	Inventaris Wob-verzoek W16-19S								
		wordt verstrekt				**weigeringsgronden**			
nr.	**document**	**reeds openbaar**	**niet**	**geheel**	**deels**	**10.1.c**	**10.2.e**	**10.2.g**	**11.1**
	NTS2016503								
1	Aanvraagformulier				x		x	x	
2	Projectvoorstel				x			x	
3	Niet-technische samenvatting	x							
4	Bijlage beschrijving dierproeven 1 oud				x			x	
5	Bijlage beschrijving dierproeven 2 oud				x			x	
6	Bijlage beschrijving dierproeven 3 oud			x					
7	DEC-advies			x					
8	Ontvangstbevestiging				x		x	x	
9	Verzoek aanvullende informatie				x		x	x	
10	Reactie verzoek aanvulling				x		x	x	
11	Bijlage beschrijving dierproeven 1 herzien				x			x	
12	Bijlage beschrijving dierproeven 2 herzien				x			x	
13	Bijlage beschrijving dierproeven 3 herzien			x					
14	Advies CCD		x						x
15	Beschikking en vergunning				x		x	x	

Centrale Commissie Dierproeven

07 APR. 2016

AVD 1080020165O3

Aanvraag
Projectvergunning Dierproeven
Administratieve gegevens

- U bent van plan om één of meerdere dierproeven uit te voeren.
- Met dit formulier vraagt u een vergunning aan voor het project dat u wilt uitvoeren. Of u geeft aan wat u in het vergunde project wilt wijzigen.
- Meer informatie over de voorwaarden vindt u op de website www.centralecommissiedierproeven.nl. of in de toelichting op de website.
- Of bel met 0900-2800028 (10 ct/min).

1 Gegevens aanvrager

1.1 Heeft u een deelnemernummer van de NVWA?
Neem voor meer informatie over het verkrijgen van een deelnemernummer contact op met de NVWA.

☒ Ja > *Vul uw deelnemernummer in* 10800
☐ Nee > *U kunt geen aanvraag doen*

1.2 Vul de gegevens in van de instellingsvergunninghouder die de projectvergunning aanvraagt.

Naam instelling of organisatie	Universiteit Utrecht
Naam van de portefeuillehouder of diens gemachtigde	[redacted]
KvK-nummer	30275924

1.3 Vul de gegevens van het postadres in.
Alle correspondentie van de CCD gaat naar de portefeuillehouder of diens gemachtigde en de verantwoordelijke onderzoeker.

Straat en huisnummer	Instantie voor Dierenwelzijn Utrecht
Postbus	12007
Postcode en plaats	3501AA Utrecht
IBAN	NL27INGB0000425267
Tenaamstelling van het rekeningnummer	Universiteit Utrecht

1.4 Vul de gegevens in van de verantwoordelijke onderzoeker.

(Titel) Naam en voorletters	[redacted] ☒ Dhr. ☐ Mw.
Functie	[redacted]
Afdeling	[redacted]
Telefoonnummer	[redacted]
E-mailadres	[redacted]

1.5 *(Optioneel)* Vul hier de gegevens in van de plaatsvervangende verantwoordelijke onderzoeker.

(Titel) Naam en voorletters	[redacted] ☐ Dhr. ☒ Mw.
Functie	Onderzoeksmedewerker
Afdeling	[redacted]
Telefoonnummer	[redacted]
E-mailadres	[redacted]

1.6	*(Optioneel)* Vul hier de gegevens in van de persoon die er verantwoordelijk voor is dat de uitvoering van het project in overeenstemming is met de projectvergunning.	(Titel) Naam en voorletters	[redacted] ☒ Dhr. ☐ Mw.
		Functie	[redacted]
		Afdeling	[redacted]
		Telefoonnummer	[redacted]
		E-mailadres	[redacted]
1.7	Is er voor deze projectaanvraag een gemachtigde?	☐ Ja > *Stuur dan het ingevulde formulier Melding Machtiging mee met deze aanvraag*	
		☒ Nee	

2 Over uw aanvraag

2.1	Wat voor aanvraag doet u?	☒ Nieuwe aanvraag > Ga verder met vraag 3
		☐ Wijziging op (verleende) vergunning die negatieve gevolgen kan hebben voor het dierenwelzijn Vul uw vergunde projectnummer in en ga verder met vraag 2.2
		☐ Melding op (verleende) vergunning die geen negatieve gevolgen kan hebben voor het dierenwelzijn Vul uw vergunde projectnummer in en ga verder met vraag 2.3
2.2	Is dit een *wijziging* voor een project of dierproef waar al een vergunning voor verleend is?	☐ Ja > Beantwoord dan in het projectplan en de niet-technische samenvatting alleen de vragen waarop de wijziging betrekking heeft en onderteken het aanvraagformulier
		☐ Nee > Ga verder met vraag 3
2.3	Is dit een *melding* voor een project of dierproef waar al een vergunning voor is verleend?	☐ Nee > Ga verder met vraag 3
		☐ Ja > Geef hier onder een toelichting en ga verder met vraag 6

3 Over uw project

3.1	Wat is de geplande start- en einddatum van het project?	Startdatum	1 - 5 - 2016
		Einddatum	1 - 9 - 2020
3.2	Wat is de titel van het project?	Mechanism-based Integrated Systems for the Prediction of Drug Induced Liver Injury	
3.3	Wat is de titel van de niet-technische samenvatting?	Mechanismen van leverschade door medicijngebruik	
3.4	Wat is de naam van de Dierexperimentencommissie (DEC) aan wie de instellingsvergunninghouder doorgaans haar projecten ter toetsing voorlegt?	Naam DEC	DEC Utrecht
		Postadres	Postbus 85500 3508 GA Utrecht
		E-mailadres	dec-utrecht@umcutrecht.nl

4 Betaalgegevens

4.1 Om welk type aanvraag gaat het?

☒ Nieuwe aanvraag Projectvergunning € 1441,- Lege

☐ Wijziging € Lege

4.2 Op welke wijze wilt u dit bedrag aan de CCD voldoen.
Bij een eenmalige incasso geeft u toestemming aan de CCD om eenmalig het bij 4.1 genoemde bedrag af te schrijven van het bij 1.2 opgegeven rekeningnummer.

☐ Via een eenmalige incasso

☒ Na ontvangst van de factuur

5 Checklist bijlagen

5.1 Welke bijlagen stuurt u mee?

Verplicht

☒ Projectvoorstel

☒ Niet-technische samenvatting

Overige bijlagen, indien van toepassing

☒ Melding Machtiging

☐

6 Ondertekening

6.1 Print het formulier uit, onderteken het en stuur het inclusief bijlagen via de beveiligde e-mailverbinding naar de CCD of per post naar:

Centrale Commissie
Dierproeven
Postbus 20401
2500 EK Den Haag

Ondertekening door de instellingsvergunninghouder of gemachtigde (zie 1.7). De ondergetekende verklaart:

- dat het projectvoorstel is afgestemd met de Instantie voor Dierenwelzijn.
- dat de personen die verantwoordelijk zijn voor de opzet van het project en de dierproef, de personen die de dieren verzorgen en/of doden en de personen die de dierproeven verrichten voldoen aan de wettelijke eisen gesteld aan deskundigheid en bekwaamheid.
- dat de dieren worden gehuisvest en verzorgd op een wijze die voldoet aan de eisen die zijn opgenomen in bijlage III van richtlijn 2010/63/EU, behalve in het voorkomende geval de in onderdeel F van de bijlage bij het bij de aanvraag gevoegde projectvoorstel gemotiveerde uitzonderingen.
- dat door het ondertekenen van dit formulier de verplichting wordt aangegaan de leges te betalen voor de behandeling van de aanvraag.
- dat het formulier volledig en naar waarheid is ingevuld.

Naam [redacted]

Functie [redacted]

Plaats Utrecht

Datum 31 - 03 - 2016

Handtekening [redacted]

Form
Project proposal

- This form should be used to write the project proposal for animal procedures.
- The appendix ‘description animal procedures’ is an appendix to this form. For each type of animal procedure, a separate appendix ‘description animal procedures’ should be enclosed.
- For more information on the project proposal, see our website (www.zbo-ccd.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’.	10800
1.2	Provide the name of the licenced establishment.	Universiteit Utrecht
1.3	Provide the title of the project.	**M**echanism-based **I**ntegrated **S**ystems for the **P**rediction of **D**rug **I**nduced **L**iver **I**njury

2 Categories

2.1 Please tick each of the following boxes that applies to your project.

- ☒ Basic research
- ☒ Translational or applied research
- ☐ Regulatory use or routine production
- ☐ Research into environmental protection in the interest of human or animal health or welfare
- ☐ Research aimed at preserving the species subjected to procedures
- ☐ Higher education or training
- ☐ Forensic enquiries

☐ Maintenance of colonies of genetically altered animals not used in other animal procedures

3 General description of the project

3.1 Background

Describe the project (motivation, background and context) with respect to the categories selected in 2.

- For legally required animal procedures, indicate which statutory or regulatory requirements apply (with respect to the intended use and market authorisation).
- For routine production, describe what will be produced and for which uses.
- For higher education or training, explain why this project is part of the educational program and describe the learning targets.

Drug induced liver injury (DILI) is a dramatic consequence of drug intake that cannot yet be reliably predicted preclinically. The EU-Innovative Medicines Initiative (IMI) MIP-DILI (co-funded by EFPHIA) aims to design a predictive translational strategy, comprised of in silico, in vitro and in vivo models. The [redacted] of Utrecht University is partner in this project because it has longstanding experience with testing drugs for immunological effects (that are related to DILI) and with translation of in vivo results to in vitro test systems.
The mechanism that underlying DILI is poorly understood. DILI may occur in various forms, e.g. from cholestasis to hepatocellular cell death and combinations of both. In a number of cases, DILI is accompanied by systemic adverse effects, including skin rashes and cytopenias (e.g. thrombocytopenia, eosinophilia). These diverse effects have been shown to involve both innate as well as adaptive immune components (Kaplowitz N, *Nature Reviews Drug Discovery*, 2005). Activation of innate immune components include inflammatory cells (e.g. neutrophils, NK cells) or factors (e.g. TNF), whereas cytopenias and skin rashes likely involve adaptive immune components (specific antibodies and sensitized T cells). Processes such as metabolic activation (leading to reactive metabolites that may form drug-protein adducts), induction of cell damage (including mitochondrial stress), and immunoregulation (that control adverse immune responses) have been demonstrated to be involved in DILI and related pathophysiological outcomes.
In this project, we use established models of immune-mediated DILI to unravel hepatotoxic mechanisms, which will be help to design predictive in vitro tests. An example of an established model has been developed and partly characterized by Shaw and colleagues in 2009 using trovafloxacin (TVX), a fluoroquinolone that has been withdrawn from the market after high incidence of liver failure in patients. In this model, TNF appears pivotal in causing severe liver damage. The availability of a clear protocol to perform a mouse model of DILI by Shaw et al. and the availability of a negative homologue levofloxacin (LVX) encouraged the MIP-DILI consortium to use TVX model as a suitable model to identify mechanisms of DILI. In this model a possible role of neutrophils in the liver pathology and an increase of several cytokines and chemokines have been demonstrated (Shaw et al., *Toxicological Sciences* 2009). Yet, it is unknown: 1- how the drug (e.g. trovafloxacin) affects liver cell to become sensitive to TNF; 2- which immune cells (innate (e.g. neutrophils and NK cells), and adaptive (e.g. T cells) or regulatory) are involved and; 3- to what extend these effects translate to long-term adaptive immune responses (e.g. T cell sensitization). In the latter, break of tolerance, which is a particular property of liver immune system, may be crucial. Interestingly, recent studies demonstrate indeed that for some drugs lack of immune tolerance may be crucial in development of DILI (Metushi IG et al., *Hepatology* 2015).
During our previous in vivo/in vitro experiments we observed that TVX and other DILI-associated compounds affected dendritic cells (DCs) (maturation and homing). These findings together with emerging evidences of the role of DC in the induction of tolerance (Goubier A et al., *Immunity* 2008; Metushi IG et al., *Hepatology* 2015) and in activation of lymphocytes (Woehrle T et al., *Blood* 2010), encouraged us to investigate if TVX and other DILI-associated compounds may lead to a disruption of tolerance. This disrupted tolerance may lead to further(uncontrolled) increase of development of cytotoxic lymphocytes that damage hepatic parenchyma. For this reason, we will investigate whether modulation of tolerance mechanisms affects DILI and associated effects in case of short-term or prolonged administration of TVX and other DILI-associated compounds. In view of the analyses of the role of the adaptive immune system in

DILI and -associated effects (e.g. sensitization, changes in spleen) we will also analyse how prolonged administration of DILI-associated compound influence sensitization to well-known bystander antigens such as OVA.
In this project we will focus on all of the above aspects of DILI and related effects. After the identification of in vivo mechanisms for trovafloxacin, other compounds associated with DILI in human will be tested in order to identify potential similarities and eventually validate in vitro/in silico tools for the identification of potential hepatotoxic compounds.
The use of mice in this model is a valid tool to investigate kinetics of DILI (e.g. recruitment of cells to the site of damage) and point to the pivotal players in the induction of liver failure and associated effects.

3.2 Purpose

Describe the project's main objective and explain why this objective is achievable.

- If the project is focussed on one or more research objectives, which research questions should be addressed during this project?
- If the main objective is not a research objective, which specific need(s) does this project respond to?

The main aim of our project is to understand the mechanisms involved in DILI, and to develop an in vivo/in vitro test strategy for the proper identification of hepatotoxic compounds during pre-clinical studies.
In vivo/in vitro correlation of the mechanisms involved in DILI will be evaluated. Both [redacted] are involved in the project to focus on in vivo-in vitro translation. [redacted] has longstanding experience with the topic, both via 3 PhD projects (for instance with industry) and via a [redacted] project (on adverse drug reactions and liver). Translation of in vivo findings to in vitro approaches is an important pillar of the MIP-DILI project. For this reason, collaboration is set up with partners inside the consortium (both industrial partners (e.g. [redacted]), and academic partners (e.g. [redacted]) and outside the consortium (e.g. [redacted] UMC Utrecht).

3.3 Relevance

What is the scientific and/or social relevance of the objectives described above?

Increased knowledge of mechanisms of DILI will contribute to better prediction of DILI of compounds. In particular translational in vitro tests (designed based on animal data) will eventually reduce the number of general toxicity studies in animals and finally also the occurrence of unexpected hepatic adverse reactions to marketed drugs. Predictive *in vitro* tests may also be used earlier in the R&D phase of drug development and thus potentially save animal studies. Altogether the outcome of the project will have important economic benefits for pharmaceutical companies (prevent drug failure of marketed drugs) and of course also for society (prevention of severe liver injury).
DILI involves multiple organs as well as a complex interaction between various immune cells and molecules. This requires a holistic in vivo approach to identify crucial mechanisms.

3.4 Research strategy

3.4.1 Provide an overview of the overall design of the project (strategy).

The project will be based on a translational and integrated approach with the following components:

1- In vivo animal experiments based on single administration of a DILI-associated compound in non-immunological and immunological modified settings. These experiments will give insight into the cells that are involved in initial liver damage.

2- In vivo animal experiments based on multiple administration of a DILI-associated compound in non-immunological and immunological modified settings. These experiments will give more insight into dysfunction of immune cells (in particular related to lack of immunotolerance) in DILI.

3- Development of in vitro models of relevant aspects of DILI to identify intracellular mechanisms and confirm the interplay among several cell types, e.g. dendritic cells (DC) and hepatocytes.

3.4.2 Provide a basic outline of the different components of the project and the type(s) of animal procedures that will be performed.

1- In vivo animal models of DILI based on single administration of a DILI-associated compound in non-immunological and immunological modified settings:

the DILI animal model that we used is described by Shaw PJ et al., 2009 in *The Journal of Pharmacology and Experimental Therapeutics*. In this model, TVX pre-treatment of mice challenged with TNF leads to liver injury (TNF will be used as a proximal mediator of LPS). Mechanisms involved in the induction of this injury are still matter of debate and the contribution of various immune cells to the damage is not yet defined. Importantly, TNF appears to be pivotal in infectious diseases as well as in most inflammatory diseases. In the last decade, several animal models of immune-mediated DILI have shown that combination of DILI-associated compounds with TNF as important inflammatory stimulus results in hepatic toxicity. Identification of the potential disruption in the inflammatory response promoted by TNF and other cytokines will be investigated.

2- In vivo animal models of DILI based on multiple administration of a DILI-associated compound:

Because we observed that TVX stimulates the innate immune system of the liver and because the activation of the innate immune system may induce an adaptive immune response we are interested to investigate the role of the adaptive immune system in DILI. In addition, DILI is often associated with delayed systemic clinical effects such as skin rash and cytopenias. The general idea is that these effects are due to stimulation of the adaptive immune system, either or not combined to specific interference with immunoregulation/immunotolerance (Metushi IG et al., Hepatology 2015). In all, this may lead to sensitization of the immune system (specific T cells and specific antibodies). From the findings by Metushi IG et al, it is hypothesized that tolerance induction may prevent the clinical effects as mentioned, and hence one of the reasons that these adverse effects occur in only few patients. We here aim to investigate whether tolerance induction is also important in preventing elicitation of adaptive immune responses in other examples of DILI.

In this project, we therefore want to test prolonged repetitive administration of different DILI-associated drugs in combination with a disruption of mechanisms involved in the onset of tolerance using specific monoclonal antibodies (to CTLA-4 and PD1, see Metushi IG et al., Hepatology 2015)).

Along with DILI-associated drugs, structural homologues that do not cause DILI will be tested.

Primary outcome parameters include immunological and histopathological changes in liver and spleen and levels of (auto)antibodies.

3- Development of in vitro models of DILI for the identification of intracellular mechanisms and confirmation of interplay among several cell types:

In vitro experiments with donor cells are dependent on in vivo findings, collected in 1 and 2. From previous in vivo experiments with TVX we already know that both neutrophils and dendritic cells are affected by TVX. To examine underlying mechanisms we aim to investigate the effect on these cells in vitro. In vitro experiments will include neutrophil recruitment tests (neutrophils migration and activation, Koenderman L et al., *Thrombosis and Haemostasis* 2010) and tests to evaluate DC stimulation (e.g. DC-induced lymphocyte proliferation, Garulli B et al., *Clinical and Vaccine Immunology* 2008). In vitro tests will represent a strategic asset to investigate intra-cellular pathways involved in the relevant physiological events linked to DILI. Moreover, those tests will eventually serve as predictive tools to exclude potential hepatotoxic compounds during pre-clinical drug development. For

these reasons, other DILI-associated compounds in human (and their non-toxic pharmacological analogues) will be tested, in order to validate the developed tests.

We aim to test a range of pharmaceuticals known to cause DILI and selected by the All these compounds were carefully selected by the MIP-DILI consortium (EFPIA companies, academia) as training compounds for investigations. Selection of drugs has been done within the consortium based on: literature findings (animal studies, in vitro findings and epidemiology (human data, from post marketing surveillance), experience by industry. Structural and pharmaceutical relationships are considered. In particular, in some cases, pairs of structural homologues (positive or negative for DILI) were selected allowing validation in vitro tests. For instance trovafloxacin was selected together with levofloxacin (as negative control).
The selected drugs that may be of interest to test in vivo are: Ximelagatran (anticoagulant, withdrawn from the market because of DILI), Amiodarone (anti-arrhythmic, causes DILI possibly dependent on LPS/TNF), Troglitazone (antidiabetic, causes DILI, mechanism not known), Tolcapone (improves side effects in Parkinson, withdrawn because of DILI, mechanism unknown), Diclofenac (causes DILI in some users, possibly dependent on LPS/TNF), Trovafloxacin (fluoroquinolone, withdrawn from market, because of DILI, possibly dependent on LPS/TNF), Fialuridine (antiviral, DILI with unknown mechanism) and proper pharmacological or chemical analogues of each drug which are not associated with DILI in man (Dabigatran, Primadone, Pioglitazone, Entacapone, Levofloxacin etc.).
In this project we will start with two other fluoroquinolones, difloxacin and tosufloxacin, which may have, based on literature, similar mechanism of action (Poon I et al., *Nature* 2014). After this first round of experiments, other ones will be tested (e.g. in combination with TNF) to investigate whether they elicit similar or other mechanisms than the fluoroquinolones.

3.4.3 Describe the coherence between the different components and the different steps of the project. If applicable, describe the milestones and selection points.

The simultaneous presence of different kind of cells and their capability to be recruited/activated from different organs at the site of injury, make the in vivo approach the essential tool for the identification of the main mechanisms involved in DILI. The findings collected during in vivo experiments will be used for the development of in vitro/in silico models. Donor mice will be used to set up these in vitro experiments and to validate the possible use of cell lines to replace primary cells. We will perform acute (single exposures) to evaluate initiating mechanisms and repetitive exposures to investigate how initial changes translate to adaptive immune responses (these require at least 7 up to 20 days to develop and to show effects of tolerance).

3.4.4 List the different types of animal procedures. Use a different appendix ‘description animal procedures’ for each type of animal procedure.

Serial number	Type of animal procedure
1	Single dose models
2	Multiple dose models
3	Development of in vitro models of DILI for the identification of intracellular mechanisms and confirmation of interplay among several cell types
4	
5	

6	
7	
8	
9	
10	

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.zbo-ccd.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’.	10800
1.2	Provide the name of the licenced establishment.	Universiteit Utrecht
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: 1 — Type of animal procedure: Single dose models

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.

Single dose model:
Since it is hypothesized that inflammation is one of the factors that determines whether DILI occurs in patients, we aim to test different DILI-associated compounds under the influence of inflammatory stimuli, such as TNF. In particular, we want to characterize the role of the immune system in development of

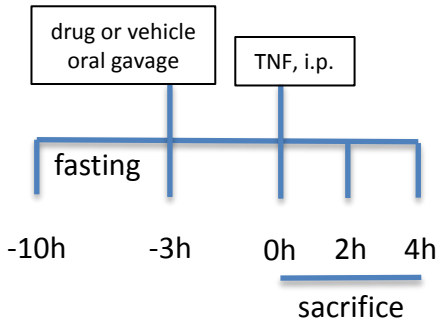
this type of DILI, thus identifying commonalities in mechanisms among different drugs. Outcome parameters will include biochemical (e.g. liver enzymes), immune and histopathological changes in various interacting organs (e.g. intestine, spleen, liver).

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

Fasting is used frequently in mouse models of DILI, in order to reduce the variance of drug availability. In addition fasting may represent an additional cellular stress that makes mice more sensitive to DILI. For this reason we will consider to introduce one group that receives TVX+TNF without fasting. This has been assessed for TVX (personal communication with consortium partners) but may differ per drug. For each drug we will evaluate the necessity of fasting in a small pilot study.

Depending on the outcome of the pilot, mice will be fasted and receive an oral gavage of:
- DILI-associated compounds
- related compounds not associated with DILI but pharmacologically or chemically resembling the DILI-associated compounds
- vehicle alone

The treatment scheme is as follows:



In the general set-up 4 groups are used:
Group 1: mice receiving Trovafloxacin (DILI-associated compound) and TNF
Group 2: mice receiving Levofloxacin (the pharmacological analogue of Trovafloxacin) and TNF
Group 3: mice receiving vehicle and TNF
Group 4: mice receiving none of the treatments
Group 5: mice receiving Trovafloxacin and TNF without fasting

In words, three hrs after the single oral administration of the drug or vehicle, mice will be injected intraperitoneally with TNF. Organs (i.e. intestine, spleen, liver and also blood) will be collected at three time points. Until now we have used the time points T=0 (so 3 hrs after dosing with TVX as the drug, but before dosing of TNF) and T=2h and T=4h (both after administration of TNF). For new drugs we will use information from literature or the consortium to define the initial dose that will then be tested for one time point only (T=4 h).

In total an experiment according to the general set-up will last 14 hrs (taking into account fasting of 7 hrs and the treatment from drug exposure to final dissections). Analyses will include i.e. flow cytometry, immunohistology, PCR, levels of particular proteins in tissues and in serum/plasma (obtained by bleeding). Flow cytometry will include both innate and adaptive immune cells. Immunohistology will include detection of localization of tissue damage (apoptosis, necrosis), influx of inflammatory cells but also localized expression of cytokines, chemokines etc. PCR will be done to detect changes in RNA expression of signalling molecules and cytokines and chemokines whereas proteins to be detected in serum include liver enzymes, cytokines and chemokines. To characterize in depth the cause of changes mechanistically we will also analyse the effects of specific inhibiting or modulating substances on the onset and regulation of DILI. We aim to use specific substances such as [redacted] Shaw PJ et al., *Toxicological Sciences* 2007), or the [redacted] (Hoque R et al., *American journal of physiology. Gastrointestinal and liver physiology* 2012)). In addition, we aim to interfere with certain immunological receptors and cells by using specific monoclonal antibodies to inhibit of deplete immune cells, [redacted] (Carr KD et al., *Immunology* 2011). The number of modulations (e.g. substances or monoclonal antibodies) depends on initial findings (e.g. changes in subsets of lymphocytes), but we estimate to include a maximum of 10 modulations per drug combination.

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

Power analysis will be performed before each experiment, based on the parameters which will be evaluated. Since damage of the liver will be our main parameter aside to immunological changes, power analysis will be based on liver enzyme levels (e.g. ALT) in blood. Based on this parameter, we have used 8 animals per group in previous experiments.

B. The animals

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

Species: For these experiments, mice will be used (e.g. C56BL/6). The choice for a mouse model is based on the similarities between the immune system of humans and mice, but also because the immune system in mice is already well characterized and several tools are available to study the mechanisms involved in DILI. The aim of the project is not to develop a new in vivo model but to define mechanistic knowledge that will help to develop in vitro models to test potential hepatotoxic compounds during R&D.
Moreover, there are several evidences in scientific literature (Lucena MI et al., Hepatology 2009) which state that sex is a determinant in the severity and predisposition to DILI. Since we do not know whether gender matters for the selected compound, we suggest to start testing female for fluoroquinolones. Depending on results (e.g. statistical variance) we will decide whether we will use both female and male mice or use the most susceptible gender in the next experiments with the fluoroquinolones. For every new drug we have to test again the gender susceptibility. [redacted]

Origin: Animals will be purchased from a registered commercial provider in EU, including the Netherlands, such as Charles River Laboratories or Harlan Laboratories.

Estimated numbers: Previously, we used 8 animals per group, based on power calculation using liver enzymes (ALT) in the serum as the parameter. In case of an experiment according the general set up (4 treatment groups, see scheme) with only one time point we therefore need 32 animals per drug. With this set up we will confirm the effective dose for DILI. Since we will test a maximum of 5 drug combinations (5 DILI and 5 matching non-DILI drugs) we will need 5 x 32 = 160 animals.
Subsequent experiments with modulating compounds will include 3 time points (to evaluate kinetics of effect) and 10 modulations (e.g. substances or monoclonal antibodies) at the most. Together, for these experiments we therefore estimate to use 3 (time points) x 10 (number of modulators) x 160 (number of animals for 5 drug combinations)= 4800 mice.
For the work described in this appendix we will need a total of 4960 mice.

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.

Replacement:
The kinetics of recruitment and the activation of immune cells to the site of damage is difficult to reproduce in vitro due to the mutual interactions of different cell types (e.g. immune cells, hepatocytes, endothelial cells) from different organs of the body (e.g. interaction between intestine, liver and spleen). To evaluate these parameters an integrated in vivo approach is needed. Mechanistic information collected from these in vivo experiments will shed light on the crucial processes involved in DILI, which is needed in order to develop in vitro tests for the identification of potential DILI-inducing new drug entities. This will add also to reduction of animals in the future. The involvement in the MIP-DILI consortium, which aims to reduce animal use for this type of toxicology, ensures the translation from in vivo to in vitro.
Reduction:
The number of animals per group will be determined by the use of power analysis on the basis of the most relevant parameter needed for the study.
The number of animals in experiment until now is based on expected effects (liver damage, particularly values of liver enzymes such as ALT in blood).
Refinements:
Mice will be housed in groups and in cages with environmental enrichment. They will be daily monitored in order to prevent any unexpected discomfort.

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

On the basis of findings (abnormal behavior and animal characteristics) and the level of discomfort (standard methods of [redacted], veterinary personnel will be consulted.

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.

NA

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.

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.

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

Classification of discomfort / humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question 1.

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

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

Pain relieving method will not be possible for 3 reasons:
-the duration of the experiment is short (based on our experience the liver injury starts to develop from 2 hrs to 4 hrs after TNF injection, at max 4 hrs animals are killed and dissected, see scheme),
-the severity of the expected adverse reaction is overall mild (in more than 75 % of animals) and moderate to severe in less than 25% of animals (depending on the drug),
-pain releiving drugs may interfere with DILI inducing drugs and inflammatory responses

☐ Yes

I. Other aspects compromising the welfare of the animals

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

No evidences are in support of potential adverse effects due to the experimental procedures.

Explain why these effects may emerge.

We do not expect any other effects.

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

Alteration of animal behaviours and evaluation of the discomfort of the animal will result in immediate consultation of the animal welfare body or the designated veterinarian

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.

Indicate the likely incidence.

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').

Mild for 75% or more of the animals, possibly moderate for maximum 25% of the animals. That is, only animals that develop liver damage after exposure to DILI-drugs may suffer from moderate to severe discomfort. The experiments will last maximally 14 hrs, but only the last 2 hrs (i.e. from 2 to 4 hrs after TNF treatment) liver injury may become severe (based on TVX results, and depending on the drug tested).

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No > Continue with Section 3: 'signatures'.

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

Excision of organs will be necessary to perform the analysis.

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

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.zbo-ccd.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’.	10800
1.2	Provide the name of the licenced establishment.	Universiteit Utrecht
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: 2 — Type of animal procedure: Multiple dose models

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.

Multiple dose models:
We here aim to investigate whether adaptive immunity and immunoregulation are important in preventing elicitation of adaptive immune responses in DILI, for instance TVX-induced DILI.

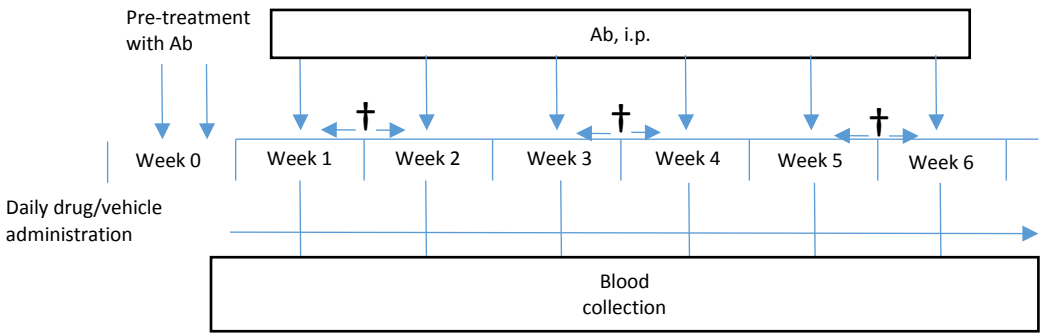
In this project, we aim to test prolonged repetitive administration of different DILI-associated drugs in combination with a disruption of mechanisms involved in the onset of tolerance using specific monoclonal antibodies [redacted]. Both [redacted] are important receptors on regulatory T cells to sustain their regulatory function. (moved to 3.4)
Importantly, innate immune responses triggered by TNF (as described in appendix 1) may contribute to adaptive immune responses. Therefore, mechanisms that are identified in single dose studies (appendix 1) will be translated to studies in this appendix, i.e. similar pharmacological modulators will be considered in multiple dose studies. Along with DILI-associated drugs, structural homologues that do not cause DILI will be tested.
Primary outcome parameters include immunological and histopathological changes in liver and spleen and levels of circulating T cells and antibodies.

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

Mice are treated with compounds via intragastric gavage once a day for six weeks. To analyse the capacity of suspected drugs to support adaptive immune responses, well-defined antigens (e.g. ovalbumin) will be co-injected during drug exposure as so-called bystander antigen. In this way, kinetics of specific immune responses can easily be analysed.
Specific monoclonal antibodies are administered i.p. before or around during exposure depending on the antibody. Injections will be done 3 times per week.
For pharmacological modulators dosing will be determined based on literature data or experience from other partners
Various mouse strains will be used, among those transgenic OT mice and KO mice [redacted]
As modulators monoclonal antibodies directed to immune receptors ([redacted]) will be used.
Blood samples will be taken several times, but not more than 8 ml/kg/14 days. Primary outcome parameters in blood are liver enzymes (e.g. ALT), cytokines and (auto-)antibodies. At 3 time points during 6 weeks treatment, mice will be dissected and various organs (e.g. liver, spleen and intestine will be collected further analyses, e.g. flow cytometry, analyses of antibody producing cells (to e.g. ovalbumin), specific antibodies, clinical parameters and gene and protein expression.
To determine the effects of modulation, vehicle controls with and without modulation will be included. DILI-associated compounds are compared to pharmacologically or chemically related compounds that are not associated with DILI.
An example of a typical experiment is outlined below.

Mice will be treated up to 6 weeks with:
1- DILI-associated compounds + no modulation
2- compounds not associated with DILI but pharmacologically or chemically resembling the DILI-associated compounds + no modulation
3- DILI-associated compounds + specific modulation
4- compounds not associated with DILI but pharmacologically or chemically resembling the DILI-associated compounds + specific modulation
5- vehicle alone + no modulation
6- vehicle alone + specific modulation
Only in groups 1 and 3, DILI or associated immune changes are expected.

Mice will be treated as follow:



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

Power analysis will be performed before each experiment, based on the parameters that will be evaluated. Since damage of the liver will be our main parameter, power analysis will be based on liver enzyme levels (ALT) in blood.

B. The animals

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

Species: For these experiments, 6-8 wk old mice will be used (e.g. C56BL/6). The choice for mouse as a model species is based on the similarities between the immune system of humans and mice, but also because the immune system in mice is well characterized and several tools are available on the market in order to study the mechanisms involved in DILI. In addition dosing regimen of various DILI-inducing drugs are known for the mouse.
The [redacted]MIP-DILI project aims to develop in vitro models in order to test potential hepatotoxic compounds and not to develop new in vivo models.
Moreover, there are several evidences in scientific literature (Lucena MI et al., Hepatology 2009) which state that sex is a determinant in the severity and predisposition to DILI. Since gender difference is not relevant for MIP-DILI (**Mechanism**-based **I**ntegrated **S**ystems for the **P**rediction of **D**rug **I**nduced **L**iver **I**njury) project we do not consider to test female and male together because this will lead to more variance in the parameters used.

Origin: Animals will be purchased from a registered commercial provider in EU, including the Netherlands, such as Charles River Laboratories or Harlan Laboratories, or in US (Jackson).

Estimated numbers: The estimated number of animals is derived from the above set up of 6 experimental groups. Based on experience and ALT levels in serum as key parameter (to perform power analyses) the required number of mice per group is 8. A maximum of 3 drug combinations will be tested in this set-up of multiple dosing. The selection of drug combinations will be based on studies done in appendix 1. Based on earlier findings and literature, we expect to do maximum 10 modulations, whereas a max of 3 time points per experiment will be analysed. In total this accounts for a maximum of: 6 (experimental groups per drug combinations) x 3 (drug combinations) x 10 (modulations) x 3 (time points) x 8 animals = 4320.

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.

Replacement:
The kinetics of recruitment and the activation of immune cells to the site of damage is difficult to reproduce in vitro due to the mutual interactions of different cell types (e.g. immune cells, hepatocytes, endothelial cells) from different organs of the body (e.g. interaction between intestine, liver and spleen). To evaluate these parameters an integrated in vivo approach is needed. Mechanistic information collected from these in vivo experiments will shed light on the crucial processes involved in DILI, which is needed in order to develop in vitro tests for the identification of potential DILI-inducing new drug entities. This will add also to reduction of animals in the future. The involvement in the [redacted]-MIP-DILI consortium, which aims to reduce animal use for this type of toxicology, ensures the translation from in vivo to in vitro.
Reduction:
The amount of animals will be limited by choosing the minimal number of control and treatment groups, necessary to achieve optimal results. The number of animals per group will be determined by the use of power analysis on the basis of the most relevant parameter needed for the study.
The number of animals in experiment until now is based on expected effects (liver damage, particularly values of liver enzymes such as ALT in blood).
Refinements:
Mice will be housed in groups and in cages with environmental enrichment. They will be daily monitored in order to prevent any unexpected discomfort.

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

Treatment will be performed by trained personnel to limit stress as a result of animal handling.

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.

NA

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.

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.

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

Classification of discomfort / humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question 1.

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

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

Pain relieving methods will not be possible as they will interfere with drug-induced effects (involving activation of innate immune system and inflammation) in the liver.

☐ Yes

I. Other aspects compromising the welfare of the animals

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

For antibiotic drugs mild diarrhoea might be observed.

Explain why these effects may emerge.

Depletion of gut flora may alter motility of the intestine leading to diarrhoea

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

Mice will be carefully monitored and in case of diarrhoea liquefied feed (weekvoer) will be supplied. Animals will be sacrificed when weight drops more than 20%

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.

If the above mentioned adverse effects will occur, animals will be sacrificed if body weight drops more than 20% over whole period of the experiment. In addition, in case severe health issues (diarhea, prolapse, dehydration, lethargy) mice will be sacrificed.

Indicate the likely incidence.

Unlikely

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’).

Moderate: max 66% of animals. In the multiple exposure study, doses will be used that by itself do not cause severe liver damage, but this damage may be enhanced by modulations. The potential damage in the liver tissue will be revealed by the blood ALT evaluation (once a week). Based on literature information, animals may suffer from the treatment and lose body weight. see J for humane endpoints.
Mild: 33% of animals not receiving a modulation treatment.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No > Continue with Section 3: ‘signatures’.

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

Excision of organs will be necessary to perform the analysis.

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

Appendix
Description animal procedures

- This appendix should be enclosed

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	10800
1.2	Provide the name of the licenced establishment.	Universiteit Utrecht
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 Type of animal procedure: Development of in vitro models of DILI for the identification of intracellular mechanisms and confirmation of interplay among several cell types

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.

We aim to develop and validate predictive in vitro tests for the identification of DILI-associated compound during preclinical drug development.
Based in mechanisms of DILI identified in vivo (experiments listed in appendixes 1 and 2), relevant in vitro tests will be explored and developed. Currently,

we are using in vitro tests to detect: -1. leukocytes recruitment (neutrophils migration and activation, Koenderman L et al., *Thrombosis and Haemostasis* 2010) ; and 2-responses to antigen stimulation (e.g. dendritic cell (DC)-induced lymphocyte proliferation, Garulli B et al., *Clinical and Vaccine Immunology* 2008); and 3- hepatocyte-DC cocultures. These three in vitro tests have been selected because the mechanistically link to in vivo findings obtained in mouse studies with TVX

Importantly, in vitro tests will represent a strategic asset to investigate intra-cellular pathways involved in the relevant physiological events linked to DILI. Those tests will serve as predictive tool to exclude potential hepatotoxic compounds during pre-clinical drug development. For these reasons, other DILI-associated compounds in human (and their non-toxic pharmacological analogues) will be tested, in order to validate the developed tests. Based on results obtained in experiments done in appendix 1 and 2, we may also set up other in vitro tests based on donor material.

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

Mice will:

1- be anesthetized in order to isolate different cell types from liver: hepatocytes and leukocytes. Anaesthetic administration is required in order to perfuse the liver with collagenase and ensure a proper yield of viable cells.
2- undergo cervical dislocation in order to isolate bone marrow and spleen: several cell cultures will be obtained using cells from those organs (e.g. bone marrow-derived DC culture, Lutz MB et al., *Journal of Immunological Methods* 1999)

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

Power analysis will be performed before each experiment, based on the parameters that will be evaluated. Depending on the mechanism investigated different parameters will be taken into account. The number of mice depends on the cell yield for each cell type.

An experiment with co-cultures of freshly isolated hepatocytes and DC with 10 compounds needs around 6480 incubations (based on three concentrations (3) x 4 (inflammatory conditions, i.e combinations of LPS and or cytokines) x 2 (incubated with or without modulator or with and without UV light) x 3 time points x 3 (triplo) x 3 (repetitions) x 10 (compounds). Per mouse we obtain 20 $x10^6$ DC, and 10^6 hepatocytes.

Example of an experimental setup:

Primary hepatocytes:
Group 1a/b: incubated with Trovafloxacin (TVX) and with/or without modulator
Group 2: incubated with Levofloxacin (LVX) and with/or without modulator
Group 3: incubated with vehicle and with/or without modulator

Monocytes and neutrophils migration will be tested using the supernatant of hepatocytes incubated as mentioned above and different time points after treatment with drug or modulating chemicals (Koenderman L et al., *Thrombosis and Haemostasis* 2010; Elliott M et al., *Nature* 2010).
For modulators see appendix 1 and 2

Per incubation $0.5x10^6$ cells are needed, so per mouse 20 incubations (based on hepatocytes) can be done. Since it is estimated that we will do 6500 incubations we will need 325 mice. In addition we need separate mice for DC isolations. These cannot be obtained from the same animals for logistic reasons (DC need to be cultured for 6-10 days to fully mature from bone marrow cells, whereas hepatocytes are cultured only for a short period). At most we will culture DC with hepatocytes in a ratio of 1 (DC) to 1 (hepatocytes). The number of DC that we obtain from one mouse is 20 x 10^6 (i.e enough for 40 incubations) which indicates that we need 160 mice. In total we need 500 mice.

Monocytes and neutrophil can be obtained from same mice so for this we do not need extra mice.

NOTE; triplo means that we will perform 3 incubations per experiment; repetitions means that we repeat the entire experiment. Triplo is to take into account the intra-experimental variation (e.g. errors in pipetting), whereas repetitions will account for extra-experimental (for instance day-to-day or donor-to-donor variation). Repetition experiments may not always be exactly the same; for instance extra controls may be added if needed.

B. The animals

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

Species: For these experiments, mice will be used (e.g. C56BL/6). The choice for a mouse model is based on the similarities between the immune system of humans and mice, but also because the immune system in mice is already well characterized and several tools are available on the market in order to study the mechanisms involved in DILI. The project aims to develop in vitro models in order to test potential hepatotoxic compounds and not to develop a new in vivo model. For this reason we would like not to consider variation in sex, age and strain which might lead to more variance in the parameter used.

Origin: Animals will be purchased from a registered commercial provider in EU, including the Netherlands, such as Charles River Laboratories or Harlan Laboratories or in US (Jackson).

Estimated numbers: The number of donor mice will be 500.

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.

Replacement:
It is still not clear if available cell lines (hepatocytes, DC etc) are resembling the behaviour of primary cells. To evaluate these parameters an integrated approach is needed. For this reason, we will compare results obtained from primary cells with the use of available cell lines (such as hepatocytes and macrophage cell-lines (which are NOT DC)). Evidences collected from these experiments will shed light on the mechanisms involved in DILI, needed to develop in vitro tests for the identification of potential DILI-inducing new drug entities. Experiments will also provide information as the applicability of cell-lines for specific questions.
Reduction:

The number of animals will be determined, based on the cell yield, and an estimation of the conditions to be tested. The test conditions are selected such that we will obtain optimum results with a minimal number of conditions.

Refinements:
Mice will be housed in groups, in cages with environmental enrichment and they will be periodically monitored in order to prevent any unexpected discomfort.

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

Experienced personnel will treat the mice, to prevent unnecessary discomfort due to animal handling. As a limited number of mice is needed to perform the studies, solitary housing can occur when for instance only one animal is needed for isolation of cells. Optimal planning will reduce this risk.

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.

nvt

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.

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.

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

Classification of discomfort / humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question 1.

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

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

☒ Yes

I. Other aspects compromising the welfare of the animals

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

No adverse effects are expected due to absence of treatment.

Explain why these effects may emerge.

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

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.

Indicate the likely incidence.

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').

mild

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No > Continue with Section 3: 'signatures'.

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

Excision of the organs is needed.

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

A. Algemene gegevens over de procedure

1. Aanvraagnummer : 2015.II.814.043
2. Titel van het project : Mechanism-based Integrated Systems for the Prediction of Drug Induced Liver Injury
3. Titel van de NTS : Hoe leverschade ontstaat door medicijngebruik.

4. Type aanvraag:
   ☒ nieuwe aanvraag projectvergunning
   ☐ wijziging van vergunning met nummer :

5. Contactgegevens DEC

Naam DEC	: DEC Utrecht
Telefoonnummer contactpersoon	: 088 – 75 59 247
Emailadres contactpersoon	: dec-utrecht@umcutrecht.nl

6. Adviestraject (data dd-mm-jjjj):
   ontvangen door DEC: 20-11-2015
   ☐ aanvraag compleet:
   ☒ in vergadering besproken: 02-12-2015 en 02-03-2016
   ☒ anderszins behandeld: per mail: 15-12-2015, 04-01-2016, 29-01-2016 en 03-02-2016
   ☒ termijnonderbreking(en) van / tot : 09-12-2015 tot 14-12-2015
   18-12-2015 tot 04-01-2016
   13-01-2016 tot 29-01-2016
   ☐ besluit van CCD tot verlenging van de totale adviestermijn met max. 15 werkdagen:
   ☐ aanpassing aanvraag:
   ☒ advies aan CCD: 22-03-2016

7. Eventueel horen van aanvrager
   - Datum: 02-03-2016
   - Plaats: Utrecht
   - Aantal aanwezige DEC-leden: 4
   - Aanwezige (namens) aanvrager: Groepsleider (PI)
   - Strekking van de vragen:
   - De DEC zou graag antwoord willen hebben op de vragen 1) waarom de onderzoekers zeven geneesmiddelen willen gebruiken en 2) wat de argumentatie is rondom de te gebruiken sekse.
   - Strekking van de antwoorden:
   - De onderzoekers lichtten toe dat ze deze geneesmiddelen hebben gekozen, omdat deze allemaal interessant zijn en allemaal gerelateerd zijn aan DILI en omdat het consortium dat onderzoek naar DILI doet een lijst heeft opgesteld van modelstoffen waarop deze

geneesmiddelen voorkomen. De onderzoekers weten niet of ze alle zeven geneesmiddelen nodig hebben, maar ze zijn bang dat ze, wanneer ze er slechts twee aanvragen bijvoorbeeld, er toch meer nodig blijken te hebben en dan opnieuw een aanvraag moeten indienen. De DEC stelt voor om te beginnen met een lager aantal en dan een go/no go-moment in te bouwen, waarbij er meerdere geneesmiddelen gebruikt kunnen worden, mocht dit nodig zijn. Verder lichten de onderzoekers toe dat ze geen goed argument hebben om slechts één sekse te gebruiken, omdat ze niet weten of er verschil zal zijn in het effect dat afhankelijk is van het geslacht. Het valt te verwachten dat er op dit punt verschillen zijn tussen mannelijke en vrouwelijke dieren en mogelijk is dat ook afhankelijk van de stof (bij de ene stof wel en bij de andere niet). De onderzoekers hebben momenteel geen data met betrekking tot vrouwelijke dieren. Deze twee punten zouden een argument kunnen zijn om te beginnen met vrouwelijke dieren.

- Het horen van de aanvrager heeft geleid tot aanpassing van de aanvraag: Ja

8. Correspondentie met de aanvrager
   - Datum: 09-12-2015
   - Strekking van de vragen:

   <u>Niet Technische Samenvatting</u>
   - 3.2, Opbrengsten project en wetenschappelijke en/of maatschappelijke belang: De DEC verzoekt u te noemen dat u uiteindelijk streeft naar een ex vivo-model. Graag aanpassen.
   - 3.5, Indeling dierproeven naar de verwachte ernst: De DEC verzoekt u 'maar kortdurend' aan de zin toe te voegen. Graag aanpassen.

   <u>Projectvoorstel</u>
   - 3.1, achtergrond: Op de 6e regel van onderen staat '*in vitro/in vitro*'. Graag aanpassen.
   - 3.3, belang: Het woord 'safe' moet vervangen worden door 'save', graag aanpassen.
   - 3.4, onderzoeksstrategie, 3.4.1, punt 3: De DEC verzoekt u in de zin 'to identify of' het woord 'of' weg te laten. Graag aanpassen.
   - 3.4, onderzoeksstrategie, 3.4.2: De DEC verzoekt u duidelijk aan te geven waarom u hebt gekozen voor de door u genoemde geneesmiddelen. Tevens ziet de DEC graag een uitleg voor het gebruik van TNF (in plaats van bijvoorbeeld IgA). Graag toelichten.

   <u>Bijlage 1</u>
   - A. Experimentele aanpak en primaire uitkomstparameters: De DEC verzoekt u om u te beperken tot het design van het experiment. De overige informatie graag verplaatsen naar 3.4. Deze opmerking geldt ook voor bijlage 2.
   - A. Experimentele aanpak en primaire uitkomstparameters: Gezien het feit dat u het belang van vasten onderzoekt, suggereert de DEC u een extra groep op te nemen van dieren die niet vasten. Indien van toepassing, aanpassen.

- B. De dieren: Graag noemen en toelichten wat het geslacht, de leeftijd en de stam zijn van de door u te gebruiken dieren. Daarbij merkt de DEC op dat de toepassing bedoeld is voor mannen en vrouwen en dus in het onderzoek volgens de DEC ook beide geslachten meegenomen zouden moeten worden. Deze opmerking geldt ook voor de andere bijlagen.
- B. De dieren: U noemt hier vijf stoffencombinaties, terwijl u er eerder zeven noemt. De DEC verzoekt u dit toe te lichten. Tevens spreekt u over modulations. De DEC vraagt zich af wat deze precies inhouden.
- K. Classificatie van ongerief: De DEC verzoekt u het ongerief bij de 25% aan te passen naar moderate.

Bijlage 2
- J. Humane eindpunten: De DEC vraagt zich af over welke periode u dit gewichtsverlies bepaalt. Graag toelichten.

Bijlage 3
- A. Experimentele aanpak en primaire uitkomstparameters, pagina 2: U noemt drie punten en spreekt vervolgens over 'these two'. Graag toelichten en aanpassen.
- D. Vervanging, vermindering en verfijning, pagina 4, bovenaan: U hebt een zin in het Nederlands geschreven. Graag aanpassen.

- Datum antwoord: 18-12-2015
- Strekking van de antwoorden:
  Verwerkt in de aanvraag.

- Datum: 13-01-2016
- Strekking van de vragen:
  Projectvoorstel
- 3.4.2 De bedoeling van de vraag van de DEC was dat u per stof zou aangeven wat de wetenschappelijke gronden zijn om die stof te gebruiken, en waarom het nodig is om ze alle 7 te gebruiken? (Het aantal geneesmiddelen bepaalt immers hoeveel dieren er nodig zijn).

Bijlage 1
- Niet beantwoord is de vraag welk geslacht en welke leeftijd (Geldt voor alle bijlagen). Er wordt niet aangegeven hoeveel groter de variatie zou zijn als beide geslachten gebruikt zouden worden en wat daarvan de gevolgen voor de aantallen zijn.

Bijlage 2
- De DEC raadt u aan om bij de humane eindpunten onder J te vermelden dat het gaat om een gewichtsverlies van 20% gedurende de gehele proef.
- J: De DEC raadt u aan om de tekst zorgvuldiger te formuleren.

- Datum: 29-01-2016
- Strekking van de antwoorden:
  Verwerkt in de aanvraag.

9. Eventuele adviezen door experts (niet lid van de DEC)
   - Aard expertise:
   - Deskundigheid expert:
   - Datum verzoek:
   - Strekking van het verzoek:
   - Datum expert advies:
   - Expert advies:

B. Beoordeling (adviesvraag en behandeling)

1. Het project is vergunningplichtig (dierproeven in de zin der wet).
2. De aanvraag betreft een nieuwe aanvraag.
3. De DEC is competent om hierover te adviseren.
4. Er zijn geen DEC-leden betrokken bij het betreffende project.

C. Beoordeling (inhoud):

1. Het project is:
   X uit wetenschappelijk oogpunt verantwoord.
   ☐ uit onderwijskundig oogpunt verantwoord.
   ☐ uit het oogpunt van productiedoeleinden verantwoord.
   X Niet wettelijk vereist.

2. De in de aanvraag aangekruiste doelcategorie is in overeenstemming met de hoofddoelstelling.

3. De DEC onderschrijft, op grond van onderstaande argumenten, het belang van de doelstelling. Het belang wordt ingeschat als substantieel.

   Veel geneesmiddelen zijn schadelijk voor de lever. **D**rug **I**nduced **L**iver **I**njury (**DILI**) is nu een van de belangrijkste oorzaken van leveraandoeningen in de Westerse wereld, niet zelden leidend tot de noodzaak van levertransplantatie. Voorspellen welke geneesmiddelen toxisch zullen zijn voor de lever is echter tot nu toe zeer moeilijk en de problemen worden in veel gevallen pas duidelijk als de drug al op de markt is.
   Het EU-programma **M**echanism-Based **I**ntegrated Systems for the **P**rediction of DILI (**MPI-DILI**) is gericht op de ontwikkeling van nieuwe testmethoden die het mogelijk zullen maken levertoxiciteit veel vroeger in het ontwikkelingsproces van geneesmiddelen te detecteren waardoor leverbeschadiging bij medicijngebruikers voorkomen kan worden. Als leverbeschadiging te verwachten is zal het kostbare ontwikkelingsproces veelal worden afgebroken.

Het EU-programma is gericht op een voorspellende, translationele strategie die bestaat uit *in silico-*, *in vitro-* en *in vivo*-modellen. Van de *in silico-* en *in vitro*-modellen wordt een aanzienlijke besparing van proefdieren verwacht.
Samengevat: voorkomen van leverschade door medicijngebruik, beperking van proefdiergebruik en kostenbesparend in medicijnontwikkeling.

4. Er worden verschillende oorzaken, of combinaties daarvan, verondersteld voor de pathofysiologie van DILI. Het kan gaan om metabolische activatie, waarbij reactieve metabolieten ontstaan die interactie met de drug kunnen aangaan; er kan celschade worden aangericht, inclusief mitochondriale stress, en de immunoregulatie kan worden verstoord met als gevolg een verstoorde immuunresponse en/of immuuntolerantie.
   Dit project richt zich op immuungerelateerde DILI. Er wordt daarbij gebruik gemaakt van gevalideerde modellen voor immuungemedieerde DILI om hepatotoxische mechanismen te ontrafelen en deze kennis te gebruiken bij de ontwikkeling van *in vitro* tests. Zo'n model is het TVX (trovafloxacin) model, waarin TNF (tumor necrosis factor) een centrale rol speelt. Het is tot dusverre echter onbekend hoe de drug levercellen (over)gevoelig maakt voor TNF, welke immuuncellen betrokken zijn, en in hoeverre op lange termijn de immuunresponse en immuuntolerantie beïnvloed worden. Door de effecten te vergelijken met die van levoflaxine (LVX), een negatieve homoloog m.b.t. DILI wordt getracht de mechanismen achter DILI te doorgronden. Dit moet dan de basis vormen voor de ontwikkeling van een *in vivo-in vitro* strategie.
   Onder 3.4.1 en 3.4.2 wordt een duidelijke onderzoeksstrategie uiteen gezet. Volgens een 3-stappenplan zal een aantal geneesmiddelen getest worden waarvan bekend is dat ze DILI veroorzaken (en die daarom veelal van de markt gehaald zijn); de effecten en mechanismen zullen vergeleken worden met die van analoga die geen DILI induceren. Gestart zal worden met een tweetal TVX-analoga (beide ook fluoroquinolones). Daarna zullen andere geneesmiddelen getest worden om verschillen en overeenkomsten in DILI-mechanismen bloot te leggen.
   Deze onderzoeksstrategie wekt bij de DEC het vertrouwen dat de beoogde resultaten binnen de gestelde termijn bereikt kunnen worden.

5. Er is sprake van de volgende bijzonderheden op het gebied van categorieën van dieren, omstandigheden of behandeling van de dieren:
   Bedreigde diersoort(en) (10e lid 4)
   ☐ Niet-menselijke primaten (10e)
   ☐ Dieren in/uit het wild (10f)
   Gefokt voor dierproeven (11)
   ☐ Zwerfdieren (10h)
   ☐ Hergebruik (1e lid 2)
   ☐ Huisvesting en verzorging
   ☐ Locatie: instelling vergunninghouder (10g)

In bijlage 1, onder B: De Dieren, wordt aandacht besteed aan het geslacht van de proefdieren. Op voorhand is niet te zeggen of mannetjes, dan wel vrouwtjes de voorkeur verdienen bij het testen van een bepaalde stof. Wel staat echter vast dat sekseverschillen bestaan in de mate van ernst en predispositie voor DILI. Afgaande op de resultaten zal steeds beslist worden welk geslacht het meest gevoelig is voor een bepaalde stof. Met dat geslacht zullen dan de volgende stappen in het onderzoek uitgevoerd worden. Deze afweging zal voor iedere nieuwe stof gemaakt moeten worden. Op grond van deze overwegingen zullen geen gemengde proefgroepen worden ingezet.

6. Het ongerief als gevolg van de dierproeven is realistisch ingeschat: 80% van de dieren ondervindt gering ongerief vooral als gevolg van de toediening van geneesmiddelen (zonder effect), en 20% matig ongerief, met name in die gevallen waarin de toegediende geneesmiddelen leverschade veroorzaken. Het gaat ~~steeds~~ in veel gevallen om kortdurende experimenten waarbij verwacht mag worden dat de dieren slechts enkele uren aan het eind van het experiment ongerief door leverschade zullen ondervinden. In de langdurige experimenten (Bijlage 2) worden humane eindpunten gehanteerd die in principe zouden moeten voorkomen dat de dieren ernstig ongerief ondervinden.

7. Er zijn geen methoden die de voorgestelde dierproeven geheel of gedeeltelijk zouden kunnen vervangen.
   Het onderzoek heeft echter wel tot doel dierproeven uiteindelijk te vervangen door *in vitro* protocollen.

8. In het project wordt optimaal tegemoet gekomen aan de vereiste van de vermindering van dierproeven.
   Daar waar variabelen in de uitleesparameters bekend zijn is een poweranalyse toegepast en zijn de juiste statistische methoden van toepassing. Voor de *in vitro* experimenten wordt het aantal dieren bepaald door de celopbrengst en het aantal condities dat in de test wordt opgenomen. Het maximale aantal te gebruiken dieren is realistisch ingeschat.

9. Het project is in overeenstemming met de vereiste van de verfijning van dierproeven in die zin dat standaard huisvesting met kooiverrijking wordt toegepast. Dat in een aantal gevallen leverschade kan ontstaan is onlosmakelijk verbonden met de aard van de experimenten. Het project is echter zo opgezet dat de proeven zo min mogelijk ongerief zullen veroorzaken.
   Er is geen sprake van een negatief milieueffect.

10. De niet-technische samenvatting is een evenwichtige weergave van het project en begrijpelijk geformuleerd.

D. Ethische afweging

Op basis van de overwegingen in deel C komt de DEC-Utrecht tot de volgende afweging over de ethische toelaatbaarheid van het project.
Het aantal dieren dat gebruikt zal worden, alsmede het geringe ongerief dat 80% van de dieren zal ondergaan en het matige ongerief voor de overige 20% zijn in verhouding tot de te verwachte baten van het project: het voorkomen van leverschade door medicijngebruik; het vervangen van dierproeven door *in vitro* methoden met het doel in ontwikkeling zijnde geneesmiddelen op DILI-activiteit te kunnen testen; en de ontwikkeling van geneesmiddelen die DILI veroorzaken tijdig te kunnen stoppen en zeker voor ze de patiënt bereiken.
Deze kosten en baten tegen elkaar afwegend acht de DEC-Utrecht dit project ethisch toelaatbaar.

E. Advies

1. Advies aan de CCD

   ☐ De DEC adviseert de vergunning niet te verlenen vanwege:

   ☐ De DEC adviseert de vergunning te verlenen onder de volgende voorwaarden

   X De DEC-Utrecht adviseert de vergunning te verlenen.

2. Het uitgebrachte advies is gebaseerd op consensus.

Dierexperimentencommissie Utrecht

8

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

Universiteit Utrecht
[redacted]
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3501 AA UTRECHT

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centralecommissiedierproeven.nl
0900 28 000 28 (10 ct/min)
info@zbo-ccd.nl

Onze referentie
Aanvraagnummer
AVD108002016503

Bijlagen
2

Datum 1 april 2016
Betreft Ontvangstbevestiging aanvraag projectvergunning Dierproeven

Geachte [redacted]

Wij hebben uw aanvraag voor een projectvergunning dierproeven ontvangen op 1 april 2016.
Het aanvraagnummer dat wij aan deze aanvraag hebben toegekend is AVD108002016503. Gebruik dit nummer wanneer u contact met de CCD opneemt.

Wacht met de uitvoering van uw project
Als wij nog informatie van u nodig hebben dan ontvangt u daarover bericht. Uw aanvraag is in ieder geval niet compleet als de leges niet zijn bijgeschreven op de rekening van de CCD. U ontvangt binnen veertig werkdagen een beslissing op uw aanvraag. Als wij nog informatie van u nodig hebben, wordt deze termijn opgeschort. In geval van een complexe aanvraag kan deze termijn met maximaal vijftien werkdagen verlengd worden. U krijgt bericht als de beslisperiode van uw aanvraag vanwege complexiteit wordt verlengd. Als u goedkeuring krijgt op uw aanvraag, kunt u daarna beginnen met het project.

Factuur
Bijgaand treft u de factuur aan voor de betaling van de leges. Wij verzoeken u de leges zo spoedig mogelijk te voldoen, zodat we uw aanvraag in behandeling kunnen nemen. Is uw betaling niet binnen dertig dagen ontvangen, dan kan uw aanvraag buiten behandeling worden gesteld. Dit betekent dat uw aanvraag niet beoordeeld wordt en u uw project niet mag starten.

Meer informatie
Heeft u vragen, kijk dan op www.centralecommissiedierproeven.nl. Of neem telefonisch contact met ons op: 0900 28 000 28 (10 ct/minuut).

Met vriendelijke groet,

Centrale Commissie Dierproeven

Deze brief is automatisch aangemaakt en daarom niet ondertekend.

Bijlagen:
- Gegevens aanvraagformulier
- Factuur

Gegevens aanvrager

Uw gegevens	
Deelnemersnummer NVWA:	10800
Naam instelling of organisatie:	Universiteit Utrecht
Naam portefeuillehouder of diens gemachtigde:	[redacted]
KvK-nummer:	30275924
Postbus:	12007
Postcode en plaats:	3501 AA UTRECHT
IBAN:	NL27INGB0000425267
Tenaamstelling van het rekeningnummer:	Universiteit Utrecht

Gegevens verantwoordelijke onderzoeker	
Naam:	[redacted]
Functie:	[redacted]
Afdeling:	[redacted]
Telefoonnummer:	[redacted]
E-mailadres:	[redacted]

Gegevens plaatsvervangende verantwoordelijke onderzoeker

Naam:	[redacted]
Functie:	Onderzoeksmedewerker
Afdeling:	[redacted]
Telefoonnummer:	[redacted]
E-mailadres:	[redacted]

Gegevens verantwoordelijke uitvoering proces

Naam:	[redacted]
Functie:	[redacted]
Afdeling:	[redacted]
Telefoonnummer:	[redacted]
E-mailadres:	[redacted]

Over uw aanvraag

Wat voor aanvraag doet u?	[x] Nieuwe aanvraag [ ] Wijziging op een (verleende) vergunning die negatieve gevolgen kan hebben voor het dierenwelzijn [ ] Melding op (verleende) vergunning die geen negatieve gevolgen kan hebben voor het dierenwelzijn

Over uw project

Geplande startdatum:	1 mei 2016
Geplande einddatum:	1 april 2020
Titel project:	Mechanism-based Integrated Systems for the Prediction of Drug Induced Liver Injury
Titel niet-technische samenvatting:	Mechanismen van leverschade door medicijngebruik
Naam DEC:	DEC Utrecht
Postadres DEC:	Postbus 85500 3508 GA Utrecht
E-mailadres DEC:	dec-utrecht@umcutrecht.nl

Betaalgegevens

De leges bedragen:	€ 1.441,-
De leges voldoet u:	na ontvangst van de factuur

Checklist bijlagen

Verplichte bijlagen:	[x] Projectvoorstel [x] Beschrijving Dierproeven [x] Niet-technische samenvatting
Overige bijlagen:	[x] DEC-advies

Ondertekening

Naam:	[redacted]
Functie:	[redacted]
Plaats:	Utrecht
Datum:	31 maart 2016

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

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3508 TA UTRECHT

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info@zbo-ccd.nl

Onze referentie
Aanvraagnummer
AVD108002016503

Bijlagen
2

Datum 1 april 2016
Betreft Factuur aanvraag projectvergunning Dierproeven

Factuur
Factuurdatum: 1 april 2016
Vervaldatum: 1 mei 2016
Factuurnummer: 16700503
Ordernummer: CB.841910.3.01.011

Omschrijving	**Bedrag**
Betaling leges projectvergunning dierproeven Betreft aanvraag AVD108002016503	€ 1.441,00

Wij verzoeken u het totaalbedrag vóór de gestelde vervaldatum over te maken op rekening NL41RBOS 056.999.6317 onder vermelding van het factuurnummer en aanvraagnummer, ten name van Centrale Commissie Dierproeven, Postbus 93144, 2509 AC te 's Gravenhage.

9

[redacted]

Van: Info-zbo
Verzonden: dinsdag 10 mei 2016 15:16
Aan: [redacted]
CC: [redacted]
Onderwerp: AVD108002016503: aanvullende informatie

Geachte [redacted]

Zojuist hebben wij u een e-mail gestuurd met daarin de verkeerde titel en het verkeerde aanvraagnummer ([redacted]).
Onze excuus daarvoor. U kunt deze e-mail verwijderen.
Onderstaand vindt u de correcte informatie.

Wij hebben een aanvraag van u in behandeling getiteld 'Mechanism-based Integrated Systems for the Prediction of Drug Induced Liver Injury' met aanvraagnummer AVD108002016503.

Wij hebben nog twee vragen over deze aanvraag.

De ongeriefsclassificatie in bijlage 3.4.4.1 lijkt niet correct. U geeft aan dat een deel van de dieren matig tot ernstig ongerief zullen ondergaan. Het ernstig ongerief komt in de ongeriefsclassificatie echter niet naar voren (75% licht, 25% matig).
U wordt verzocht aan te geven welk percentage dieren ernstig ongerief zullen ondergaan. Kunt u, indien er in bijlage 3.4.4.1 sprake is van ernstig ongerief, aangeven of dit voor bijlage 3.4.4.2. ook het geval is.

Ook de ongeriefsclassificatie in bijlage 3.4.4.3. lijkt niet correct. Op basis van de in de aanvraag verstrekte informatie zou dit terminaal moeten zijn i.p.v. licht: Levercellen worden geïsoleerd terwijl de dieren onder narcose zijn. Daarna worden de dieren gedood en wordt het beenmerg en de milt geïsoleerd. Er lijken geen voorafgaande handelingen te zijn. Kunt u bevestigen dat dit correct is. Indien de dieren wel handelingen ondergaan voor zij onder narcose worden gebracht, wordt u verzocht deze te vermelden.

Opsturen informatie
U heeft 14 dagen de tijd om te antwoorden. De CCD wil uw aanvraag echter graag in haar eerstvolgende vergadering bespreken. Wij zouden daarom de antwoorden graag uiterlijk donderdag 12 mei 2016 ontvangen.

Wanneer een beslissing
De beslistermijn op uw aanvraag wordt opgeschort tot het moment dat bovengenoemde informatie is ontvangen. Na ontvangst van uw reactie/de ontbrekende informatie nemen wij uw aanvraag verder in behandeling. Als u goedkeuring krijgt op uw aanvraag, kunt u daarna beginnen met het project.

Met vriendelijke groet,

[redacted]

Centrale Commissie Dierproeven **www.centralecommissiedierproeven.nl**

.....
Postbus 20401 | 2500 EK | Den Haag
.....
T: 0900 2800028
E: info@zbo-ccd.nl (Let op: nieuw e-mail adres)

10

[redacted]

Van: [redacted]

Verzonden: vrijdag 13 mei 2016 9:27
Aan: 'Info-zbo'
Onderwerp: RE: AVD108002016503: Aanvullende informatie

Categorieën: Dossier: [redacted]

Geachte CCD,

3.4.4.1. Acute ernstige leverschade leidt niet binnen 4 uur tot ernstig ongerief. De schade kan in die korte tijd ernstig zijn, maar het ongerief niet.
3.4.4.2: Mocht er in bijlage 2 ernstig ongerief zijn, dan betreft het waarschijnlijk een humaan eindpunt en zal het ernstige ongerief hoogstens kortdurend zijn.
3.4.4.3: Als er zonder voorafgaande handeling eerst onder anesthesie levercellen worden geïsoleerd en de dieren daarna onder dezelfde anesthesie worden gedood, dan is de categorie terminaal van toepassing. Als het dier eerst onder anesthesie gedood wordt en de levercellen na de dood worden geïsoleerd, dan is het ongerief licht. Voor de DEC maakt dat voor de ethische afweging niets uit. In beide gevallen bestaat het ongerief er uit dat het dier onder anesthesie wordt gebracht.

Met vriendelijke groeten,

[redacted]

UMC Utrecht

[redacted] Postbus 85500 | 3508 GA UTRECHT
[redacted] www.umcutrecht.nl

Van: Info-zbo [mailto:info@zbo-ccd.nl]
Verzonden: dinsdag 10 mei 2016 15:17
Aan: dec-utrecht
Onderwerp: AVD108002016503: Aanvullende informatie

Geachte DEC,

Wij hebben een aanvraag van u in behandeling waarover u ons van advies heeft voorzien. Het gaat om het project getiteld' Mechanism-based Integrated Systems for the Prediction of Drug Induced Liver Injury' met aanvraagnummer AVD108002016503.

Wij hebben de aanvrager zojuist nog een aantal vragen gesteld over deze aanvraag.

De ongeriefsclassificatie in bijlage 3.4.4.1 lijkt niet correct. U geeft aan dat een deel van de dieren matig tot ernstig ongerief zullen ondergaan. Het ernstig ongerief komt in de ongeriefsclassificatie echter niet naar voren (75% licht, 25% matig).
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Mocht u ons over deze onderwerpen nog aanvullend willen adviseren, zouden wij graag uiterlijk donderdag 12 mei uw aanvullend advies willen ontvangen.

Met vriendelijke groet,

[redacted]

Centrale Commissie Dierproeven **www.centralecommissiedierproeven.nl**

.....
Postbus 20401 | 2500 EK | Den Haag
.....
T: 0900 2800028
E: info@zbo-ccd.nl (Let op: nieuw e-mail adres)

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.zbo-ccd.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’.	10800
1.2	Provide the name of the licenced establishment.	Universiteit Utrecht
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: 1 — Type of animal procedure: Single dose models

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.

Single dose model:
Since it is hypothesized that inflammation is one of the factors that determines whether DILI occurs in patients, we aim to test different DILI-associated compounds under the influence of inflammatory stimuli, such as TNF. In particular, we want to characterize the role of the immune system in development of

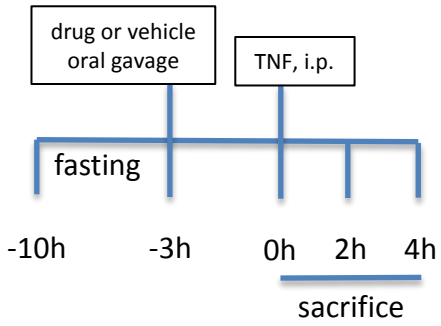
this type of DILI, thus identifying commonalities in mechanisms among different drugs. Outcome parameters will include biochemical (e.g. liver enzymes), immune and histopathological changes in various interacting organs (e.g. intestine, spleen, liver).

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

Fasting is used frequently in mouse models of DILI, in order to reduce the variance of drug availability. In addition fasting may represent an additional cellular stress that makes mice more sensitive to DILI. For this reason we will consider to introduce one group that receives TVX+TNF without fasting. This has been assessed for TVX (personal communication with consortium partners) but may differ per drug. For each drug we will evaluate the necessity of fasting in a small pilot study.

Depending on the outcome of the pilot, mice will be fasted and receive an oral gavage of:
- DILI-associated compounds
- related compounds not associated with DILI but pharmacologically or chemically resembling the DILI-associated compounds
- vehicle alone

The treatment scheme is as follows:



In the general set-up 4 groups are used:
Group 1: mice receiving Trovafloxacin (DILI-associated compound) and TNF
Group 2: mice receiving Levofloxacin (the pharmacological analogue of Trovafloxacin) and TNF
Group 3: mice receiving vehicle and TNF
Group 4: mice receiving none of the treatments
Group 5: mice receiving Trovafloxacin and TNF without fasting

In words, three hrs after the single oral administration of the drug or vehicle, mice will be injected intraperitoneally with TNF. Organs (i.e. intestine, spleen, liver and also blood) will be collected at three time points. Until now we have used the time points T=0 (so 3 hrs after dosing with TVX as the drug, but before dosing of TNF) and T=2h and T=4h (both after administration of TNF). For new drugs we will use information from literature or the consortium to define the initial dose that will then be tested for one time point only (T=4 h).

In total an experiment according to the general set-up will last 14 hrs (taking into account fasting of 7 hrs and the treatment from drug exposure to final dissections). Analyses will include i.e. flow cytometry, immunohistology, PCR, levels of particular proteins in tissues and in serum/plasma (obtained by bleeding). Flow cytometry will include both innate and adaptive immune cells. Immunohistology will include detection of localization of tissue damage (apoptosis, necrosis), influx of inflammatory cells but also localized expression of cytokines, chemokines etc. PCR will be done to detect changes in RNA expression of signalling molecules and cytokines and chemokines whereas proteins to be detected in serum include liver enzymes, cytokines and chemokines. To characterize in depth the cause of changes mechanistically we will also analyse the effects of specific inhibiting or modulating substances on the onset and regulation of DILI. We aim to use specific substances such as [redacted] In addition, we aim to interfere with certain immunological receptors and cells by using specific monoclonal antibodies to inhibit of deplete immune cells, [redacted] The number of modulations (e.g. substances or monoclonal antibodies) depends on initial findings (e.g. changes in subsets of lymphocytes), but we estimate to include a maximum of 10 modulations per drug combination.

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

Power analysis will be performed before each experiment, based on the parameters which will be evaluated. Since damage of the liver will be our main parameter aside to immunological changes, power analysis will be based on liver enzyme levels (e.g. ALT) in blood. Based on this parameter, we have used 8 animals per group in previous experiments.

B. The animals

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

Species: For these experiments, mice will be used (e.g. C56BL/6). The choice for a mouse model is based on the similarities between the immune system of humans and mice, but also because the immune system in mice is already well characterized and several tools are available to study the mechanisms involved in DILI. The aim of the project is not to develop a new in vivo model but to define mechanistic knowledge that will help to develop in vitro models to test potential hepatotoxic compounds during R&D.
Moreover, there are several evidences in scientific literature (Lucena MI et al., Hepatology 2009) which state that sex is a determinant in the severity and predisposition to DILI. Since we do not know whether gender matters for the selected compound, we suggest to start testing female for fluoroquinolones. Depending on results (e.g. statistical variance) we will decide whether we will use both female and male mice or use the most susceptible gender in the next experiments with the fluoroquinolones. For every new drug we have to test again the gender susceptibility. [redacted]
MIP-DILI project (**Mechanism**-based **I**ntegrated **S**ystems for the **P**rediction of **D**rug **I**nduced **L**iver **I**njury).

Origin: Animals will be purchased from a registered commercial provider in EU, including the Netherlands, such as Charles River Laboratories or Harlan Laboratories.

Estimated numbers: Previously, we used 8 animals per group, based on power calculation using liver enzymes (ALT) in the serum as the parameter. In case of an experiment according the general set up (4 treatment groups, see scheme) with only one time point we therefore need 32 animals per drug. With this set up we will confirm the effective dose for DILI. Since we will test a maximum of 5 drug combinations (5 DILI and 5 matching non-DILI drugs) we will need 5 x 32 = 160 animals.
Subsequent experiments with modulating compounds will include 3 time points (to evaluate kinetics of effect) and 10 modulations (e.g. substances or monoclonal antibodies) at the most. Together, for these experiments we therefore estimate to use 3 (time points) x 10 (number of modulators) x 160 (number of animals for 5 drug combinations)= 4800 mice.
For the work described in this appendix we will need a total of 4960 mice.

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.

Replacement:
The kinetics of recruitment and the activation of immune cells to the site of damage is difficult to reproduce in vitro due to the mutual interactions of different cell types (e.g. immune cells, hepatocytes, endothelial cells) from different organs of the body (e.g. interaction between intestine, liver and spleen). To evaluate these parameters an integrated in vivo approach is needed. Mechanistic information collected from these in vivo experiments will shed light on the crucial processes involved in DILI, which is needed in order to develop in vitro tests for the identification of potential DILI-inducing new drug entities. This will add also to reduction of animals in the future. The involvement in the MIP-DILI consortium, which aims to reduce animal use for this type of toxicology, ensures the translation from in vivo to in vitro.
Reduction:
The number of animals per group will be determined by the use of power analysis on the basis of the most relevant parameter needed for the study.
The number of animals in experiment until now is based on expected effects (liver damage, particularly values of liver enzymes such as ALT in blood).
Refinements:
Mice will be housed in groups and in cages with environmental enrichment. They will be daily monitored in order to prevent any unexpected discomfort.

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

On the basis of findings (abnormal behavior and animal characteristics) and the level of discomfort (standard methods of [redacted], veterinary personnel will be consulted.

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.

NA

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.

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.

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

Classification of discomfort / humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question 1.

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

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

Pain relieving method will not be possible for 3 reasons:
-the duration of the experiment is short (based on our experience the liver injury starts to develop from 2 hrs to 4 hrs after TNF injection, at max 4 hrs animals are killed and dissected, see scheme),
-the severity of the expected adverse reaction is overall mild (in more than 89 % of animals) and possibly severe in less than 11% of animals (depending on the drug and time of cytokine exposure),
-pain releiving drugs may interfere with DILI inducing drugs and inflammatory responses

☐ Yes

I. Other aspects compromising the welfare of the animals

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

No evidences are in support of potential adverse effects due to the experimental procedures.

Explain why these effects may emerge.

We do not expect any other effects.

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

Alteration of animal behaviours and evaluation of the discomfort of the animal will result in immediate consultation of the animal welfare body or the designated veterinarian

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.

Indicate the likely incidence.

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').

Mild for 89% or more of the animals, possibly severe for 11% of the animals. That is, only animals that receive the DILI-associated compound are expected to develop severe damage after exposure to the drugs; and only between 2 and 4 hrs after the injection of the cytokine (1/3 of the animals receive the potentially toxic compound, and of these only 1/3, will last until 4 hrs). The experiments will last maximally 14 hrs, but only the last 2 hrs (i.e. from 2 to 4 hrs after TNF treatment) liver injury may become severe, based on TVX results, and depending on the drug tested.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No > Continue with Section 3: 'signatures'.

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

Excision of organs will be necessary to perform the analysis.

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

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.zbo-ccd.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’.	10800
1.2	Provide the name of the licenced establishment.	Universiteit Utrecht
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: 2 — Type of animal procedure: Multiple dose models

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.

Multiple dose models:
We here aim to investigate whether adaptive immunity and immunoregulation are important in preventing elicitation of adaptive immune responses in DILI, for instance TVX-induced DILI.

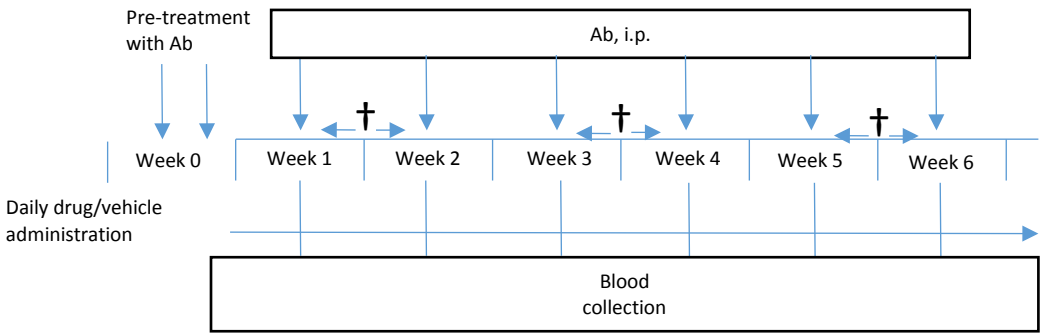
In this project, we aim to test prolonged repetitive administration of different DILI-associated drugs in combination with a disruption of mechanisms involved in the onset of tolerance using specific monoclonal antibodies [redacted] are important receptors on regulatory T cells to sustain their regulatory function. (moved to 3.4)
Importantly, innate immune responses triggered by TNF (as described in appendix 1) may contribute to adaptive immune responses. Therefore, mechanisms that are identified in single dose studies (appendix 1) will be translated to studies in this appendix, i.e. similar pharmacological modulators will be considered in multiple dose studies. Along with DILI-associated drugs, structural homologues that do not cause DILI will be tested.
Primary outcome parameters include immunological and histopathological changes in liver and spleen and levels of circulating T cells and antibodies.

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

Mice are treated with compounds via intragastric gavage once a day for six weeks. To analyse the capacity of suspected drugs to support adaptive immune responses, well-defined antigens (e.g. ovalbumin) will be co-injected during drug exposure as so-called bystander antigen. In this way, kinetics of specific immune responses can easily be analysed.
Specific monoclonal antibodies are administered i.p. before or around during exposure depending on the antibody. Injections will be done 3 times per week.
For pharmacological modulators dosing will be determined based on literature data or experience from other partners
Various mouse strains will be used, among those transgenic OT mice and KO mice [redacted]
As modulators monoclonal antibodies directed to immune receptors (e.g. costimulatory receptors) will be used.
Blood samples will be taken several times, but not more than 8 ml/kg/14 days. Primary outcome parameters in blood are liver enzymes (e.g. ALT), cytokines and (auto-)antibodies. At 3 time points during 6 weeks treatment, mice will be dissected and various organs (e.g. liver, spleen and intestine will be collected further analyses, e.g. flow cytometry, analyses of antibody producing cells (to e.g. ovalbumin), specific antibodies, clinical parameters and gene and protein expression.
To determine the effects of modulation, vehicle controls with and without modulation will be included. DILI-associated compounds are compared to pharmacologically or chemically related compounds that are not associated with DILI.
An example of a typical experiment is outlined below.

Mice will be treated up to 6 weeks with:
1- DILI-associated compounds + no modulation
2- compounds not associated with DILI but pharmacologically or chemically resembling the DILI-associated compounds + no modulation
3- DILI-associated compounds + specific modulation
4- compounds not associated with DILI but pharmacologically or chemically resembling the DILI-associated compounds + specific modulation
5- vehicle alone + no modulation
6- vehicle alone + specific modulation
Only in groups 1 and 3, DILI or associated immune changes are expected.

Mice will be treated as follow:



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

Power analysis will be performed before each experiment, based on the parameters that will be evaluated. Since damage of the liver will be our main parameter, power analysis will be based on liver enzyme levels (ALT) in blood.

B. The animals

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

Species: For these experiments, 6-8 wk old mice will be used (e.g. C56BL/6). The choice for mouse as a model species is based on the similarities between the immune system of humans and mice, but also because the immune system in mice is well characterized and several tools are available on the market in order to study the mechanisms involved in DILI. In addition dosing regimen of various DILI-inducing drugs are known for the mouse.
The [redacted]-MIP-DILI project aims to develop in vitro models in order to test potential hepatotoxic compounds and not to develop new in vivo models.
Moreover, there are several evidences in scientific literature (Lucena MI et al., Hepatology 2009) which state that sex is a determinant in the severity and predisposition to DILI. Since gender difference is not relevant for MIP-DILI (**Mechanism**-based **I**ntegrated **S**ystems for the **P**rediction of **D**rug **I**nduced **L**iver **I**njury) project we do not consider to test female and male together because this will lead to more variance in the parameters used.

Origin: Animals will be purchased from a registered commercial provider in EU, including the Netherlands, such as Charles River Laboratories or Harlan Laboratories, or in US (Jackson).

Estimated numbers: The estimated number of animals is derived from the above set up of 6 experimental groups. Based on experience and ALT levels in serum as key parameter (to perform power analyses) the required number of mice per group is 8. A maximum of 3 drug combinations will be tested in this set-up of multiple dosing. The selection of drug combinations will be based on studies done in appendix 1. Based on earlier findings and literature, we expect to do maximum 10 modulations, whereas a max of 3 time points per experiment will be analysed. In total this accounts for a maximum of: 6 (experimental groups per drug combinations) x 3 (drug combinations) x 10 (modulations) x 3 (time points) x 8 animals = 4320.

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.

Replacement:
The kinetics of recruitment and the activation of immune cells to the site of damage is difficult to reproduce in vitro due to the mutual interactions of different cell types (e.g. immune cells, hepatocytes, endothelial cells) from different organs of the body (e.g. interaction between intestine, liver and spleen). To evaluate these parameters an integrated in vivo approach is needed. Mechanistic information collected from these in vivo experiments will shed light on the crucial processes involved in DILI, which is needed in order to develop in vitro tests for the identification of potential DILI-inducing new drug entities. This will add also to reduction of animals in the future. The involvement in the [redacted]-MIP-DILI consortium, which aims to reduce animal use for this type of toxicology, ensures the translation from in vivo to in vitro.
Reduction:
The amount of animals will be limited by choosing the minimal number of control and treatment groups, necessary to achieve optimal results. The number of animals per group will be determined by the use of power analysis on the basis of the most relevant parameter needed for the study.
The number of animals in experiment until now is based on expected effects (liver damage, particularly values of liver enzymes such as ALT in blood).
Refinements:
Mice will be housed in groups and in cages with environmental enrichment. They will be daily monitored in order to prevent any unexpected discomfort.

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

Treatment will be performed by trained personnel to limit stress as a result of animal handling.

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.

NA

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.

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.

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

Classification of discomfort / humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question 1.

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

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

Pain relieving methods will not be possible as they will interfere with drug-induced effects (involving activation of innate immune system and inflammation) in the liver.

☐ Yes

I. Other aspects compromising the welfare of the animals

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

For antibiotic drugs mild diarrhoea might be observed.

Explain why these effects may emerge.

Depletion of gut flora may alter motility of the intestine leading to diarrhoea

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

Mice will be carefully monitored and in case of diarrhoea liquefied feed (weekvoer) will be supplied. Animals will be sacrificed when weight drops more than 20%

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.

If the above mentioned adverse effects will occur, animals will be sacrificed if body weight drops more than 20% over whole period of the experiment. In addition, in case severe health issues (diarhea, prolapse, dehydration, lethargy) mice will be sacrificed.

Indicate the likely incidence.

Unlikely

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’).

Moderate: max 67% of animals. In the multiple exposure study, doses will be used that by itself do not cause severe liver damage, but this damage may be enhanced by modulations. The potential damage in the liver tissue will be revealed by the blood ALT evaluation (once a week). Based on literature information, animals may suffer from the treatment and lose body weight. see J for humane endpoints.
Mild: 33% of animals not receiving a modulation treatment.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No > Continue with Section 3: ‘signatures’.

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

Excision of organs will be necessary to perform the analysis.

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

Appendix
Description animal procedures

- This appendix should be enclosed

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	10800
1.2	Provide the name of the licenced establishment.	Universiteit Utrecht
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 Type of animal procedure: Development of in vitro models of DILI for the identification of intracellular mechanisms and confirmation of interplay among several cell types

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.

We aim to develop and validate predictive in vitro tests for the identification of DILI-associated compound during preclinical drug development.
Based in mechanisms of DILI identified in vivo (experiments listed in appendixes 1 and 2), relevant in vitro tests will be explored and developed. Currently,

we are using in vitro tests to detect: -1. leukocytes recruitment (neutrophils migration and activation, Koenderman L et al., *Thrombosis and Haemostasis* 2010) ; and 2-responses to antigen stimulation (e.g. dendritic cell (DC)-induced lymphocyte proliferation, Garulli B et al., *Clinical and Vaccine Immunology* 2008); and 3- hepatocyte-DC cocultures. These three in vitro tests have been selected because the mechanistically link to in vivo findings obtained in mouse studies with TVX

Importantly, in vitro tests will represent a strategic asset to investigate intra-cellular pathways involved in the relevant physiological events linked to DILI. Those tests will serve as predictive tool to exclude potential hepatotoxic compounds during pre-clinical drug development. For these reasons, other DILI-associated compounds in human (and their non-toxic pharmacological analogues) will be tested, in order to validate the developed tests. Based on results obtained in experiments done in appendix 1 and 2, we may also set up other in vitro tests based on donor material.

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

Mice will:

1- be anesthetized in order to isolate different cell types from liver: hepatocytes and leukocytes. Anaesthetic administration is required in order to perfuse the liver with collagenase and ensure a proper yield of viable cells.
2- undergo cervical dislocation in order to isolate bone marrow and spleen: several cell cultures will be obtained using cells from those organs (e.g. bone marrow-derived DC culture, Lutz MB et al., *Journal of Immunological Methods* 1999)

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

Power analysis will be performed before each experiment, based on the parameters that will be evaluated. Depending on the mechanism investigated different parameters will be taken into account. The number of mice depends on the cell yield for each cell type.

An experiment with co-cultures of freshly isolated hepatocytes and DC with 10 compounds needs around 6480 incubations (based on three concentrations (3) x 4 (inflammatory conditions, i.e combinations of LPS and or cytokines) x 2 (incubated with or without modulator or with and without UV light) x 3 time points x 3 (triplo) x 3 (repetitions) x 10 (compounds). Per mouse we obtain 20 $x10^6$ DC, and 10^6 hepatocytes.

Example of an experimental setup:

Primary hepatocytes:
Group 1a/b: incubated with Trovafloxacin (TVX) and with/or without modulator
Group 2: incubated with Levofloxacin (LVX) and with/or without modulator
Group 3: incubated with vehicle and with/or without modulator

Monocytes and neutrophils migration will be tested using the supernatant of hepatocytes incubated as mentioned above and different time points after treatment with drug or modulating chemicals (Koenderman L et al., *Thrombosis and Haemostasis* 2010; Elliott M et al., *Nature* 2010).
For modulators see appendix 1 and 2

Per incubation $0.5x10^6$ cells are needed, so per mouse 20 incubations (based on hepatocytes) can be done. Since it is estimated that we will do 6500 incubations we will need 325 mice. In addition we need separate mice for DC isolations. These cannot be obtained from the same animals for logistic reasons (DC need to be cultured for 6-10 days to fully mature from bone marrow cells, whereas hepatocytes are cultured only for a short period). At most we will culture DC with hepatocytes in a ratio of 1 (DC) to 1 (hepatocytes). The number of DC that we obtain from one mouse is 20 x 10^6 (i.e enough for 40 incubations) which indicates that we need 160 mice. In total we need 500 mice.

Monocytes and neutrophil can be obtained from same mice so for this we do not need extra mice.

NOTE; triplo means that we will perform 3 incubations per experiment; repetitions means that we repeat the entire experiment. Triplo is to take into account the intra-experimental variation (e.g. errors in pipetting), whereas repetitions will account for extra-experimental (for instance day-to-day or donor-to-donor variation). Repetition experiments may not always be exactly the same; for instance extra controls may be added if needed.

B. The animals

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

Species: For these experiments, mice will be used (e.g. C56BL/6). The choice for a mouse model is based on the similarities between the immune system of humans and mice, but also because the immune system in mice is already well characterized and several tools are available on the market in order to study the mechanisms involved in DILI. The project aims to develop in vitro models in order to test potential hepatotoxic compounds and not to develop a new in vivo model. For this reason we would like not to consider variation in sex, age and strain which might lead to more variance in the parameter used.

Origin: Animals will be purchased from a registered commercial provider in EU, including the Netherlands, such as Charles River Laboratories or Harlan Laboratories or in US (Jackson).

Estimated numbers: The number of donor mice will be 500.

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.

Replacement:
It is still not clear if available cell lines (hepatocytes, DC etc) are resembling the behaviour of primary cells. To evaluate these parameters an integrated approach is needed. For this reason, we will compare results obtained from primary cells with the use of available cell lines (such as hepatocytes and macrophage cell-lines (which are NOT DC)). Evidences collected from these experiments will shed light on the mechanisms involved in DILI, needed to develop in vitro tests for the identification of potential DILI-inducing new drug entities. Experiments will also provide information as the applicability of cell-lines for specific questions.
Reduction:

The number of animals will be determined, based on the cell yield, and an estimation of the conditions to be tested. The test conditions are selected such that we will obtain optimum results with a minimal number of conditions.

Refinements:
Mice will be housed in groups, in cages with environmental enrichment and they will be periodically monitored in order to prevent any unexpected discomfort.

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

Experienced personnel will treat the mice, to prevent unnecessary discomfort due to animal handling. As a limited number of mice is needed to perform the studies, solitary housing can occur when for instance only one animal is needed for isolation of cells. Optimal planning will reduce this risk.

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.

nvt

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.

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.

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

Classification of discomfort / humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question 1.

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

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

☒ Yes

I. Other aspects compromising the welfare of the animals

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

No adverse effects are expected due to absence of treatment.

Explain why these effects may emerge.

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

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.

Indicate the likely incidence.

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').

Non-recovery, animals will not be treated before sacrifice.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No > Continue with Section 3: 'signatures'.

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

Excision of the organs is needed.

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

Centrale Commissie Dierproeven

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Onze referentie
Aanvraagnummer
AVD108002016503

Uw referentie

Bijlagen
1

Datum 02 juni 2016
Betreft Beslissing Aanvraag projectvergunning dierproeven

Geachte [redacted]

Op 1 april 2016 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project 'Mechanism-based integrated systems for the prediction of drug induced liver injury' met aanvraagnummer AVD108002016503. Wij hebben uw aanvraag beoordeeld.

Beslissing
Wij keuren uw aanvraag goed op grond van artikel 10a van de Wet op de Dierproeven (hierna: de wet). U kunt met uw project 'Mechanism-based integrated systems for the prediction of drug induced liver injury' starten. De vergunning wordt afgegeven van 02 juni 2016 tot en met 1 september 2020.

Voorwaarden
Aan deze vergunning is de voorwaarde verbonden zoals genoemd in de vergunning en hieronder toegelicht.

In uw aanvraag geeft u aan niet beide geslachten te willen gebruiken, omdat er in de literatuur aanwijzingen zijn dat het geslacht van DILI patiënten bepalend is voor de ernst en predispositie voor DILI. Uit de literatuur blijkt echter niet dat deze verschillen ook in de muis tot uiting komen. Uit het DEC advies blijkt dat u aan de DEC heeft toegelicht dat u geen goede argumenten heeft om slechts één geslacht te gebruiken. Wij zijn daarom van mening dat er niet voldoende grond is om het project met alleen vrouwelijke dieren uit te voeren. Wij hebben daarom een voorwaarde toegevoegd aan deze vergunning waarbij u mannelijke en vrouwelijke dieren in evenredige aantallen dient te gebruiken. Indien gedurende het project blijkt dat er geslachts-specifieke effecten zijn, kunt u deze informatie als wijziging rapporteren aan de CCD. Deze rapportage kan voor de CCD aanleiding zijn om de voorwaarde van gelijk gebruik van beide geslachten te wijzigen of in te trekken. Deze voorwaarde is toegevoegd om het aantal in voorraad gedode dieren te beperken.

Beoordeling achteraf
Na afloop van het project zal er een beoordeling plaatsvinden, zoals bedoeld in artikel 10a1 lid 1d en lid 3 van de wet. Meer informatie over de eisen bij een beoordeling achteraf vindt u in de bijlage.

Procedure
Bij uw aanvraag heeft u een advies van de Dierexperimentencommissie DEC Utrecht gevoegd. Dit advies is ontvangen op 1 april 2016. Bij de beoordeling van uw aanvraag is het advies betrokken

overeenkomstig artikel 10a, lid 3 van de wet. Op 13 mei 2016 heeft de DEC ons van aanvullend advies voorzien over de ongeriefsclassificaties. Bij de beoordeling van uw aanvraag is zowel het advies als het aanvullend advies betrokken overeenkomstig artikel 10a, lid 3 van de wet. Dit advies en de in de bijlage opgenomen beschrijving van de artikelen van de wet- en regelgeving zijn de grondslag van dit besluit. Wij nemen het advies van de Dierexperimentencommissie grotendeels over met uitzondering van bovenstaande afwijkingen. Met het oog op artikel 10a, lid 1 van de wet wordt een algemene voorwaarde toegevoegd.

Wij hebben u op 10 mei 2016 om aanvullende informatie gevraagd over de in de aanvraag beschreven ongeriefsclassificaties. Op 12 mei 2016 heeft u digitaal gereageerd op onze vragen. Wij kunnen ons vinden in de nadere verduidelijking van uw aanvraag.

Bezwaar
Als u het niet eens bent met deze beslissing, kunt u binnen zes weken na verzending van deze brief schriftelijk een bezwaarschrift indienen. Een bezwaarschrift kunt u sturen naar Centrale Commissie Dierproeven, afdeling Juridische Zaken, postbus 20401, 2500 EK Den Haag.

Bij het indienen van een bezwaarschrift vragen we u in ieder geval de datum van de beslissing waartegen u bezwaar maakt en het aanvraagnummer te vermelden. U vindt deze gegevens in de rechter kantlijn in deze brief.

Bezwaar schorst niet de werking van het besluit waar u het niet mee eens bent. Dat betekent dat dat besluit wel in werking treedt en geldig is. U kunt tijdens deze procedure een voorlopige voorziening vragen bij de Voorzieningenrechter van de rechtbank in de woonplaats van de aanvrager. U moet dan wel kunnen aantonen dat er sprake is van een spoedeisend belang.

Voor de behandeling van een voorlopige voorziening is griffierecht verschuldigd. Op http://www.rechtspraak.nl/Organisatie/Rechtbanken/Pages/default.aspx kunt u zien onder welke rechtbank de vestigingsplaats van de aanvrager valt.

Meer informatie
Heeft u vragen, kijk dan op www.centralecommissiedierproeven.nl. Of neem telefonisch contact met ons op: 0900 28 000 28 (10 ct/minuut).

De Centrale Commissie Dierproeven
namens deze:

[redacted]

ir. G. de Peuter
Algemeen Secretaris

Dit besluit is genomen met inachtneming van het Besluit mandaat, volmacht en machtiging van de Centrale Commissie Dierproeven CCD 2014 zoals de Centrale Commissie Dierproeven heeft vastgesteld op 19 december 2014, ref 2014-04 en is gepubliceerd in de Staatscourant van 2 januari 2015, Nr. 163

Bijlagen
- Vergunning
  Hiervan deel uitmakend: - DEC-advies
  - Weergave wet- en regelgeving

Projectvergunning

gelet op artikel 10a van de Wet op de dierproeven

Verleent de Centrale Commissie Dierproeven aan
Naam: Universiteit Utrecht
Postbus: 12007
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Deelnemersnummer: 10800

deze projectvergunning voor het tijdvak 02 juni 2016 tot en met 1 september 2020, voor het project 'Mechanism-based integrated systems for the prediction of drug induced liver injury' met aanvraagnummer AVD108002016503, volgens advies van Dierexperimentencommissie DEC Utrecht.

De functie van de verantwoordelijk onderzoeker is [redacted]
De aanvraag omvat de volgende bescheiden:

1. een aanvraagformulier projectvergunning dierproeven, ontvangen op 1 april 2016;
2. de bij het aanvraagformulier behorende bijlagen:
   a. Projectvoorstel, zoals ontvangen bij digitale indiening op 1 april 2016;
   b. Niet-technische Samenvatting van het project, zoals ontvangen bij digitale indiening op 1 april 2016;
   c. Advies van Dierexperimentencommissie, zoals ontvangen op 1 april 2016;
   d. De aanvullingen op uw aanvraag, zoals ontvangen op 12 mei 2016;
   e. Aanvullend advies van Dierexperimentencommissie, zoals ontvangen op 13 mei 2016.

Dierproeven

Naam dierproef	Diersoort	Aantal dieren	Ernst
3.4.4.1. Single dose models	Muizen	4960	Licht: 89% Ernstig: 11%
3.4.4.2. Multiple dose models	Muizen	4320	Licht: 33% Matig:67%
3.4.4.3. Development of in vitro models of DILI for the identification of intracellular mechanisms and confirmation of interplay among several cell types	Muizen	500	Terminaal

Na afloop van dit project wordt een beoordeling achteraf uitgevoerd. Deze beoordeling zal uiterlijk 1 december 2020 plaatsvinden.

Voorwaarden
Op grond van artikel 10a1 lid 2 van de Wod zijn aan een projectvergunning voorwaarden te stellen

Mannelijke en vrouwelijke dieren moeten in evenredige aantallen gebruikt worden. Indien gedurende het project blijkt dat er geslachts-specifieke effecten zijn, kunt u deze informatie als wijziging rapporteren aan de CCD. Deze informatie kan voor de CCD aanleiding zijn om bovenstaande voorwaarde van gelijk gebruik van beide geslachten te wijzigen of in te trekken. Indien voorafgaand aan de proeven al informatie in de literatuur beschikbaar is waaruit blijkt dat een model of proces geslachtsafhankelijk zou zijn, is het ook mogelijk om deze informatie te gebruiken om wetenschappelijk te onderbouwen dat het gebruik van zowel mannelijke als vrouwelijke dieren zou leiden tot een grote toename van het benodigd aantal dieren en dit aan ons te rapporteren.

Algemene voorwaarde
1) In artikel 10, lid 1a van de wet, wordt bepaald dat het verboden is een dierproef te verrichten voor een doel dat, naar de algemeen kenbare, onder deskundigen heersende opvatting, ook kan worden

bereikt anders dan door middel van een dierproef, of door middel van een dierproef waarbij minder dieren kunnen worden gebruikt of minder ongerief wordt berokkend dan bij de in het geding zijnde proef het geval is. Nieuwe onderzoeken naar alternatieven kunnen tot gevolg hebben dat inzichten en/of omstandigheden van het aangevraagde project in de vergunningsperiode wijzigen, gedurende de looptijd van deze vergunning.
Indien bovenstaande zich voordoet dient aanvrager dit in overleg met de IvD te melden bij de CCD. De CCD kan in een dergelijke situatie aan de vergunning nieuwe voorwaarden verbinden en gestelde voorwaarden wijzigen of intrekken.

Weergave wet- en regelgeving

Dit project en wijzigingen

Volgens artikel 10c van de Wet op de dierproeven (hierna de wet) is het verboden om andere dierproeven uit te voeren dan waar de vergunning voor is verleend. De dierproeven mogen slechts worden verricht in het kader van een project, volgens artikel 10g. Uit artikel 10b volgt dat de dierproeven zijn ingedeeld in de categorieën terminaal, licht, matig of ernstig. Als er wijzigingen in een dierproef plaatsvinden, moeten deze gemeld worden aan de Centrale Commissie Dierproeven. Hebben de wijzigingen negatieve gevolgen voor het dierenwelzijn, dan moet volgens artikel 10a5 de wijziging eerst voorgelegd worden en mag deze pas doorgevoerd worden na goedkeuren door de Centrale Commissie Dierproeven.
Artikel 10b schrijft voor dat het verboden is een dierproef te verrichten die leidt tot ernstige mate van pijn, lijden, angst of blijvende schade die waarschijnlijk langdurig zal zijn en niet kan worden verzacht, tenzij hiervoor door de Minister van Economische Zaken een ontheffing is verleend.

Verzorging

De fokker, leverancier en gebruiker moeten volgens artikel 13f van de wet over voldoende personeel beschikken en ervoor zorgen dat de dieren behoorlijk worden verzorgd, behandeld en gehuisvest. Er moeten ook personen zijn die toezicht houden op het welzijn en de verzorging van de dieren in de inrichting, personeel dat met de dieren omgaat moet toegang hebben tot informatie over de in de inrichting gehuisveste soorten en personeel moet voldoende geschoold en bekwaam zijn. Ook moeten er personen zijn die een eind kunnen maken aan onnodige pijn, lijden, angst of blijvende schade die tijdens een dierproef bij een dier wordt veroorzaakt. Daarnaast zijn er personen die zorgen dat een project volgens deze vergunning wordt uitgevoerd en als dat niet mogelijk is zorgen dat er passende maatregelen worden getroffen.
In artikel 9 staat dat de persoon die het project en de dierproef opzet deskundig en bekwaam moet zijn.
In artikel 8 van het Dierproevenbesluit 2014 staat dat personen die dierproeven verrichten, de dieren verzorgen of de dieren doden, hiervoor een opleiding moeten hebben afgerond.
Voordat een dierproef die onderdeel uitmaakt van dit project start, moet volgens artikel 10a3 van de wet de uitvoering afgestemd worden met de instantie voor dierenwelzijn.

Pijnbestrijding en verdoving

In artikel 13 van de wet staat dat een dierproef onder algehele of plaatselijke verdoving wordt uitgevoerd tenzij dat niet mogelijk is, dan wel bij het verrichten van een dierproef worden pijnstillers toegediend of andere goede methoden gebruikt die de pijn, het lijden, de angst of de blijvende schade bij het dier tot een minimum beperken. Een dierproef die bij het dier gepaard gaat met zwaar letsel dat hevige pijn kan veroorzaken, wordt niet zonder verdoving uitgevoerd. Hierbij wordt afgewogen of het toedienen van verdoving voor het dier traumatischer is dan de dierproef zelf en het toedienen van verdoving onverenigbaar is met het doel van de dierproef. Bij een dier wordt geen stof toegediend waardoor het dier niet meer of slechts in verminderde mate in staat is pijn te tonen, wanneer het dier niet tegelijkertijd voldoende verdoving of pijnstilling krijgt toegediend, tenzij wetenschappelijk gemotiveerd. Dieren die pijn kunnen lijden als de verdoving eenmaal is uitgewerkt, moeten preventief en postoperatief behandeld worden met pijnstillers of andere geschikte pijnbestrijdingsmethoden, mits die verenigbaar zijn met het doel van de dierproef. Zodra het doel van de dierproef is bereikt, moeten passende maatregelen worden genomen om het lijden van het dier tot een minimum te beperken.

Einde van een dierproef

Artikel 13a van de wet bepaalt dat een dierproef is afgelopen wanneer voor die dierproef geen verdere waarnemingen hoeven te worden verricht of, voor wat betreft nieuwe genetisch gemodificeerde dierenlijnen, wanneer bij de nakomelingen niet evenveel of meer, pijn, lijden, angst, of blijvende schade wordt waargenomen of verwacht dan bij het inbrengen van een naald. Er wordt dan door een dierenarts of een andere ter zake deskundige beslist of het dier in leven zal worden gehouden. Een dier wordt gedood als aannemelijk is dat het een matige of ernstige vorm van pijn, lijden, angst of blijven schade zal blijven ondervinden. Als een dier in leven wordt gehouden, krijgt het de verzorging en huisvesting die past bij zijn gezondheidstoestand.
Volgens artikel 13b moet de dood als eindpunt van een dierproef zoveel mogelijk worden vermeden en vervangen door in een vroege fase vaststelbare, humane eindpunten. Als de dood als eindpunt onvermijdelijk is, moeten er zo weinig mogelijk dieren sterven en het lijden zo veel mogelijk beperkt blijven.

Uit artikel 13c volgt dat het doden van dieren door een deskundig persoon moet worden gedaan, wat zo min mogelijk pijn, lijden en angst met zich meebrengt. De methode om te doden is vastgesteld in de Europese richtlijn artikel 6.
In artikel 13d is vastgesteld dat proefdieren geadopteerd kunnen worden, teruggeplaatst in hun habitat of in een geschikt dierhouderijsysteem, als de gezondheidstoestand van het dier het toelaat, er geen gevaar is voor volksgezondheid, diergezondheid of milieu en er passende maatregelen zijn genomen om het welzijn van het dier te waarborgen.

Beoordeling achteraf
Volgens artikel 10a1, lid 1d en lid 3 van de wet moeten projecten waarbij niet-menselijke primaten worden gebruikt, projecten die als ernstig ingedeelde dierproeven omvatten of een dierproef die leidt tot ernstige mate van pijn, lijden, angst of blijvende schade die waarschijnlijk langdurig zal zijn en niet kan worden verzacht, achteraf beoordeeld worden. In dit project worden dierproeven toegepast die vallen in de categorie ernstig volgens artikel 10b van de wet en wordt daarom voorzien van een beoordeling achteraf. Deze beoordeling zal uiterlijk 1 september 2020 plaatsvinden. Er zal dan beoordeeld worden of de doelstellingen van het project werden bereikt. Daarnaast wordt bekeken of de schade die de dieren hebben ondervonden, het aantal en soorten proefdieren en de ernst van de dierproeven conform de vergunning waren.