. Furthermore, the research strategy, allows reduction of the number of animals per group. Due to the complexity of the experiments, only limited numbers of animals can be in experiment per day, allowing the analysis of results in between, possibly leading to a decision to terminate a group. This will be based on sequential analysis, meaning that if it is sure that with the additional number of animals to be tested in a group will not lead to significant improvement or decline (the difference already being too big), the group will be terminated.

Refinement
see below

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

Pre-challenge surgery will always be performed under anaesthesia and appropriate peri-operative care including analgesia. Animals are housed according to EU regulations and provided appropriate cage space, bedding material and species specific environmental enrichment. Whenever scientifically sound, the animals will be kept under anaesthesia during the entire procedure. Furthermore, research designs will be optimized to obtain as much information as (technically) possible, taking into account possible increasing discomfort. Obtaining physiological data by telemetry requires one surgery, but after that less handling and discomfort is required. Similarly, placement of a cannula induces discomfort once, but does prevent repeated skin puncturing or restraint.
If necessary, for any animal showing signs of discomfort, measures will be taken to relieve this discomfort and, if necessary, treatment of the animals will be altered. If an animal shows signs of severe discomfort (Humane endpoints), they will be humanely killed after consultation of the animal technicians, responsible researcher and/ or a designated veterinarian. In cases where (time to) death is a read out parameter, the duration of such signs will be kept as short as possible (~4 hours, in most studies), or in selected cases up to 24 hours. Survivors will be euthanized at a predefined time point within this time frame.
Studies will be performed in contained facilities specifically equipped for handling highly toxic chemicals.

Repetition and duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

Not applicable

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

☐ No

☒ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

Before any intervention, animals will be housed according to the guidelines, or also whenever possible.
In cases in which animals are instrumented by surgery, they will be housed individually to prevent damage to cannulas or electrodes.
In case of individual housing, social interaction is limitedly possible, due to the use of open cages which allow sniffing and reaching out to neighboring cages. Appropriate cage enrichment will be available for long term housing, such as wooden chewing blocks, paper rolls, play houses, shelters etc., always taking

into account that the animals cannot hurt themselves.
Some experimental housing situations (e.g. metabolic cages) warrant individual housing without bedding or enrichment. Periods of housing in these situations are kept as short as possible (max ~36 hrs)

G. 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 H.

☐ Yes > Describe this establishment.

Not applicable

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

Not applicable

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

☒ 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 anaesthesia, such as isoflurane (4-5% induction, 1-2% maintenance) anesthesia, depending on surgical procedure and animal species. Specific details will be determined in consultation with the Animal Welfare Body.
Appropriate peri-operative care will be provided with analgesics and if necessary antibiotics up to 48 hours. 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.

I. Other aspects compromising the welfare of the animals

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. Treatment should minimize such effects, however, it might not, and additionally positive control groups are included that will not be treated.
In the unlikely event a surgery would be not successful, this could lead to compromised recovery, measures to be taken in such a case are described in paragraph (J).

Explain why these effects may emerge.

The effects described are the direct result from a toxic challenge. The observation of these effects, and efficacy of medical intervention, 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 24 -36 hours in this phase, depending on the aim of the model use).

J. 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 K.

☒ 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, or a less effective pharmaceutical dose, leading to a longer period of lower 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 end point 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 End Point), the animals will be sacrificed.
In earlier phases, a failure from the animals to recover from surgery, i.e. has not returned to initial body weight at the day of exposure after surgery might be an indication for exclusion from the experiment. A normal short body weight dip of around 10% can occur, but normally the animals rapidly return to their pre-operative weight within a 1-5 day recovery period.
Furthermore a drop in heart rate under ~35% of baseline level is an indication for early termination of the experiment. Specific end points will be specified per individual study plan, and discussed with the Animal Welfare Board.

Scoring for overall condition after surgery and chemical challenge:
0: Good (no clinical signs, normal weight gain, smooth fur)
1: Adequate (normal effects of low invasive surgery, normal feeding and drinking, stable weight, no weight gain)
2: Average (average signs of low invasive surgery, slightly decreased eating and drinking, bad appearance of fur, slight weight decrease (<20%), recovering weight)
3: Poor (bad appearance of fur, weight decrease <30%, lethargy, poor coordination, no eating and drinking)
4: Bad (no eating, drinking, weight decrease >30%, progressive weight loss >2 days (surgery phase), 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 (in case of surgery). Progression to score 4, or score 3 for 2 days (in case of surgery) indicates a very bad recovery, and will indicate removal of the animal of the study by euthanasia as indicated, in consultation with the Animal Welfare Body.

Indicate the likely incidence.

Without treatment, at lethal and sublethal challenges, the animals will reach the humane end point, or lose consciousness rapidly, as an endpoint of the study in the majority of cases. However, in this phase a certain degree of efficacy is expected from the medical countermeasures to be tested, which should allow the animals to reach a predefined endpoint in time without substantial clinical morbidity. In this phase, ~50% of the animals is expected to reach the HEP as the end of the experiment instead of a predefined endpoint in time.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

	Annex III	Species				Discomfort			
	Procedure	Mice	Rats	Guinea pigs	Minpigs	1 Non recovery	2 Mild	3 Moderate	4 Severe
Preparation	Cannulation		x	x	x			x	
	Butterfly access				x		x		
	EEG electrode placement		x	x	x			x	
	ECG electrode placement		x	x	x			x	
	Skin microdialysis probe placement		x	x	x	x		x+	
	Brain Microdialysis guide placement		x	x				x	
	Implantation of osmotic minipump	x	x	x			x		
Challenge	subcutaneous injection	x	x	x			x		
	intraperitioneal injection	x	x	x			x		
	intravenous injection	x	x	x	x		x		
	intramuscular injection		x	x	x		x		
	dermal application (percutaneous)		x	x	x		x		
	Inhalation	x	x	x	x		x		
Treatment	subcutaneous injection	x	x	x			x		
	intravenous injection	x	x	x	x		x		
	intramuscular injection		x	x	x		x		
	Intraosseus	x	x	x	x			x	
	intraperitioneal	x	x	x			x		
Monitoring	Anesthetized model		x	x	x	x			
	Blood sampling	x	x	x	x		x		
	Telemetry	x	x	x	x		x		
	Subclinical	x	x	x	x		x		
	Sublethal	x	x	x	x			x	x *
	Lethal	x	x	x	x			x	x *
	Euthanasia	x	x	x	x	x			

Non-recovery: for challenge and treatment under anaesthesia immediately after instrumentation
moderate : for short term acute challenges, with no or ineffective treatment where loss of consciousness appears within ~60 minutes after onset of clinical signs.
+: In certain cases, skin microdialysis is conducted in freely moving animals (and not in anesthetized

non-recovery animals); in such cases the estimated discomfort is "moderate".
*: Within the scientific boundaries of the project, rapid death might be an outcome parameter in a positive control group. It is recognized that this intuitively could be classified as "severe" discomfort. However, the anticipated speed of the progression of toxicity, and in most cases rapid loss of consciousness in case of challenging awake animals, eventual discomfort could be rated as "moderate". In case of lower dose challenges, or a (partially) effective medical countermeasure, the effects arise more slowly, or decline and will be of lower severity. This can lead to a maximum of 'severe' discomfort, when a longer duration of observation is required for the model (> 8 hours). However, in such a case the HEP will be taken into account, and the duration of effects will be kept as short as possible (hours). In that case, the discomfort of the animals could also be classified as "moderate".

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

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

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 HEP.

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.

☒ Yes