	Inventaris Wob-verzoek W16-04s								
		wordt verstrekt				**weigeringsgronden**			
nr.	**document**	**reeds openbaar**	**niet**	**geheel**	**deels**	**10.1.c**	**10.2.e**	**10.2.g**	**11.1**
	NTS 20151209				x		x	x	
1	Aanvraagformulier				x		x	x	
2	Brief mbt factuurinformatie				x		x	x	
3	Projectvoorstel				x		x	x	
4	Niet-technische samenvatting	x							
5	Bijlagen dierproeven oud				x		x	x	
6	DEC-advies				x		x	x	
7	Ontvangstbevestiging				x		x	x	
8	Vraag en reactie 25-08-2015				x		x	x	
9	Bijlagen dierproeven nieuw				x		x	x	
10	advies CCD		x						x
11	Beschikking en vergunning				x		x	x	

1.

1 1 AUG 2015

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.zbo-ccd.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* 10300
☐ Nee > *U kunt geen aanvraag doen*

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

Naam instelling of organisatie	Stichting Katholieke Universiteit Nijmegen
Naam van de portefeuillehouder of diens gemachtigde	[redacted]
KvK-nummer	4 1 0 5 5 6 2 9

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	Geert Grootepleln 10
Postbus	9101
Postcode en plaats	6500HB Nijmegen
IBAN	NL90ABNA0231209983
Tenaamstelling van het rekeningnummer	UMC St Radboud

1.4 Vul de gegevens in van de verantwoordelijke onderzoeker.

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

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

(Titel) Naam en voorletters	☑ Dhr. ☐ Mw.
Functie	
Afdeling	
Telefoonnummer	
E-mailadres	

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]
Functie
Afdeling
Telefoonnummer
E-mailadres

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 08-09-2015
Einddatum 08-09-2020

3.2 Wat is de titel van het project?

Development of new tumor targeting agents for molecular imaging and therapy of cance

3.3 Wat is de titel van de niet-technische samenvatting?

Ontwikkeling van tracers om tumoren zichtbaar te maken en kanker gericht te behandel

3.4 Wat is de naam van de Dierexperimentencommissie (DEC) aan wie de instellingsvergunninghouder doorgaans haar projecten ter toetsing voorlegt?

Naam DEC RU DEC
Postadres Postbus 9101, 6500 HB Nijmegen [redacted]
E-mailadres [redacted]

4 Betaalgegevens

4.1 Om welk type aanvraag gaat het?

- [x] Nieuwe aanvraag Projectvergunning € 741,00 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
- [x] Na ontvangst van de factuur

5 Checklist bijlagen

5.1 Welke bijlagen stuurt u mee?

Verplicht

- [x] Projectvoorstel
- [x] Niet-technische samenvatting

Overige bijlagen, indien van toepassing

- [ ] Melding Machtiging
- [x] DEC advies, document factuurgegevens

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 1.6). 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	Instantie voor dierenwelzijn
Plaats	Nijmegen
Datum	0 8 - 0 8 - 2 0 1 5
Handtekening	[redacted]

2.

Radboud universitair medisch centrum
Concernstaf, sectie Kwaliteit en Veiligheid

Postbus 9101, 6500 HB Nijmegen
Huispost 628
Geert Grooteplein 10

[redacted]

www.radboudumc.nl

KvK 41055629/4

Geert Grooteplein 10
Postbus 9101
6500 HB Nijmegen

Datum
9 augustus 2015

Instantie voor Dierenwelzijn

Onderwerp
Factuurinformatie Projectaanvraag

Geachte CCD,

Hierbij sturen wij u de administratieve gegevens behorend bij de ingediende projectaanvraag. Wij verzoeken u de factuur te versturen naar de IvD als gemachtigde van de vergunninghouder. Hiervoor AUB het bij u bekend e-mailadres gebruiken (instantievoordierenwelzijn@radboudumc.nl).

Om verwerking door de financiële afdeling mogelijk te maken verzoeken wij u tevens ***op de factuur*** de volgende gegevens te vermelden:

Factuuradres:	Radboudumc 28 F&A crediteuren Postbus 9101 6500HB, Nijmegen
Kostenplaats en kostensoort:	040823-461220
CDL projectnummer:	2015-0071
Verantwoordelijk onderzoeker:	[redacted]

Bij voorbaat dank.

Met vriendelijke groeten

[redacted]

Instantie voor Dierenwelzijn
instantievoordierenwelzijn@radboudumc.nl

Form
Project proposal

- This form should be used to write the project propsal of 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'.	10300
1.2	Provide the name of the licenced establishment.	Stichting Katholieke Universiteit Nijmegen
1.3	Provide the title of the project.	Development of new tumor targeting agents for molecular imaging and therapy of cancer

2 Categories

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

[] Basic Research
[X] Translational or applied research
[] Regulatory use of routine production
[] Research into environmental protection in the interest of human or animal health or welfare dier
[] 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.

Cancer is the leading cause of death in the Netherlands. To improve patient survival, both advances in diagnosis and therapy are needed. Development of new molecular tracers may improve both diagnosis and cancer therapy, and will lead to better treatment outcome for cancer patients. Currently, new agents are being developed that specifically target tumor cells. These agents include monoclonal antibodies (e.g. cetuximab (anti-EGFR), bevacizumab (anti-VEGF), girentuximab (anti-CAIX), labetuzumab (anti-CEA)), antibody fragments (e.g. Fab'-fragments, nanobodies), peptides (e.g. RGD, Ac-TZ14011), and small molecules directed against specific tumor-associated targets, like the Prostate-Specific Membrane Antigen (PSMA). In order to characterize or visualize tumors, these tumor-targeting agents are labeled with for example a radioactive or fluorescent tag, which can be detected with a dedicated camera (e.g. PET, SPECT, fluorescence). Thus, these agents can be used to study biological processes in cancer, such as proliferation, metabolism, angiogenesis, and receptor expression, for tumor characterization and imaging to improve tumor detection, diagnosis, treatment, prediction of outcome, and treatment response monitoring.

Since the introduction of monoclonal antibodies(1) research has focused on both diagnostic and therapeutic potential of tagged antibodies. By targeting specific tumor-associated antigens, antibodies may show high and specific tumor accumulation, resulting in high tumor-to-nontumor uptake ratios. This enables the use of antibodies as tumor targeting vehicles carrying diagnostic or therapeutic agents.(2) Labeled antibodies have been used in (our) preclinical imaging research during the past 15 years, [e.g.(3-5)] which has resulted not only in increased knowledge about tumor biology, but has also led to the initiation of multiple clinical trials.[e.g.(6-8)] [redacted] Also, other tumor-targeting agents like peptides, nanobodies and small molecules have been a topic of extensive preclinical research. [redacted] s.

In addition to *imaging* of cancer, tumor-targeting tracers can also be used to *treat* cancer. For this purpose the agent is labeled with a cytotoxic drug or a high activity dose of an alpha, beta, or gamma emitters. Currently, antibody (fragments) and peptides are explored as vehicles to carry

cytotoxic loads to the tumor. For example, targeted radioimmunotherapy has been widely investigated in preclinical research by us and others, with Lu-177-labeled agents to treat prostate cancer (11), clear cell renal cell cancer (12), and colorectal cancer (6). In addition, the same agents used for therapy can be used for response monitoring and treatment adjustment, in a theranostic approach (13). This research is supported by several Dutch Cancer Society grants [redacted]

Initially, these tumor-targeting tracers are developed and tested extensively *in vitro*. The agents with optimal *in vitro* characteristics (stability, integrity, radiochemical purity, affinity, internalization kinetics, detectability, a.o) will be selected for *in vivo* testing to determine, optimize and exploit their in vivo tumor targeting properties. The *in vivo* behavior of the tumor-targeting agents (pharmacokinetics, biodistribution, accumulation in the tumor, therapeutic efficacy) can only be assessed in animal models. Knowledge obtained in these animal studies will be used to translate the application of the newly developed agents into clinic. Currently, most of our research is focused on prostate, kidney, head and neck, colorectal, ovarian, and breast cancer.

1. Kohler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature. 1975;256(5517):495-7.
2. Fleuren ED, Versleijen-Jonkers YM, Heskamp S, van Herpen CM, Oyen WJ, van der Graaf WT, et al. Theranostic applications of antibodies in oncology. Molecular oncology. 2014;8(4):799-812.
3. [redacted]

[redacted]

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?

New and existing tumor-targeting agents will be (further) developed to improve tumor detection, characterization, and treatment of cancer. This may serve as valuable input to optimize and individualize cancer treatment. Therefore the aims of the project are:

1) In vivo characterization of new tumor-targeting agents for molecular imaging of cancer
2) In vivo characterization of new tumor-targeting agents for therapy of cancer

Experiments described in this project will answer the question whether the new agents are suitable to detect, characterize, image, and/or treat cancer in a preclinical setting. This type of research has proven very valuable for subsequent clinical translation of new imaging and/or therapeutic agents [e.g. (6-8)].

In the past decades, we have obtained broad experience with this strategy of development and preclinical *in vivo* characterization of tumor-targeting agents [e.g. (1-13)]. Our research is published in international peer-reviewed journals, and ample external funding has been obtained to conduct this research (e.g. Dutch Cancer Society, CTMM, NanoNext, ZonMW).

3.3 Relevance

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

In the experiments described in this project we will study biological molecular processes in tumors, like angiogenesis, receptor expression, tumor metabolism, proliferation, apoptosis, etc. The tumor-targeting tracers that we develop and characterize *in vivo* will further increase our knowledge on tumor biology. A similar research strategy characterizing and visualizing comparable tracers has led to more accurate clinical diagnostics and therapy. Newly developed tracers will improve this further. This may allow personalized medicine and patient-tailored therapy, to optimize the therapeutic effect and to minimize unwanted side effects and overtreatment for the individual patient. Finally, this may lead to improved treatment outcome and quality of life for cancer patients.

3.4 Research Strategy

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

The development, characterization and implementation of new tumor-targeting probes can be subdivided into four stages. Only stages 2 and 3 include animal experiments, but for an overview of the research strategy all four stages are briefly described below:

In the first stage, the newly developed tracers or drugs will be characterized by *in vitro* experiments, to determine their (bio)chemical properties (stability, integrity, radiochemical purity, detectability, lipophilicity, a.o.) and tumor-targeting potential (e.g. binding to tumor cells, affinity (Kon, Koff) , cytotoxicity, internalization kinetics). This precedes the actual animal experiments. To minimize the number of animal experiments that need to be performed, only tracers that show optimal **in vitro** characteristics will be selected for *in vivo* testing.
In the second stage the *in vivo* pharmacokinetics and biodistribution of these agents will be studied in animal models (mice and rats). For this purpose, imaging (e.g. microPET, microSPECT, fluorescence) and/or biodistribution studies will be carried out to determine tumor targeting, tumor retention, clearance, and biodistribution of the tumor-targeting agents.
In the third stage, tumor targeting agents will be tested for their therapeutic efficacy, tolerability, and side effects (stage 3).
Diagnostic imaging and therapeutic agents that show promising *in vivo* behavior in our animal models are selected for translation into the clinic (stage 4).

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.

The newly developed agents will be assessed *in vivo* for their imaging or therapeutic potential by studying their pharmacokinetics and tumor targeting properties. For this purpose, mice bearing subcutaneous or orthotopic human tumor xenografts will be used. In certain studies, also non-tumor bearing mice may be used. For example, when assessing only the blood clearance of a tumor-targeting agent, it is not necessary to induce a tumor. In a typical experiment, first, the tumor-targeting agent (e.g. labeled girentuximab in a model of renal cell carcinoma, or a labeled small molecule PSMA inhibitor in a model of prostate cancer) will be administered to the animal (typically intraveneously). Then, experimental procedures like blood sampling, imaging, and/or biodistribution studies will be carried out to determine the pharmacokinetics and tumor targeting properties of the new agent.

The potential of new tumor targeting agents for cancer therapy can also be assessed in tumor or non-tumor bearing animals. Non-tumor bearing animals are frequently used in studies with toxicity as the primary endpoint, in order to prevent that tumor growth is limiting the duration of the follow up (e.g. renal toxicity may occur weeks to months after therapy). In a typical experiment, first, the therapeutic agent will be administered (either a single dose or multiple weekly i.p. injections) and the therapeutic effect and toxicity will be monitored longitudinally. Therapeutic efficacy may be assessed by measuring tumor size by caliper measurements or non-invasive imaging techniques. Toxicity may be assessed by measuring body weight and/or by analysis of hematological and specific organ toxicity in blood samples.

The majority of these studies will be carried out in immunodeficient mice, as these are the lowest vertebrates in which human cancer xenografts grow without being rejected. However, some experimental models are not available in mice (e.g. liver metastases of colorectal cancer), or can only

be mimicked in rats (e.g. hyperthermic intraperitoneal chemotherapy of peritoneal carcinomatosis). In these cases, rats will be used and the same procedures as described above will be applied.

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

After the development and in vitro characterization of tracer, the animal studies involve:
1) In vivo assessment of
   a) tumor targeting
   b) tracer accumulation
   c) clearance
   d) pharmacokinetics
2) Optimization of agents for imaging and/or therapy

After each step we will evaluate whether the tracer is suitable for imaging and/or therapy of cancer. If the tracer shows unfavorable in vivo behavior this will serve as a no go for further evaluation. In case the probe requires further optimization, new in vitro studies will be carried out (e.g. with respect to stability, affinity), followed by in vivo characterization (e.g. tumor targeting, accumulation, circulatory halflife), imaging and/or therapy studies.

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	Characterization of in vivo behaviour of new tumor targeting agents in mice
2	Assessment of therapeutic efficacy of new tumor targeting agents in mice
3	Characterization of in vivo behaviour of new tumor targeting agents in rats
4	Assessment of therapeutic efficacy of new tumor targeting agents in rats

5.

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'.	10300	
1.2	Provide the name of the licenced establishment.	Stichting Katholieke Universiteit Nijmegen	
1.3	List the different types of animal procedures. Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.	Serial number 1	Type of animal procedure Characterization of in vivo behaviour of new tumor targeting agents in mice

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

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

The aim of the experiments is to determine the in vivo behaviour (e.g. tumor targeting, retention, clearance, etc) of new tumor targeting agents in animal models. For this purpose, the agent will be admistered (e.g. i.v., i.p., etc) to the animal and subsequently the pharmacokinetics and biodistribution of the agent can be studied by means of biodistribution and/or imaging. In addition, the pharmacokinetics can be studied by for example blood sampling or urine collection.
A standard approach to study the in vivo behaviour of a new tumor targeting agent includes the following experiment:
1. Dose optimization: determine which dose of the tumor targeting agent results in the highest tumor uptake with relative low uptake in normal tissue
2. Pharmacokinetics: Determine the pharmacokinetics of the tracer (e.g. how rapidly accumulates the tracer in the tumor and clears from normal tissue and circulation)
3. Imaging of the distribution of the tumor targeting agent in vivo

To quantitatively determine the in vivo behaviour of the tumor targeting agent we will use the following primary end points
- Tumor and normal tissue uptake measured by biodistribution study. In this set up the animal will be euthanized at a certain time point (e.g. 1h, 24h, 72h) after injection of the tumor targeting agent. The animals will be dissected and tumor and normal organs will be collected. The uptake in tissues of interest will be quantified by using for example a gamma counter. Aliquots of the injected tracer will be counted simultaneously.
- Tumor and normal tissue uptake measured by imaging. In this set up, the injection of the radiotracer will be followed by an imaging procedure (e.g. optical, SPECT or PET) under anesthesia. Since it is not necessary to euthanize the animal, this procedure can be repeated at several time points after injection. After the scan, a 3D image will be reconstructed and a region of interest will be drawn around the tumor and tissue of interest to quantitatively determine the activity concentration.
- Pharmacokinetics of a tracer will be determined by taking blood samples (e.g. via cheek puncture, tail cut) or urine samples. The tracer concentration can be measured by for example ELISA or by measuring the radioactive or fluoresent signal (e.g. in a gamma counter).
These outcome parameters have successfully been used in previous studies performed by our research group and by other international research groups. (1-5)

.

4. Tolmachev V, Varasteh Z, Honarvar H, Hosseinimehr SJ, Eriksson O, Jonasson P, et al. Imaging of platelet-derived growth factor receptor beta expression in glioblastoma xenografts using affibody molecule 111In-DOTA-Z09591. J Nucl Med. 2014;55(2):294-300.

5. Luo H, Hong H, Slater MR, Graves SA, Shi S, Yang Y, et al. PET of c-Met in cancer with 64Cu-labeled Hepatocyte Growth Factor. J Nucl Med. 2015.

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

In a standard experiment the first step is to inject or transplant the animals with a tumor. In case the tumor requires growth factors or hormones to grow, a growth factor pellet will be transplanted subcutaneously. When the tumor reaches a size of approximately 0.1 cm3 (on average this takes 2-3 weeks), the animal will be injected with the tumor targeting agent. After injection, blood samples can be collected to measure the pharmacokinetics of the tracer. Depending on how fast the tracer accumulates in the tumor and clears form the circulation, biodistribution and imaging studies will be performed at several time points (e.g. 1h, 24h, and 72h) after injection to quantitatively determine the biodistribution of the tracer. On average, these type of studies will take three weeks. However, in case of slow growing tumors or longitudinal studies, the total time an animal is in the experiment can be longer (maximum 6 months).

In more detail, the following experimental steps can be carried out:
- Injection of tumor cells (one or two sided, i.p., i.v., s.c., i.m., orthotopically, intracardially, etc). If necessary, discomfort will be minimized by performing the procedure under generally anesthesia and administering analgesics (e.g. carprofen, temgesic, etc). Frequency: in most cases only one injection of tumor cells will be performed (one tumor per animal). In certain studies (e.g. imaging studies with a receptor positive and receptor negative tumor in one animal), two types of tumor cells will be injected. Depending on how fast the tumors grow, they will be injected at the same time, or there will be a certain time interval (e.g. 1 week) between the two tumor cell injections. The injection takes less then a minute. Orthotopic injection may last longer, depending on site of injection.
- Transplantation of tumor tissue. This procedure will be carried out under general anesthesia and if necessary analgesics will be administered. Frequency: in most cases only one tumor transplantation will be performed (one tumor per animal). In certain studies (e.g. imaging studies with a receptor positive and receptor negative tumor in one animal), two types of tumors will be transplanted. Depending on how fast the tumors grow, they will be transplanted at the same time, or there will be a certain time interval (e.g. 1 week) between the two tumor cell transplantation. Subcutaneous transplantations will take approximately 10 minutes. Orthotopic transplantations may last longer, depending on site of transplantation.
- Particular tumors require hormones or growth factor in order to grow in mice (for example certain breast cancer cells require estrogen to grow). These will be administered by transplanting a growth factor pellet subcutaneously. This procedure will be performed once and under general anesthesia. It will take approximately 10 min. When the injection/transplantation of the tumor cells also requires anesthesia, the transplanation of the pellet will be performed in the same session.
- Administration of tumor targeting agent (i.p., i.v., orally, s.c., etc.) In general, tumor targeting agents will be injected intraveneously. However, in certain studies we can choose a different administration route. For example, in case of intraperitoneal tumor growth of ovarian cancer, the tracer can be injected intraperitoneally as well. Frequency: In most studies the tumor targeting agent will be injected once. However, in case of longitudinal studies, agents can be injected multiple times (e.g. every other day, weekly, etc.). The maximum number of injections of a tumor targeting agent is six. The duration of injection is approximately 1 minute (depending on the type of injection). The guidelines published in "A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes" will be followed (reference 1).

- Blood sampling (for example via cheek puncture, orbital puncture, tail cut). This can be performed multiple times. The exact method and frequency will depend on the amount of blood that is required to answer the research question. In case the clearance of the tumor targeting agent is studied, 15-20 ul blood will be taken via a tail cut. This can be performed up to 7 times per week (maximum 10 samples). In case blood is collected for further laboratory analysis of for example leucocytes, hemoglobin or creatinin, 100-150 ul blood (max 7.5% of blood volume) will be taken via cheekpuncture. This can be performed once per week, with a maximum of 10 samples in total. The duration depends on the procedure (cheek puncture: 5 min, tail cut: 3 min). The guidelines published in "A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes" will be followed (reference 1).
- Weighing of animals. The frequency depends on the research question and the general condition of the animal (standard 1-2 per week, but if necessary the animals will be weighed daily). The duration is less then one minute.
- Measuring of tumor size using a caliper. In case tumor size is relevant for the research question, it will be measured 2-3 times per week, and if necessary it will be measured daily. The duration is 2 minutes.
- Imaging under general anesthesia. The frequency depends on the research question. However, the maximum number of times an animal is allowed to recover from anesthesia after imaging is 3 times within one week. The duration of the scan depends on the imaging modalities and ranges from 1 second to 3 hours. Most scans can be acquired in less than 1 hour. In case the radiotracer 18F-FDG is used for PET imaging, the animals will be fasted for a maximum of 12 h prior to injection (the maximum number of times fasting will be used is five times).

Reference 1: Karl-Heinz Diehl et al. Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes. Journal of Applied Toxicology 2001:21;15–23

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

In order to minimize the number of animals all tracers will first be evaluated in vitro. Only tracers that show favorable in vitro characteristics (e.g. high affinity, internalization, immunoreactivity) will be selected for in vivo studies.
For each experiment a power calculation will be performed based on data from literature and previous experiments to determine the minimum amount of animals needed. We will also take into account drop out of animals during the experiment due to experimental procedures, for example surgery, tumor growth, etc. If necessary, a biostatistician will be consulted to assist in these power calculations.

B. The animals

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

In most studies we will use immunodeficient athymic mice, because in these models human tumors can be grown without immunological rejection. For some studies, mice tumor cell lines will be used and they will be grown in syngeneic models (e.g. studies with the B16 melanoma cell line derived from C57BL/6, will be performed in C57Bl/6 mice). The most frequently used animal strains are:
Mice: BALB/c nude, BALB/c rj/nu, SCID, C57Bl/6, BALB/c and Swiss, C3H mice, FVB mice, CBA mice, NSG mice
However, other strains can be used if literature research or previous experiments indicate that this is required to answer the research question.

For most studies, animals will be 6-8 weeks old at start of the experiment. However, for specific research questions, e.g. imaging agents that specifically target tumors in young or elderly mice, animals of the appropriate age will be used.

Based on animal experiments that were performed by our research group in 2012-2014 we expect to use a maximum of 4,000 mice. This calculation is based on the following:
In order to study the in vivo behaviour of a new tumor targeting agent, and to optimize it for imaging and therapy purposes, several experiments will be carried out.
- First, a dose-optimization study will be performed to determine which tracer dose will result in the highest tumor uptake with relative low uptake in normal tissue. In general we need six groups to optimize this. Depending on the power calculation, the number of animals per group is on average five. This results in approximately 30 animals per dose optimization study.
- Next, the pharmacokinetics will be studied by performing biodistribution studies at different time points. On average we will need eight groups of animals for this, which results in approximately 40 mice per pharmacokinetics study.
- In the third stage imaging will be performed. For this we will need approximately 10 animals.
Based on this, we estimate that we will need approximately 80 mice to fully characterize and optimize a new tumor targeting agent for imaging and therapy.
During the last three years, we have developed tumor targeting agents for several tumor associated antigens, eg [redacted]
For each target, different types of radiotracers can be designed, such as monoclonal antibodies or antibody fragments, nanobodies, affibody molecules, peptides, and small molecules. These agents differ in several aspects like affinity, size, tumor accumulation, clearance, etc. In addition, monomers, dimers, or heterodimers can be produced in order to increase the affinity and tumor targeting potential of these agents.
We expect to develop ten new radiotracers per year. In order to fully characterize and optimize these agents (dose optimization, pharmacokinetics, imaging) we will need 800 mice per year. In total this will result in 4,000 animals in five years.

Species	Origin	Maximum number of animals	Life stage
mice		4000	6-8 weeks

C. Re-use

Will the animals be re-used?

[X] 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:
Any new agent will first be characterized extensively in vitro. Only agents with promising characteristics will be applied in animals to study their in vivo behaviour. The aim of the studies is to determine the in vivo behaviour of tumor targeting agents (e.g. tumor targeting, clearance, etc). This can only be performed in living animals and there are currently no alternative models to get these data without the use of animals.
Reduction:
Before each experiment we will perform a literature search to find all information relevant for the animal experiment. In addition, a power calculation will be performed based on data from literature and previous experiments to determine the minimum amount of animals needed. We will also take into account drop out of animals during the experiment due to experimental procedures, for example surgery, tumor growth, etc. In case necessary, a biostatisticaion will be involved in the power calculation.
Refinement:
The experiments will be carried out in mice and rats because there are no alternatives to study the in vivo behaviour of tumor targeting agents in lower animal species. Most experimental procedures will only cause moderate discomfort or less. The use of anesthesia and analgesics is in general not required and may cause even more discomfort that the experimental procedures it self. However, if necessary (e.g. after orthotopic or intracardial injection of tumor cells), anesthesia and analgesics will be applied. During imaging procedures and surgical procedures (when the animal is anesthesized), they will be placed on a warmth mattress. to prevent heat loss. To reduce anxiety, mice will be housed in groups and not individually. The general health of the animals will be monitored daily by the researcher and/or the biotechnician. The methods that we use are well establised and the experiments will be performed by experienced researches. [redacted]

[redacted]

4. Baum RP, Prasad V, Muller D, Schuchardt C, Orlova A, Wennborg A, et al. Molecular imaging of HER2-expressing malignant tumors in breast cancer patients using synthetic 111In- or 68Ga-labeled affibody molecules. J Nucl Med. 2010;51(6):892-7.

[redacted]

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

In order to prevent unneccesary discomfort or stress the general health of the animals will be checked daily by the researches or the biotechnicians.
In case animals reach a humane end point the responsible researcher will be contacted.
To reduce axiety, mice will be housed in groups and not individually.
If necessary, anesthesia and analgesics wil be applied to reduce discomfort.
For imaging and biodistribution, only a very low dose (tracer dose) of the tumor targeting agent is required. Therefore, we do not expect that this will cause discomfort or side effects.

Environmental effects:
During and after the experiments, radioactive or biohazardous waste will be collected seperately and removed according to the university guidelines.

Repetition and Duplication

E. Repetition

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

Not applicable

Accommodation and care

F. Accommodation and care

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

[X] 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?

[X] 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 I.
[X] Yes > Will anaesthesia, analgesiaor other pain relieving methods be used?

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

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

I. Other aspects compromising the welfare of the animals

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

The most frequently occuring types of discomfort are:
- Stress
- Discomfort due to tumor growth
- Injections or surgical procedures causing pain and stress
- Recovery from anesthesia
- Euthanasia

The tumor targeting agents themself will not cause side effects or discomfort, since the injected dose is very low (tracer dose)

Explain why these effects may emerge.

Stress can be caused by the handling of the animals, injections, and other experimental procedures. In addition, the animals can suffer from the recovery from anesthesia after the imaging procedure.

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

To reduce stress, animals will be housed in groups and not indvidually. In addition, before the experiments starts the animals will be housed for a week in order to acclimatise to their new environment.

The tumor growth can cause discomfort, especially in case of large tumors, ulcerating tumors or invasive tumors. In most studies, the experiment will start when the tumor is only 0.1 cm3 and therefore the tumor not likely to cause discomfort due to size, ulceration or invasive growth. In general, animals will be taken out of the experiment before tumorscause severe discomfort. However, in case tumors unexpectedly cause severe ulceration or invasive growth, the animal may experience severe discomfort and will be taken out of the experiment. We expect that this will happen in less than 1% of the animals.

Recovery from anesthesia is causing discomfort to the animal. However, this is required in case of imaging of a living animal. In order to minimize this discomfort, the maximum number of times an animal recovers from anesthesia is three times a week and after the last scan, the animal will be euthanized before it recovers from anesthesia. During the anesthesia the animal will be placed on a warmth mattress to prevent heat loss.

Based on extensive experiments with many different types of radiotracers, we do not expect that these tracers themselves will cause discomfort to the animals.

The experiments will be carried out by experienced researchers who have been trained to perform these type of studies.

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.
[X] Yes > Describe the criteria that will be used to identify the humane endpoints.

Animals will be taken out of the experiments and humanely euthanized if one the following humane endpoints is reached:
- Tumor size > 2 cm3
- Ulceration or invasive tumor growth causing discomfort to the animal
- Abnormal motility due to tumor growth (severely reduced motility)
- Other signs of clinical discomfort (for example, dehydration, 15% weight loss in less than 2 days, or a decrease of 20% compared to the weight at start of the experiment).

Indicate the likely incidence.

In most imaging and biodistribution studies the experiments will start when the tumor is only 0.1 cm3 and thus it is very unlikely that the humane end points are reached. Based on previous experience we expect this to be less than 1%.

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).

We expect that 95% of the animals will experience moderate discomfort, because either tumor growth is induced or the animals will have to recover from anesthesia which is required for the imaging procedures. The remaining 5% of the animals will experience mild discomfort, since we will not induce a tumor and perform imaging. In the exceptional situation that a tumor causes unexpected severe discomfort due to for example ulceration or invasive growth, the animal may experience severe discomfort. This will happen in less than 1% of the animals.

End of experiment

L. Method of killing

L. Method of killing

Will the animals be killed during or after the procedures?

[] No > Continue with Section 3: 'Signatures'.

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

At the end of the study we will determine the biodistribution of the agent ex vivo. In addition, tumors or normal tissue can be analyzed immunohistochemicaly.

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.

[X] 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'.	10300	
1.2	Provide the name of the licenced establishment.	Stichting Katholieke Universiteit Nijmegen	
1.3	List the different types of animal procedures. Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.	Serial number 2	Type of animal procedure Assessment of therapeutic efficacy of new tumor targeting agents in mice

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

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

The aim of the experiments is to determine the therapeutic efficacy of new tumor targeting agents in animal models for the treatment of cancer. Tumor targeting agents can be for example antibodies, peptides or small molecules. They can be used 'naked' or be labeled with beta- or alpha-emitting radionuclides (e.g. Lu-177, Y-90 or Bi-213) or cytotoxic drugs. In these experiments, the therapeutic efficacy of these agents will be compared with either the unlabeled compound or conventional anti-cancer treatment (e.g. chemotherapy, irradiation, surgical resection of the tumor, or cell-based therapies)

The primary outcome measurements of these experiments are:
1) Toxicity
Toxicity will be assessed by measuring body weight and by analyzing blood and/or urine for hematological toxicity (hemoglobin, leucocytes, thrombocytes) and renal toxicity (creatinine). Body weight is a reliable indicator of the general health of the animal. In addition, chemotherapy or targeted radiotherapy can cause bonemarrow and kidney toxicity. This can be measured by analyzing blood samples for several hematological and renal parameters. Asssessing the toxicity can not be performed in animal procedure 1, since in these studies only a tracer amount of the tumor targeting agent is administered, while the therapeutic dose is much higher (up to a 100 fold). (1-5)
2) Tumor growth
Tumor growth can be measured by caliper measurements (in case of a subcutaneous tumor) or by non-invasive imaging techniques such as PET, SPECT or MRI (in case of an orthotopic tumor model such as prostate cancer, intraperitoneal tumors, liver metastases, etc). Both methods have shown to reliably monitor tumor growth. (1-5)
3) Survival
Next to tumor growth, the survival is an important primary outcome measure to proof the therapeutic efficacy. Certain therapeutic agents may cause severe toxicity and therefore only measuring tumor growth is not sufficient. For example, tumor growth can be significantly inhibited during the first three weeks, but when the treatment causes severe hematological or renal toxicity, this may result in death of the animals. Thus, the overall effect of the treatment is not beneficial for the animal. In order to study survival, we will longitudinally monitor the animals untill they die or have to be sacrificed based on the humane endpoint (e.g. tumor > 2 cm3, weight loss more than 25% compared to baseline, etc). (1-5)
4) Assessment of molecular processes induced by treatment
Finally, the tumor targeting agent may induce molecular processess in the tumor that can be measured by non-invasive imaging or by analyzing the tumors by western blot or immunohistochemical stainings. An example of such a molecular process is a change in tumor cell proliferation that can be measured by 18F-FLT PET, or a change in glucose metabolism that can be measured with 18F-FDG PET. Changes in 18F-FLT or 18F-FDG often proceed the changes in tumor size as can be measured by caliper measurements and can therefore be used to predict response to treatment. (6, 7)
The experiments will be carried out in the following order:
The first step is to determine which dose results in optimal therapeutic efficacy without inducing severe toxicity (e.g. determination of the maximum tolerable dose (MTD)). The toxicity can be assessed by measuring body weight and by taking blood samples to assess hematological (platelets,

leucocytes, thrombocytes) and renal toxicity (creatinine, urea). Next the therapeutic effect is monitored by measuring tumor growth and survival. Tumor growth can be monitored by caliper measurements or non-invasive imaging techniques (e.g. CT, MRI, PET, SPECT, optical, ultrasound). Finally, we can also study which molecular processes occur in tumors that were treated. Processes of interest are for example proliferation, apoptosis, angiogenesis, expression of growth factor receptor, activation of intracellular signaling pathways. These processes can be measured by non-invasive imaging techniques, or by collecting tumor samples for ex vivo analysis (immunohistochemistry, western blot, etc) at the end of the study.

[redacted]

7. Hong YS, Kim HO, Kim KP, Lee JL, Kim HJ, Lee SJ, et al. 3'-Deoxy-3'-18F-fluorothymidine PET for the early prediction of response to leucovorin, 5-fluorouracil, and oxaliplatin therapy in patients with metastatic colorectal cancer. J Nucl Med. 2013;54(8):1209-16.

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

In order to determine the therapeutic effect of tumor targeting agents, the following procedures can be carried out. The study will start with inoculation of a tumor. When the tumor reaches a size of approximately 0.1 cm3 (2-3 weeks) the therapy will be started. Tumor size will be monitored by caliper measurements or non-invasive imaging techniques. Toxicity will be assessed by measuring body weight and by taking blood samples to analyze hematological and renal toxicity. Finally, the molecular processess in tumor (e.g. proliferation, apoptosis, etc) may be monitored by non-invasive imaging techniques, or by removing the tumor at the end of the study for ex vivo analysis (immunohistochemistry, westerblot, etc). The maximum time an animal is in an experiment is 12 months.

In more detail the following procedures will be carried out:
- Injection of tumor cells (one or two sided, i.p., i.v., s.c., i.m., orthotopically, intracardially, etc). If necessary, discomfort will be minimized by performing the procedure under generally anesthesia and administering analgesics (e.g. carprofen, temgesic, etc). Frequency: in most cases only one injection of tumor cells will be performed (one tumor per animal). In certain studies (e.g. imaging studies with a receptor positive and receptor negative tumor in one animal), two types of tumor cells will be injected. Depending on how fast the tumors grow, they will be injected at the same

time, or there will be a certain time interval (e.g. 1 week) between the two tumor cell injections. The injection takes less then a minute. Orthotopic injection may last longer, depending on site of injection.
- Transplantation of tumor tissue. This procedure will be carried out under general anesthesia and if necessary analgesics will be administered. Frequency: in most cases only one tumor transplantation will be performed (one tumor per animal). In certain studies (e.g. imaging studies with a receptor positive and receptor negative tumor in one animal), two types of tumors will be transplanted. Depending on how fast the tumors grow, they will be transplanted at the same time, or there will be a certain time interval (e.g. 1 week) between the two tumor cell transplantation. Subcutaneous transplantations will take approximately 10 minutes. Orthotopic transplantations may last longer, depending on site of transplantation.
- Particular tumors require hormones or growth factor in order to grow in mice (for example certain breast cancer cells require estrogen to grow). These will be administered by transplanting a growth factor pellet subcutaneously. This procedure will be performed once and under general anesthesia. It will take approximately 10 min. When the injection/transplantation of the tumor cells also requires anesthesia, the transplanation of the pellet will be performed in the same session
- Administration of tumor targeting agent (i.p., i.v., orally, s.c., etc.) In general, tumor targeting agents will be injected intraveneously. However, in certain studies we can choose a different administration route. For example, in case of intraperitoneal tumor growth of ovarian cancer, the tracer can be injected intraperitoneally as well. Frequency: In most studies the tumor targeting agent will be injected once. However, in case of longitudinal studies, agents can be injected multiple times (e.g. every other day, weekly, etc). The maximum number of injections in six. The duration of injection is approximately 1 minute (depending on the type of injection). The guidelines published in "A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes" will be followed (reference 1).

- Administration of conventional anti-cancer therapy such as chemotherapy and antibody-based therapy (i.p., i.v., etc). Conventional anti-cancer therapy is generally injected intraperitoneally. However, certain agents can also be administered i.v., s.c., orally, etc. For example, small-molecule inhibitors like BRAF inhibitors require oral administration. Frequency: depends on the duration of the study. Therapy can be injected once, or in case of longitudinal studies, agents can be injected multiple times (e.g. every other day, weekly, etc). The duration of injection is approximately 1 minute (depending on the type of injection).The guidelines published in "A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes" will be followed (reference 1).
- Irradiation: Mice will undergo irradiation under general anesthesia (2 Gy - 10 Gy). Frequency: in general one dose of 10 Gy will be administered or radiation is administered fractionated (e.g. 3x3Gy). The duration of one irradiation session is approximately 10 minutes. Irradiation can be whole body (2 Gy) or only on the tumor (2 - 10 Gy).
- Blood sampling (for example via cheek puncture, orbital puncture, tail cut). This will be performed multiple times. The exact method and frequency will depend on the amount of blood that is required to answer the research question. In case the clearance of the tumor targeting agent is studied, 15-20 ul blood will be taken via a tail cut. This can be performed up to 7 times per week (maximum 10 samples). In case blood is collected for further laboratory analysis of for example leucocytes, hemoglobin or creatinin, 100-150 ul blood (max 7.5% of blood volume) will be taken via cheekpuncture. This can be performed once per week, with a maximum of 10 samples in total.The duration depends on the procedure (cheek puncture: 5 min, tail cut: 3 min). The guidelines published in "A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes" will be followed (reference 1).
- Weighing of animals. The frequency depends on the research question and the general condition of the animal. However, standard bodyweight is measured 2-3 times a week, and if the general condition of the animal is getting worse the animals will be weighed daily. The duration is less then one minute.
- Measuring of tumor size. In case tumor size is relevant for the research question, it will be measured 2-3 times per week, with a maximum of 7 times per week. The duration is 2 minutes.

- Imaging under general anesthesia. The frequency depends on the research question. However, the maximum number of times an animal is allowed to recover from anesthesia after imaging is 3 times within one week. The duration of the scan depends on the imaging modalities and ranges from 1 second to 2 hours. Most scans are acquired in less than 1 hour. In case the radiotracer 18F-FDG is used for PET imaging, the animals will be fasted for a maximum of 12 h prior to injection. (the maximum number of times fasting will be used is five times).

Reference 1: Karl-Heinz Diehl et al. Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes. Journal of Applied Toxicology 2001:21;15–23

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

In order to minimize the number of animals all tracers will first be evaluated in vitro. Only tracers that show favorable in vitro characteristics (e.g. high affinity, internalization, immunoreactivity) will be selected for in vivo studies.
For each experiment a power calculation will be performed based on data from literature and previous experiments to determine the minimum amount of animals needed. We will also take into account drop out of animals during the experiment due to experimental procedures, for example surgery, tumor growth, etc. If necessary, a biostatistician will be consulted to assist in these power calculations.

B. The animals

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

In most studies we will use immunodeficient athymic mice, because in these models human tumors can be grown without immunological rejection. For some studies, mice tumor cell lines will be used and they will be grown in syngeneic models (e.g. studies with the B16 melanoma cell line derived from C57BL/6, will be performed in C57Bl/6 mice). The most frequently used animal strains are:
Mice: BALB/c nude, BALB/c rj/nu, SCID, C57Bl/6, BALB/c and Swiss, C3H mice, FVB mice, CBA mice, NSG mice
However, other strains can be used if literature research or previous experiments indicate that this is required to answer the research question.
For most studies, animals will be 6-8 weeks old at start of the experiment. However, for specific research questions, e.g. imaging agents that specifically target tumors in young or elderly mice, animals of the appropriate age will be used.
Based on animal experiments that were performed by our research group in 2012-2014 we expect to use a maximum of 1,000 mice. This calculation is based on the following:
In order to study the therapeutic efficacy we will perform the following studies:
- First, a dose-finding study will be performed to determine at what dose level tumor growth can be inhibited without causing severe toxicity. In general we will need five groups to determine this. Depending on the power calculation, the number of animals per group is on average five. This results in approximately 25 animals per dose-finding study.

- Next, the effect of therapy on tumor growth, survival, and toxicity will be assessed. On average we will need five groups of animals for this. Depending on the power calculation, the number of animals per group is on average 10, which results in approximately 50 mice per study.
- In the third stage we can assess the effect of treatment on molecular processess in the tumor (e.g. proliferation, apoptosis, growth factor receptor expression, etc). For this we will need approximately five groups of animals. Depending on the power calculation, the number of animals per group is on average 5, which results in approximately 25 mice per study.
Based on this, we estimate that we will need approximately 100 mice to fully characterize and optimize a new tumor targeting agent for cancer therapy.
During the last three years, we have developed several tumor targeting agents directed against tumor associated antigens like HER2, TROP-2, CEA, and CAIX. In addition, we are developing new tumor targeting agents directed against PSMA.
We expect to develop two new tumor targeting agents per year. In order to study the therapeutic efficacy and toxicity of these agents we will need 200 mice per jaar. In total this will result in 1,000 mice in five years.

Species	Origin	Maximum number of animals	Life stage
mice		1000	6-8 weeks

C. Re-use

Will the animals be re-used?

[X] 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:

Any new agent will first be characterized extensively in vitro. Only agents with promising characteristics will be applied in animals to study their in vivo behaviour. The aim of the studies is to determine the in vivo behaviour of tumor targeting agents (e.g. tumor targeting, clearance, etc). This can only be performed in living animals and there are currently no alternative models to get these data without the use of animals.
Reduction:
Before each experiment we will perform a literature search to find all information relevant for the animal experiment. In addition, a power calculation will be performed based on data from literature and previous experiments to determine the minimum amount of animals needed. We will also take into account drop out of animals during the experiment due to experimental procedures, for example surgery, tumor growth, etc. In case necessary, a biostatisticaion will be involved in the power calculation.
Refinement:
The experiments will be carried out in mice and rats because there are no alternatives to study the in vivo behaviour of tumor targeting agents in lower animal species. Most experimental procedures will only cause moderate discomfort or less. The use of anesthesia and analgesics is in general not required and may cause even more discomfort that the experimental procedures it self. However, if necessary (e.g. after orthotopic or intracardial injection of tumor cells), anesthesia and analgesics will be applied. During imaging procedures and surgical procedures (when the animal is anesthesized), they will be placed on a warmth mattress. to prevent heat loss. To reduce anxiety, mice will be housed in groups and not individually. The general health of the animals will be monitored daily by the researcher and/or the biotechnician. The methods that we use are well establised and the experiments will be performed by experienced researches. Previous research by our group and others has shown the translational value of this type of studies. Several tumor targeting agents that have been developed in animal models have been translated successfully into the clinic (1-6)

4.Baum RP, Prasad V, Muller D, Schuchardt C, Orlova A, Wennborg A, et al. Molecular imaging of HER2-expressing malignant tumors in breast cancer patients using synthetic 111In- or 68Ga-labeled affibody molecules. J Nucl Med. 2010;51(6):892-7.

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

In order to prevent unneccesary discomfort or stress the general health of the animals will be checked daily by the researches or the biotechnicians. In case animals reach a humane end point the responsible researcher will be contacted.

To reduce axiety, mice will be housed in groups and not individually.
If necessary, anesthesia and analgesics wil be applied to reduce discomfort.

Environmental effects:
During and after the experiments, radioactive or biohazardous waste will be collected seperately and removed according to the university guidelines.

Repetition and Duplication

E. Repetition

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

Not applicable

Accommodation and care

F. Accommodation and care

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

[X] 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?

[X] 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 I.
[X] Yes > Will anaesthesia, analgesiaor other pain relieving methods be used?

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

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

I. Other aspects compromising the welfare of the animals

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

The most frequently occuring types of discomfort are:
- Stress (code 02)
- Discomfort due to tumor growth (code 03)
- Discomfort due to toxicity of treatment (code 03)
- Injections or surgical procedures causing pain and stress (code 03)
- Recovery from anesthesia (code 03)
- Euthanasia (code 02)

Explain why these effects may emerge.

Stress can be caused by the handling of the animals, injections, and other experimental procedures. In addition, the animals can suffer from the recovery from anesthesia after the imaging procedure.

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

To reduce stress, animals will be housed in groups and not indvidually. In addition, before the experiments starts the animals will be housed for a week in order to acclimatise to their new environment.
The tumor growth can cause discomfort, especially in case of large tumors, ulcerating tumors or invasive tumors. In most studies, the experiment will start when the tumor is only 0.1 cm3 and therefore the tumor not likely to cause discomfort due to size, ulceration or invasive growth. However, in some studies, tumor size will be monitored longitudinally, for example when survival is studied. In this case, mice will be checked daily by the researcher or biotechnician and in case the tumors causes severe discomfort, the animal will be taken out of the experiment according to the humane endpoints.

New tumor targeting agents can cause toxicity to the animal and this will be carefully monitored. The animals will be checked daily by the researcher or biotechnician and body weight will be measured up to 7 times a week. In case mice suffer from severe toxicity, the animal will be taken out of the experiment according to the humane endpoints.

Recovery from anesthesia is causing discomfort to the animal. However, this is required in case of imaging of a living animal. In order to minimize this discomfort, the maximum number of times an animal recovers from anesthesia is three times a week and after the last scan, the animal will be euthanized before it recovers from anesthesia.During the anesthesia the animal will be placed on a warmth mattress to prevent heat loss.

The experiments will be carried out by experienced researchers who have been trained to perform these type of studies.

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.
[X] Yes > Describe the criteria that will be used to identify the humane endpoints.

Animals will be taken out of the experiments and humanely euthanized if one the following humane endpoints is reached:
- Tumor size of 2 cm3
- Ulceration or invasive tumor growth causing discomfort to the animal
- Abnormal motility due to tumor growth (severely reduced motility)
- Other signs of clinical discomfort (for example, dehydration, 15% weight loss in less than 2 days, or a decrease of 20% compared to the weight at start of the experiment).

Treatment induced toxicity will cause changes in the humane endpoint as described above. Therefore, no treatment specific endpoints are described.

Indicate the likely incidence.

The most likely primary end point to be met in these studies is tumor size of 2 cm3 or ulceration or invasive tumor growth that causes discomfort to the animal. However, in most cases experiments start when the tumors are approximately 0.1 cm3 and it is unlikely that the tumor reach one of the humane endpoints. However, in some studies the mice will be followed for longer periods (e.g. to measure survival) and they may reach one of these end points. We expect that this will occur in 20% of the animals.

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).

We expect that 85% of the animals will experience moderate discomfort, because either tumor growth is induced or the animals will have to recover from anesthesia which is required for the imaging procedures. The remaining 15% of the animals will experience severe discomfort and will reach the humane end point, for example in therapy experiments due to tumor growth up to 10% of the body weight (2 cm3)

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

L. Method of killing

[] No > Continue with Section 3: 'Signatures'.

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

At the end of the study we will collect tumor tissue to analyze molecular processes in the tumor.

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.

[X] 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'.	10300	
1.2	Provide the name of the licenced establishment.	Stichting Katholieke Universiteit Nijmegen	
1.3	List the different types of animal procedures. Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.	Serial number 3	Type of animal procedure Characterization of in vivo behaviour of new tumor targeting agents in rats

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

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

The aim of the experiments is to determine the in vivo behaviour (e.g. tumor targeting, retention, clearance, etc) of new tumor targeting agents in animal models. For this purpose, the agent will be admistered (e.g. i.v., i.p., etc) to the animal and subsequently the pharmacokinetics and biodistribution of the agent can be studied by means of biodistribution and/or imaging. In addition, the pharmacokinetics can be studied by for example blood sampling or urine collection.
A standard approach to study the in vivo behaviour of a new tumor targeting agent includes the following experiment:
1. Dose optimization: determine which dose of the tumor targeting agent results in the highest tumor uptake with relative low uptake in normal tissue
2. Pharmacokinetics: Determine the pharmacokinetics of the tracer (e.g. how rapidly accumulates the tracer in the tumor and clears from normal tissue and circulation)
3. Imaging of the distribution of the tumor targeting agent in vivo
To quantitatively determine the in vivo behaviour of the tumor targeting agent we will use the following primary end points
- Tumor and normal tissue uptake measured by biodistribution study. In this set up the animal will be euthanized at a certain time point (e.g. 1h, 24h, 72h) after injection of the tumor targeting agent. The animals will be dissected and tumor and normal organs will be collected. The uptake in tissues of interest will be quantified by using for example a gamma counter. Aliquots of the injected tracer will be counted simultaneously.
- Tumor and normal tissue uptake measured by imaging. In this set up, the injection of the radiotracer will be followed by an imaging procedure (e.g. optical, SPECT or PET) under anesthesia. Since it is not necessary to euthanize the animal, this procedure can be repeated at several time points after injection. After the scan, a 3D image will be reconstructed and a region of interest will be drawn around the tumor and tissue of interest to quantitatively determine the activity concentration.
- Pharmacokinetics of a tracer will be determined by taking blood samples (e.g. via cheek puncture, tail cut) or urine samples. The tracer concentration can be measured by for example ELISA or by measuring the radioactive or fluoresent signal (e.g. in a gamma counter).
These outcome parameters have successfully been used in previous studies performed by our research group and by other international research groups. (1-5)

4.Tolmachev V, Varasteh Z, Honarvar H, Hosseinimehr SJ, Eriksson O, Jonasson P, et al. Imaging of platelet-derived growth factor receptor beta expression in glioblastoma xenografts using affibody molecule 111In-DOTA-Z09591. J Nucl Med. 2014;55(2):294-300.

5.Luo H, Hong H, Slater MR, Graves SA, Shi S, Yang Y, et al. PET of c-Met in cancer with 64Cu-labeled Hepatocyte Growth Factor. J Nucl Med. 2015.

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

In a standard experiment the first step is to inject or transplant the animals with a tumor. In case the tumor requires growth factors or hormones to grow, a growth factor pellet will be transplanted subcutaneously. When the tumor reaches a size of approximately 0.1 cm3 (on average this takes 2-3 weeks), the animal will be injected with the tumor targeting agent. After injection, blood samples can be collected to measure the pharmacokinetics of the tracer. Depending on how fast the tracer accumulates in the tumor and clears form the circulation, biodistribution and imaging studies will be performed at several time points (e.g. 1h, 24h, and 72h) after injection to quantitatively determine the biodistribution of the tracer. On average, these type of studies will take three weeks. However, in case of slow growing tumors or longitudinal studies, the total time an animal is in the experiment can be longer (maximum 6 months).
In more detail, the following procedures can be carried out:
- Injection of tumor cells (one or two sided, i.p., i.v., s.c., i.m., orthotopically, intracardially, etc). If necessary, discomfort will be minimized by performing the procedure under generally anesthesia and administering analgesics (e.g. carprofen, temgesic, etc). Frequency: in most cases only one injection of tumor cells will be performed (one tumor per animal). In certain studies (e.g. imaging studies with a receptor positive and receptor negative tumor in one animal), two types of tumor cells will be injected. Depending on how fast the tumors grow, they will be injected at the same time, or there will be a certain time interval (e.g. 1 week) between the two tumor cell injections. The injection takes less then a minute. Orthotopic injection may last longer, depending on site of injection.
- Transplantation of tumor tissue. This procedure will be carried out under general anesthesia and if necessary analgesics will be administered. Frequency: in most cases only one tumor transplantation will be performed (one tumor per animal). In certain studies (e.g. imaging studies with a receptor positive and receptor negative tumor in one animal), two types of tumors will be transplanted. Depending on how fast the tumors grow, they will be transplanted at the same time, or there will be a certain time interval (e.g. 1 week) between the two tumor cell transplantation. Subcutaneous transplantations will take approximately 10 minutes. Orthotopic transplantations may last longer, depending on site of transplantation.
- Particular tumors require hormones or growth factor in order to grow in mice (for example certain breast cancer cells require estrogen to grow). These will be administered by transplanting a growth factor pellet subcutaneously. This procedure will be performed once and under general anesthesia. It will take approximately 10 min. When the injection/transplantation of the tumor cells also requires anesthesia, the transplanation of the pellet will be performed in the same session.
- Administration of tumor targeting agent (i.p., i.v., orally, s.c., etc.) In general, tumor targeting agents will be injected intraveneously. However, in certain studies we can choose a different administration route. For example, in case of intraperitoneal tumor growth of ovarian cancer, the tracer can be injected intraperitoneally as well. The maximum number of injections is six. Frequency: In most studies the tumor targeting agent will be injected once. However, in case of longitudinal studies, agents can be injected multiple times (e.g. every other day, weekly, etc). The duration of injection is approximately 1 minute (depending on the type of injection). The guidelines published in "A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes" will be followed (reference 1).
- Blood sampling (for example via cheek puncture, orbital puncture, tail cut). This can be performed multiple times. The exact method and frequency will depend on the amount of blood that is required to answer the research question. In case the clearance of the tumor targeting agent is studied, 20-30 ul blood will be taken via a tail cut. This can be performed up to 7 times per week (maximum 10 samples). In case blood is collected for further laboratory analysis of for example leucocytes, hemoglobin or creatinin, 200-250 ul blood (max 7.5% of blood volume) will be taken via

cheekpuncture. This can be performed once per week, with a maximum of 10 samples in total.The duration depends on the procedure (cheek puncture: 5 min, tail cut: 3 min). The guidelines published in "A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes" will be followed (reference 1).
- Weighing of animals. The frequency depends on the research question and the general condition of the animal (standard 1-2 per week, but if necessary the animals will be weighed daily). The duration is less then one minute.
- Measuring of tumor size using a caliper. In case tumor size is relevant for the research question, it will be measured 2-3 times per week, and if necessary it will be measured daily. The duration is 2 minutes.
- Imaging under general anesthesia. The frequency depends on the research question. However, the maximum number of times an animal is allowed to recover from anesthesia after imaging is 3 times within one week. The duration of the scan depends on the imaging modalities and ranges from 1 second to 3 hours. Most scans can be acquired in less than 1 hour. In case the radiotracer 18F-FDG is used for PET imaging, the animals will be fasted for a maximum of 12 h prior to injection (fasting will be performed with a maximum of 5 times).
Reference 1: Karl-Heinz Diehl et al. Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes. Journal of Applied Toxicology 2001:21;15–23

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

In order to minimize the number of animals all tracers will first be evaluated in vitro. Only tracers that show favorable in vitro characteristics (e.g. high affinity, internalization, immunoreactivity) will be selected for in vivo studies.
For each experiment a power calculation will be performed based on data from literature and previous experiments to determine the minimum amount of animals needed. We will also take into account drop out of animals during the experiment due to experimental procedures, for example surgery, tumor growth, etc. If necessary, a biostatistician will be consulted to assist in these power calculations.

B. The animals

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

In general, new tumor targeting agents can be tested in mice. However, sometimes rat models are preferred over mice models because of their larger size. For example, we have developed a reliable rat model for colorectal cancer liver metastases, where the tumor cells are injected in the portal vein. This is a realistic metastatic colorectal cancer model, which is difficult to set up in mice because of the very small size of the portal vein. In most studies we will use immunodeficient animals because in these models human tumors can be grown without immunological rejection. For some studies, rat tumor cell lines will be used and they will be grown in syngeneic models (e.g. studies with the CC531 colon carcinoma cell line derived from Wag/Rij, will be performed in Wag/Rij rats). The most frequently used animal strains are:
Rat: Wistar, Lewig, Wag/Rij, BN, Sprague Dawley
However, other strains can be used if literature research or previous experiments indicate that this is required to answer the research question.
For most studies, animals will be 6-8 weeks old at start of the experiment. However, for specific research questions, e.g. imaging agents that specifically target tumors in young or elderly mice, animals of the appropriate age will be used.

Based on animal experiments that were performed by our research group in 2012-2014 we expect to use a maximum of 400 rats. This calculation is based on the following:
In order to study the in vivo behaviour of a new tumor targeting agent, and to optimize it for imaging and therapy purposes, several experiments will be carried out.
- First, a dose-optimization study will be performed to determine which tracer dose will result in the highest tumor uptake with relative low uptake in normal tissue. In general we need six groups to optimize this. Depending on the power calculation, the number of animals per group is on average five. This results in approximately 30 animals per dose optimization study.
- Next, the pharmacokinetics will be studied by performing biodistribution studies at different time points. On average we will need eight groups of animals for this, which results in approximately 40 rats per pharmacokinetics study.
- In the third stage imaging will be performed. For this we will need approximately 10 animals.
Based on this, we estimate that we will need approximately 80 to fully characterize and optimize a new tumor targeting agent for imaging and therapy.
During the last three years, we have developed tumor targeting agents for several tumor associated antigens, eg: HER2, IGF-1R, EGFR, TROP-2, CEA, and CAIX. In addition, we are developing several new tumor targeting agents directed against for example immune-checkpoints, PSMA and Plexin D1.
For each target, different types of radiotracers can be designed, such as monoclonal antibodies or antibody fragments, nanobodies, affibody molecules, peptides, and small molecules. These agents differ in several aspects like affinity, size, tumor accumulation, clearance, etc. In addition, monomers, dimers, or heterodimers can be produced in order to increase the affinity and tumor targeting potential of these agents.
We expect to develop one new radiotracers per year that has to be tested in rats. In order to fully characterize and optimize these agents (dose optimization, pharmacokinetics, imaging) we will need 80 rats per year. In total this will result in 400 animals in five years.

Species	Origin	Maximum number of animals	Life stage
rat		400	6-8 weeks

C. Re-use

Will the animals be re-used?

[X] 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:
Any new agent will first be characterized extensively in vitro. Only agents with promising characteristics will be applied in animals to study their in vivo behaviour. The aim of the studies is to determine the in vivo behaviour of tumor targeting agents (e.g. tumor targeting, clearance, etc). This can only be performed in living animals and there are currently no alternative models to get these data without the use of animals.
Reduction:
Before each experiment we will perform a literature search to find all information relevant for the animal experiment. In addition, a power calculation will be performed based on data from literature and previous experiments to determine the minimum amount of animals needed. We will also take into account drop out of animals during the experiment due to experimental procedures, for example surgery, tumor growth, etc. In case necessary, a biostatisticaion will be involved in the power calculation.
Refinement:
The experiments will be carried out in rats because there are no alternatives to study the in vivo behaviour of tumor targeting agents in lower animal species.In general, new tumor targeting agents can be tested in mice. However, sometimes rat models are preferred over mice models because of their larger size. For example, we have developed a reliable rat model for colorectal cancer liver metastases, where the tumor cells are injected in the portal vein. This is a realistic metastatic colorectal cancer model, which is difficult to set up in mice because of the very small size of the portal vein.
Most experimental procedures will only cause moderate discomfort or less. The use of anesthesia and analgesics is in general not required and may cause even more discomfort that the experimental procedures it self. However, if necessary (e.g. after orthotopic or intracardial injection of tumor cells), anesthesia and analgesics will be applied. During imaging procedures and surgical procedures (when the animal is anesthesized), they will be placed on a warmth mattress. to prevent heat loss. To reduce anxiety, mice will be housed in groups and not individually. The general health of the animals will be monitored daily by the researcher and/or the biotechnician. The methods that we use are well establised and the experiments will be performed by experienced researches. [redacted] shown the translational value of this type of studies. Several tumor targeting agents that have been developed in animal models have been translated successfully into the clinic (1-6)

[redacted]

4.Baum RP, Prasad V, Muller D, Schuchardt C, Orlova A, Wennborg A, et al. Molecular imaging of HER2-expressing malignant tumors in breast cancer patients using synthetic 111In- or 68Ga-labeled affibody molecules. J Nucl Med. 2010;51(6):892-7.

[redacted]

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

In order to prevent unneccesary discomfort or stress the general health of the animals will be checked daily by the researches or the biotechnicians.
In case animals reach a humane end point the responsible researcher will be contacted.
To reduce axiety, mice will be housed in groups and not individually.
If necessary, anesthesia and analgesics wil be applied to reduce discomfort.
For imaging and biodistribution, only a very low dose (tracer dose) of the tumor targeting agent is required. Therefore, we do not expect that this will cause discomfort or side effects.
Environmental effects:
During and after the experiments, radioactive or biohazardous waste will be collected seperately and removed according to the university guidelines.

Repetition and Duplication

E. Repetition

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

Not applicable

Accommodation and care

F. Accommodation and care

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

[X] 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?

[X] 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 I.
[X] Yes > Will anaesthesia, analgesiaor other pain relieving methods be used?

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

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

I. Other aspects compromising the welfare of the animals

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

The most frequently occuring types of discomfort are:

- Stress
- Discomfort due to tumor growth
- Injections or surgical procedures causing pain and stress
- Recovery from anesthesia
- Euthanasia

The tumor targeting agents themself will not cause side effects or discomfort, since the injected dose is very low (tracer dose)

Explain why these effects may emerge.

Stress can be caused by the handling of the animals, injections, and other experimental procedures. In addition, the animals can suffer from the recovery from anesthesia after the imaging procedure.

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

To reduce stress, animals will be housed in groups and not indvidually. In addition, before the experiments starts the animals will be housed for a week in order to acclimatise to their new environment.The tumor growth can cause discomfort, especially in case of large tumors, ulcerating tumors or invasive tumors. In most studies, the experiment will start when the tumor is only 0.1 cm3 and therefore the tumor not likely to cause discomfort due to size, ulceration or invasive growth. In general, animals will be taken out of the experiment before tumors cause severe discomfort. However, in case tumors unexpectedly cause severe ulceration or invasive growth, the animal may experience severe discomfort and will be taken out of the experiment. We expect that this will happen in less than 1% of the animals.
Recovery from anesthesia is causing discomfort to the animal. However, this is required in case of imaging of a living animal. In order to minimize this discomfort, the maximum number of times an animal recovers from anesthesia is three times a week and after the last scan, the animal will be euthanized before it recovers from anesthesia. During the anesthesia the animal will be placed on a warmth mattress to prevent heat loss.
Based on extensive experiments with many different types of radiotracers, we do not expect that these tracers themselves will cause discomfort to the animals.
The experiments will be carried out by experienced researchers who have been trained to perform these type of studies.

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.
[X] Yes > Describe the criteria that will be used to identify the humane endpoints.

Animals will be taken out of the experiments and humanely euthanized if one the following humane endpoints is reached:
• Tumor size > 2 cm3
• Ulceration or invasive tumor growth causing discomfort to the animal
• Abnormal motility due to tumor growth (severely reduced motility)
• Other signs of clinical discomfort (for example, dehydration, 15% weight loss in less than 2 days, or a decrease of 20% compared to the weight at start of the experiment).

Indicate the likely incidence.

In most imaging and biodistribution studies the experiments will start when the tumor is only 0.1 cm3 and thus it is very unlikely that the humane end points are reached. Based on previous experience we expect this to be less than 1%.

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).

We expect that 95% of the animals will experience moderate discomfort, because either tumor growth is induced or the animals will have to recover from anesthesia which is required for the imaging procedures. The remaining 5% of the animals will experience mild discomfort, since we will not induce a tumor and perform imaging. In the exceptional situation that a tumor causes unexpected severe discomfort due to for example ulceration or invasive growth, the animal may experience severe discomfort. This will happen in less than 1% of the animals.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

[] No > Continue with Section 3: 'Signatures'.
[X] Yes > Explain why it is necessary to kill the animals during or after the procedures.

At the end of the study we will determine the biodistribution of the agent ex vivo. In addition, tumors or normal tissue can be analyzed immunohistochemicaly.

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

[] No > Descripe the method of killing that will be used and provide justifications for this choice.

[X] Yes

Appendix
Description animal procedures

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1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	10300	
1.2	Provide the name of the licenced establishment.	Stichting Katholieke Universiteit Nijmegen	
1.3	List the different types of animal procedures. Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.	Serial number 4	Type of animal procedure Assessment of therapeutic efficacy of new tumor targeting agents in rats

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

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

The aim of the experiments is to determine the therapeutic efficacy of new tumor targeting agents in animal models for the treatment of cancer. Tumor targeting agents can be for example antibodies, peptides or small molecules. They can be used 'naked' or be labeled with beta- or alpha-emitting radionuclides (e.g. Lu-177, Y-90 or Bi-213) or cytotoxic drugs. In these experiments, the therapeutic efficacy of these agents will be compared with either the unlabeled compound or conventional anti-cancer treatment (e.g. chemotherapy, irradiation, surgical resection of the tumor, or cell-based therapies)
The primary outcome measurements of these experiments are:
1) Toxicity
Toxicity will be assessed by measuring body weight and by analyzing blood and/or urine for hematological toxicity (hemoglobin, leucocytes, thrombocytes) and renal toxicity (creatinine). Body weight is a reliable indicator of the general health of the animal. In addition, chemotherapy or targeted radiotherapy can cause bonemarrow and kidney toxicity. This can be measured by analyzing blood samples for several hematological and renal parameters. Asssessing the toxicity can not be performed in animal procedure 1, since in these studies only a tracer amount of the tumor targeting agent is administered, while the therapeutic dose is much higher (up to a 100 fold). (1-5)
2) Tumor growth
Tumor growth can be measured by caliper measurements (in case of a subcutaneous tumor) or by non-invasive imaging techniques such as PET, SPECT or MRI (in case of an orthotopic tumor model such as prostate cancer, intraperitoneal tumors, liver metastases, etc). Both methods have shown to reliably monitor tumor growth. (1-5)
3) Survival
Next to tumor growth, the survival is an important primary outcome measure to proof the therapeutic efficacy. Certain therapeutic agents may cause severe toxicity and therefore only measuring tumor growth is not sufficient. For example, tumor growth can be significantly inhibited during the first three weeks, but when the treatment causes severe hematological or renal toxicity, this may result in death of the animals. Thus, the overall effect of the treatment is not beneficial for the animal. In order to study survival, we will longitudinally monitor the animals untill they die or have to be sacrificed based on the humane endpoint (e.g. tumor > 2 cm3, weight loss more than 25% compared to baseline, etc). (1-5)
4) Assessment of molecular processes induced by treatment
Finally, the tumor targeting agent may induce molecular processess in the tumor that can be measured by non-invasive imaging or by analyzing the tumors by western blot or immunohistochemical stainings. An example of such a molecular process is a change in tumor cell proliferation that can be measured by 18F-FLT PET, or a change in glucose metabolism that can be measured with 18F-FDG PET. Changes in 18F-FLT or 18F-FDG often proceed the changes in tumor size as can be measured by caliper measurements and can therefore be used to predict response to treatment. (6, 7)
The experiments will be carried out in the following order:
The first step is to determine which dose results in optimal therapeutic efficacy without inducing severe toxicity (e.g. determination of the maximum tolerable dose (MTD)). The toxicity can be assessed by measuring body weight and by taking blood samples to assess hematological (platelets, leucocytes, thrombocytes) and renal toxicity (creatinine, urea). Next the therapeutic effect is monitored by measuring tumor growth and survival.

Tumor growth can be monitored by caliper measurements or non-invasive imaging techniques (e.g. CT, MRI, PET, SPECT, optical, ultrasound). Finally, we can also study which molecular processes occur in tumors that were treated. Processes of interest are for example proliferation, apoptosis, angiogenesis, expression of growth factor receptor, activation of intracellular signaling pathways. These processes can be measured by non-invasive imaging techniques, or by collecting tumor samples for ex vivo analysis (immunohistochemistry, western blot, etc) at the end of the study.

[redacted]

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

In order to determine the therapeutic effect of tumor targeting agents, the following procedures can be carried out. The study will start with inoculation of a tumor. When the tumor reaches a size of approximately 0.1 cm3 (2-3 weeks) the therapy will be started. Tumor size will be monitored by caliper measurements or non-invasive imaging techniques. Toxicity will be assessed by measuring body weight and by taking blood samples to analyze hematological and renal toxicity. Finally, the molecular processess in tumor (e.g. proliferation, apoptosis, etc) may be monitored by non-invasive imaging techniques, or by removing the tumor at the end of the study for ex vivo analysis (immunohistochemistry, westerblot, etc). The maximum time an animal is in the experiment is 12 months.
In more detail the following procedures will be carried out:
- Injection of tumor cells (one or two sided, i.p., i.v., s.c., i.m., orthotopically, intracardially, etc). If necessary, discomfort will be minimized by performing the procedure under generally anesthesia and administering analgesics (e.g. carprofen, temgesic, etc). Frequency: in most cases only one injection of tumor cells will be performed (one tumor per animal). In certain studies (e.g. imaging studies with a receptor positive and receptor negative tumor in one animal), two types of tumor cells will be injected. Depending on how fast the tumors grow, they will be injected at the same time, or there will be a certain time interval (e.g. 1 week) between the two tumor cell injections. The injection takes less then a minute. Orthotopic injection may last longer, depending on site of injection.

- Transplantation of tumor tissue. This procedure will be carried out under general anesthesia and if necessary analgesics will be administered. Frequency: in most cases only one tumor transplantation will be performed (one tumor per animal). In certain studies (e.g. imaging studies with a receptor positive and receptor negative tumor in one animal), two types of tumors will be transplanted. Depending on how fast the tumors grow, they will be transplanted at the same time, or there will be a certain time interval (e.g. 1 week) between the two tumor cell transplantation. Subcutaneous transplantations will take approximately 10 minutes. Orthotopic transplantations may last longer, depending on site of transplantation.
- Particular tumors require hormones or growth factor in order to grow in mice (for example certain breast cancer cells require estrogen to grow). These will be administered by transplanting a growth factor pellet subcutaneously. This procedure will be performed once and under general anesthesia. It will take approximately 10 min. When the injection/transplantation of the tumor cells also requires anesthesia, the transplanation of the pellet will be performed in the same session - Administration of tumor targeting agent (i.p., i.v., orally, s.c., etc.) In general, tumor targeting agents will be injected intraveneously. However, in certain studies we can choose a different administration route. For example, in case of intraperitoneal tumor growth of ovarian cancer, the tracer can be injected intraperitoneally as well. Frequency: In most studies the tumor targeting agent will be injected once. However, in case of longitudinal studies, agents can be injected multiple times (e.g. every other day, weekly, etc). The maximum number of injections is six. The duration of injection is approximately 1 minute (depending on the type of injection). The guidelines published in "A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes" will be followed (reference 1).

- Administration of conventional anti-cancer therapy such as chemotherapy and antibody-based therapy (i.p., i.v., etc). Conventional anti-cancer therapy is generally injected intraperitoneally. However, certain agents can also be administered i.v., s.c., orally, etc. For example, small-molecule inhibitors like BRAF inhibitors require oral administration. Frequency: depends on the duration of the study. Therapy can be injected once, or in case of longitudinal studies, agents can be injected multiple times (e.g. every other day, weekly, etc). The duration of injection is approximately 1 minute (depending on the type of injection).The guidelines published in "A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes" will be followed (reference 1).
- Irradiation: Mice will undergo irradiation under general anesthesia (2 Gy - 10 Gy). Frequency: in general one dose of 10 Gy will be administered or radiation is administered fractionated (e.g. 3x3Gy). The duration of one irradiation session is approximately 10 minutes. Irradiation can be whole body (2 Gy) or only on the tumor (2 - 10 Gy).
- Blood sampling (for example via cheek puncture, orbital puncture, tail cut). This will be performed multiple times. The exact method and frequency will depend on the amount of blood that is required to answer the research question. In case the clearance of the tumor targeting agent is studied, 20-30 ul blood will be taken via a tail cut. This can be performed up to 7 times per week (maximum 10 samples). In case blood is collected for further laboratory analysis of for example leucocytes, hemoglobin or creatinin, 200-250 ul blood (max 7.5% of blood volume) will be taken via cheekpuncture. This can be performed once per week, with a maximum of 10 samples in total.The duration depends on the procedure (cheek puncture: 5 min, tail cut: 3 min). The guidelines published in "A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes" will be followed (reference 1).
- Weighing of animals. The frequency depends on the research question and the general condition of the animal. However, standard bodyweight is measured 2-3 times a week, and if the general condition of the animal is getting worse the animals will be weighed daily. The duration is less then one minute.
- Measuring of tumor size. In case tumor size is relevant for the research question, it will be measured 2-3 times per week, with a maximum of 7 times per week. The duration is 2 minutes.
- Imaging under general anesthesia. The frequency depends on the research question. However, the maximum number of times an animal is allowed to recover from anesthesia after imaging is 3 times within one week. The duration of the scan depends on the imaging modalities and ranges from 1

second to 2 hours. Most scans are acquired in less than 1 hour. In case the radiotracer 18F-FDG is used for PET imaging, the animals will be fasted for a maximum of 12 h prior to injection. (the maximum number of times fasting will be used is five times).
Reference 1: Karl-Heinz Diehl et al. Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes. Journal of Applied Toxicology 2001:21;15–23

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

In order to minimize the number of animals all tracers will first be evaluated in vitro. Only tracers that show favorable in vitro characteristics (e.g. high affinity, internalization, immunoreactivity) will be selected for in vivo studies.
For each experiment a power calculation will be performed based on data from literature and previous experiments to determine the minimum amount of animals needed. We will also take into account drop out of animals during the experiment due to experimental procedures, for example surgery, tumor growth, etc. If necessary, a biostatistician will be consulted to assist in these power calculations.

B. The animals

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

In general, new tumor targeting agents can be tested in mice. However, sometimes rat models are preferred over mice models because of their larger size. For example, we have developed a reliable rat model for colorectal cancer liver metastases, where the tumor cells are injected in the portal vein. This is a realistic metastatic colorectal cancer model, which is difficult to set up in mice because of the very small size of the portal vein. I n most studies we will use immunodeficient rats, because in these models human tumors can be grown without immunological rejection. For some studies, rat tumor cell lines will be used and they will be grown in syngeneic models (e.g. studies with the CC531 colon carcinoma cell line derived from Wag/Rij, will be performed in Wag/Rij rats). The most frequently used animal strains are:
Rats: Wistar, Lewis, Wag/Rij, BN, Sprague Dewley
However, other strains can be used if literature research or previous experiments indicate that this is required to answer the research question.
For most studies, animals will be 6-8 weeks old at start of the experiment. However, for specific research questions, e.g. imaging agents that specifically target tumors in young or elderly mice, animals of the appropriate age will be used.
Based on animal experiments that were performed [redacted] research group in 2012-2014 [redacted]. This calculation is based on the following:
In order to study the therapeutic efficacy we will perform the following studies:
- First, a dose-finding study will be performed to determine at what dose level tumor growth can be inhibited without causing severe toxicity. In general we will need five groups to determine this. Depending on the power calculation, the number of animals per group is on average five. This results in approximately 25 animals per dose-finding study.
- Next, the effect of therapy on tumor growth, survival, and toxicity will be assessed. On average we will need five groups of animals for this. Depending on the power calculation, the number of animals per group is on average 10, which results in approximately 50 mice per study.

- In the third stage we can assess the effect of treatment on molecular processess in the tumor (e.g. proliferation, apoptosis, growth factor receptor expression, etc). For this we will need approximately five groups of animals. Depending on the power calculation, the number of animals per group is on average 5, which results in approximately 25 rats per study.
Based on this, we estimate that we will need approximately 100 rats to fully characterize and optimize a new tumor targeting agent for cancer therapy.
During the last three years, we have developed several tumor targeting agents directed against tumor associated antigens like HER2, TROP-2, CEA, and CAIX. In addition, we are developing new tumor targeting agents directed against PSMA.
We expect to develop one new tumor targeting agents for therapy per year. In order to study the therapeutic efficacy and toxicity of these agents we will need 100 rats per jaar. In total this will result in 500 rats in five years.

Species	Origin	Maximum number of animals	Life stage
Rat		500	6-8 weeks

C. Re-use

Will the animals be re-used?

[X] 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:

Any new agent will first be characterized extensively in vitro. Only agents with promising characteristics will be applied in animals to study their in vivo behaviour. The aim of the studies is to determine the in vivo behaviour of tumor targeting agents (e.g. tumor targeting, clearance, etc). This can only be performed in living animals and there are currently no alternative models to get these data without the use of animals.
Reduction:
Before each experiment we will perform a literature search to find all information relevant for the animal experiment. In addition, a power calculation will be performed based on data from literature and previous experiments to determine the minimum amount of animals needed. We will also take into account drop out of animals during the experiment due to experimental procedures, for example surgery, tumor growth, etc. In case necessary, a biostatisticaion will be involved in the power calculation.
Refinement:
The experiments will be carried out in mice and rats because there are no alternatives to study the in vivo behaviour of tumor targeting agents in lower animal species. Most experimental procedures will only cause moderate discomfort or less. The use of anesthesia and analgesics is in general not required and may cause even more discomfort that the experimental procedures it self. However, if necessary (e.g. after orthotopic or intracardial injection of tumor cells), anesthesia and analgesics will be applied. During imaging procedures and surgical procedures (when the animal is anesthesized), they will be placed on a warmth mattress. to prevent heat loss. To reduce anxiety, mice will be housed in groups and not individually. The general health of the animals will be monitored daily by the researcher and/or the biotechnician. The methods that we use are well establised and the experiments will be performed by experienced researches. [redacted] others has shown the translational value of this type of studies. Several tumor targeting agents that have been developed in animal models have been translated successfully into the clinic (1-6)

[redacted].

4.Baum RP, Prasad V, Muller D, Schuchardt C, Orlova A, Wennborg A, et al. Molecular imaging of HER2-expressing malignant tumors in breast cancer patients using synthetic 111In- or 68Ga-labeled affibody molecules. J Nucl Med. 2010;51(6):892-7.

[redacted]

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

In order to prevent unneccesary discomfort or stress the general health of the animals will be checked daily by the researches or the biotechnicians. In case animals reach a humane end point the responsible researcher will be contacted.
To reduce axiety, mice will be housed in groups and not individually.

If necessary, anesthesia and analgesics wil be applied to reduce discomfort.
Environmental effects:
During and after the experiments, radioactive or biohazardous waste will be collected seperately and removed according to the university guidelines.

Repetition and Duplication

E. Repetition

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

Not applicable

Accommodation and care

F. Accommodation and care

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

[X] 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?

[X] 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 I.
[X] Yes > Will anaesthesia, analgesiaor other pain relieving methods be used?

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

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

I. Other aspects compromising the welfare of the animals

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

The most frequently occuring types of discomfort are:
- Stress (code 02)
- Discomfort due to tumor growth (code 03)
- Discomfort due to toxicity of treatment (code 03)
- Injections or surgical procedures causing pain and stress (code 03)
- Recovery from anesthesia (code 03)
- Euthanasia (code 02)

Explain why these effects may emerge.

Stress can be caused by the handling of the animals, injections, and other experimental procedures. In addition, the animals can suffer from the recovery from anesthesia after the imaging procedure.

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

To reduce stress, animals will be housed in groups and not indvidually. In addition, before the experiments starts the animals will be housed for a week in order to acclimatise to their new environment.
The tumor growth can cause discomfort, especially in case of large tumors, ulcerating tumors or invasive tumors. In most studies, the experiment will start when the tumor is only 0.1 cm3 and therefore the tumor not likely to cause discomfort due to size, ulceration or invasive growth. However, in some studies, tumor size will be monitored longitudinally, for example when survival is studied. In this case, mice will be checked daily by the researcher or biotechnician and in case the tumors causes severe discomfort, the animal will be taken out of the experiment according to the humane endpoints.
New tumor targeting agents can cause toxicity to the animal and this will be carefully monitored. The animals will be checked daily by the researcher or biotechnician and body weight will be measured up to 7 times a week. In case mice suffer from severe toxicity, the animal will be taken out of the experiment according to the humane endpoints.
Recovery from anesthesia is causing discomfort to the animal. However, this is required in case of imaging of a living animal. In order to minimize this discomfort, the maximum number of times an animal recovers from anesthesia is three times a week and after the last scan, the animal will be euthanized before it recovers from anesthesia.During the anesthesia the animal will be placed on a warmth mattress to prevent heat loss.

The experiments will be carried out by experienced researchers who have been trained to perform these type of studies.

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.
[X] Yes > Describe the criteria that will be used to identify the humane endpoints.

Animals will be taken out of the experiments and humanely euthanized if one the following humane endpoints is reached:
• Tumor size of 2 cm3
• Ulceration or invasive tumor growth causing discomfort to the animal
• Abnormal motility due to tumor growth (severely reduced motility)
• Other signs of clinical discomfort (for example, dehydration, 15% weight loss in less than 2 days, or a decrease of 20% compared to the weight at start of the experiment).
Treatment induced toxicity will cause changes in the humane endpoint as described above. Therefore, no treatment specific endpoints are described.

Indicate the likely incidence.

To reduce stress, animals will be housed in groups and not indvidually. In addition, before the experiments starts the animals will be housed for a week in order to acclimatise to their new environment.
The tumor growth can cause discomfort, especially in case of large tumors, ulcerating tumors or invasive tumors. In most studies, the experiment will start when the tumor is only 0.1 cm3 and therefore the tumor not likely to cause discomfort due to size, ulceration or invasive growth. However, in some studies, tumor size will be monitored longitudinally, for example when survival is studied. In this case, mice will be checked daily by the researcher or biotechnician and in case the tumors causes severe discomfort, the animal will be taken out of the experiment according to the humane endpoints.
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The experiments will be carried out by experienced researchers who have been trained to perform these type of studies.

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).

We expect that 85% of the animals will experience moderate discomfort, because either tumor growth is induced or the animals will have to recover from anesthesia which is required for the imaging procedures. The remaining 15% of the animals will experience severe discomfort and will reach the humane end point, for example in therapy experiments due to tumor growth up to 10% of the body weight (2 cm3)

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

L. Method of killing

[] No > Continue with Section 3: 'Signatures'.

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

At the end of the study we will collect tumor tissue to analyze molecular processes in the tumor.

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.

[X] Yes

6.

DEC-advies

A. Algemene gegevens over de procedure

1. Aanvraagnummer 2015-0071
2. Titel van het project: “Development of new tumor targeting agents for molecular imaging and therapy of cancer”.
3. Titel van de NTS: “Ontwikkeling van tracers om tumoren zichtbaar te maken en kanker gericht te behandelen”.
4. Type aanvraag:
   - ☐ nieuwe aanvraag projectvergunning
5. Contactgegevens DEC:
   - Naam DEC: RUDEC
   - Telefoonnummer contactpersoon: 0[redacted] bereikbaar op maandag, dinsdag, en donderdag van 9:00 tot 15:00 uur
   - Mailadres contactpersoon: [redacted]
6. Adviestraject:
   - ☐ ontvangen door DEC: 23-04-2015
   - ☐ in vergadering besproken: 12-05-2015
   - ☐ schriftelijke vragen gesteld: 18-05-2015
   - ☐ antwoorden en aangepaste aanvraag ontvangen op 19-05-2015
   - ☐ schriftelijke vraag gesteld: 21-05-2015
   - ☐ antwoord en aangepaste aanvraag ontvangen op 24-05-2015
   - ☐ anderszins behandeld: aangepaste aanvraag en finaal advies zijn op 27 juli 2015 in een schriftelijke e-mailronde voorgelegd aan de DEC-leden voor instemming.
   - ☐ termijnonderbreking(en) van 18-05-2015 tot 19-05-2015 en van 21-05-2015 tot 24-05-2015
   - ☐ besluit van CCD tot verlenging van de totale adviestermijn met maximaal 15 werkdagen: n.v.t.
   - ☐ advies aan CCD: 07-08-2015
7. Eventueel horen van aanvrager
   - Datum
   - Plaats
   - Aantal aanwezige DEC-leden
   - Aanwezige (namens) aanvrager
   - Strekking van de vraag / vragen
   - Strekking van het (de) antwoord(en)
   - Het horen van de aanvrager heeft geleid tot aanpassing van de aanvraag
8. Correspondentie met de aanvrager
   - Datum: 18-05-2015
   - Strekking van de vragen:
   -
   - **Project Proposal:**
   - - 3.1 Aan het eind van de tweede alinea is het woord ‘Other’ blijven staan.

- - 3.4.1. In de laatste zin van de alinea die de eerste fase beschrijft wordt in vitro bedoeld terwijl er in vivo staat.
- **Description of Animal Procedures:**
- - DAP2 en DAP4, onderdeel K: Het cumulatief ongerief voor de meeste dieren is matig. In welk percentage van de 1000 muizen of 500 ratten verwachten de onderzoekers ernstig ongerief?
- Datum antwoord: 19-05-2015
- Strekking van de antwoorden:
-
- Project Proposal:
- - 3.1 Het woord 'Other' is verwijderd.
- - 3.4.1 In deze zin is 'in vivo' vervangen door 'in vitro'.
- Description of Animal Procedures:
- - DAP2 en DAP4, onderdeel K: Onderdeel K is als volgt aangepast: We expect that 85% of the animals will experience moderate discomfort, because either tumor growth is induced or the animals will have to recover from anesthesia which is required for the imaging procedures. The remaining 15% of the animals will reach the humane end point, for example in therapy experiments due to tumor growth up to 10% of the body weight (2 cm3).

- Datum: 21-05-2015
- Strekking van de vraag: Verzoek om punt 3.5 van NTS te controleren op juistheid
- Antwoord en aanpassing op 24-05-2015

- De antwoorden hebben geleid tot aanpassing van 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.
2. De aanvraag betreft een nieuwe aanvraag.
3. De DEC is competent om hierover te adviseren.

C. Beoordeling (inhoud):

1. Het project is:

- ☐ uit wetenschappelijk oogpunt verantwoord

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

3. De DEC onderschrijft het belang van de doelstelling, namelijk 'In vivo karakterisering van nieuwe tumor-specifieke tracers voor diagnostiek en therapie van kanker'. De te behalen onderzoeksresultaten zullen er toe leiden dat nieuwe tracers voor de kliniek beschikbaar komen welke tumoren specifiek aan kunnen tonen en bestrijden. De fundamentele kennis over tumorbiologie zal worden vergroot. Dit onderzoeksveld, waarbij ook het niet-invasief volgen van het succes van therapie een belangrijk onderdeel vormt, is sterk in opkomst en staat mondiaal zeer in de belangstelling. De resultaten kunnen op termijn derhalve leiden tot betere detectie, karakterisering en behandeling van kanker bij mensen. Gezien de ernst en de wijdverbreidheid van de aandoeningen waar het hier om gaat en gezien de grote behoefte aan effectieve en individueel gerichte behandelingen acht de DEC het belang van de doelstelling essentieel.
4. De gekozen strategie en experimentele aanpak zijn rechtlijnig en hebben in de afgelopen jaren reeds hun waarde bewezen. Ze kunnen dan ook zeker leiden tot het behalen van de doelstelling binnen het kader van het project. De gekozen aanpak leidt tot betrouwbare uitspraken over de biodistributie en het therapeutische effect van verbeterde of nieuwe tumor-gerichte stoffen in muizen en ratten. De aanvragers nemen al jaren internationaal een vooraanstaande positie in dit onderzoeksveld in en hebben reeds voor meerdere middelen de basis gelegd voor een succesvolle introductie in de kliniek.
5. Er is geen sprake van bijzonderheden op het gebied van categorieën van dieren, omstandigheden of behandeling van de dieren.
6. Het ongerief als gevolg van de dierproeven is realistisch ingeschat en geclassificeerd. De schatting is gebaseerd op jarenlange evaluaties van eigen onderzoek. Het ongerief wordt met name veroorzaakt door het opwekken van tumoren, de tumorgroei, het toedienen van tumor-gerichte agentia en het herhaaldelijk bijkomen uit anesthesie noodzakelijk voor imaging procedures. Als gevolg hiervan ondergaat 4% van de muizen en 2% van de ratten licht ongerief (zij blijven tumor-vrij). De overgrote meerderheid van de dieren, 93% van de muizen en 90% van de ratten, ondergaat matig ongerief. Een kleine minderheid, 3% van de muizen en 8% van de ratten ondergaat ernstig ongerief als gevolg van therapie-experimenten.
7. Er zijn geen methoden die de voorgestelde dierproeven geheel of gedeeltelijk zouden kunnen **vervangen.** De doelstelling van het project kan niet gerealiseerd worden zonder proefdieren of door gebruik van minder complexe diersoorten. Het voorafgaande in vitro werk is uitgebreid en reeds afgerond alvorens een tracer in een dierproef wordt gebruikt. Het in vivo gedrag van de tumor-gerichte stoffen kan alleen in dieren worden uitgetest die qua fysiologie voldoende overeenkomsten met mensen hebben. Het overgrote deel van de proeven wordt met, veelal immunodeficiente, muizen gedaan om humane tumoren te kunnen laten groeien. Daar waar een model een groter proefdier vereist wordt een rat (naar schatting 15% van het totaal aantal gevraagde dieren) gebruikt.
8. In het project wordt optimaal tegemoet gekomen aan de vereiste van de **vermindering** van dierproeven. Het maximale aantal te gebruiken dieren en de grootte van de groepen in de experimenten is realistisch ingeschat en proportioneel ten opzichte van de gekozen strategie en de looptijd. De DEC is het eens met het beschreven onderzoeksmodel en de onderbouwing van het aantal benodigde dieren. In het project worden een aantal tracers ontwikkeld en onderzocht op hun geschiktheid voor diagnose en therapie voor verschillende tumoren. Dit is een continu proces dat over vele jaren zou moeten leiden tot theranostische agentia voor elke specifieke tumor. Het definiëren van milestones is nog niet aan de orde. Wel is het evident dat een beproefde en gestandaardiseerde wijze van karakteriseren van de tracers na elke stap de ruimte

laat om de volgende stap te heroverwegen. De DEC is van oordeel dat het project kan worden uitgevoerd met maximaal 5000 muizen en 900 ratten.

9. Het project is in overeenstemming met de vereiste van de **verfijning** van dierproeven. De experimentele handelingen bij de dieren zullen worden uitgevoerd door hierin getrainde onderzoekers, waardoor de stress voor de dieren zoveel mogelijk wordt beperkt. In alle gevallen waarin dat nodig is worden verdoving en pijnbestrijding toegepast. Wanneer de dieren gedurende langere tijd verdoofd worden zullen zij op een warmtemat geplaatst worden om warmteverlies tegen te gaan. De gezondheid van de dieren wordt dagelijks gecheckt door de onderzoeker of een biotechnicus. Op die manier kunnen dieren die een humaan eindpunt bereiken op humane wijze gedood worden om onnodig lijden van de dieren te voorkomen. De DEC is ervan overtuigd dat de dierproeven zo humaan mogelijk worden uitgevoerd.
   Negatieve effecten op het milieu worden voorkomen door adequate huisvesting van de dieren en de vereiste voorzorgsmaatregelen bij het hanteren van onderzoeksmateriaal en het radioactieve of biologisch gevaarlijk afval.

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

D. Ethische afweging

Op basis van de onder C genoemde overwegingen komt de DEC tot de volgende ethische afweging.

Dit onderzoek is gericht op de ontwikkeling van tumor-specifieke tracers welke geschikt zijn om te gebruiken in de diagnose en therapie van kanker. Dit onderzoeksveld is sterk in ontwikkeling en deze onderzoeksgroep speelt hierin een gekende rol. Het is dan ook zeer aannemelijk dat het project zal leiden tot mogelijkheden tot betere detectie en behandeling van kanker bij de mens. Hierbij moet zeker het uitzicht op vroege detectie van de effectiviteit van therapieën, waardoor bij niet-aanslaan tijdig kan worden gestopt om patiënten niet onnodig te belasten, niet onbenoemd blijven. Gezien de ernst en de wijdverbreidheid van de aandoeningen waar het hier om gaat en gezien de grote behoefte aan ‘gepersonaliseerde’ therapie acht de DEC het belang van de beschreven doelstelling essentieel. De concrete doelstellingen zijn ook haalbaar en kunnen niet zonder dieren worden behaald.

Tegenover dit aanzienlijke belang staat dat 92% van de dieren matig en 4% ernstig ongerief zal ondervinden voornamelijk door het aanbrengen, volgen en behandelen van tumoren. De commissie is er van overtuigd dat bij de dierproeven adequaat invulling zal worden gegeven aan de vereisten op het gebied van de vervanging, vermindering en/of verfijning. Het resterende ongerief is onvermijdelijk, wil men de doelstellingen kunnen realiseren. De experimenten komen qua design overeen met wat in het onderzoeksveld gebruikelijk is.

De DEC is van oordeel dat het hier boven geschetste belang de onvermijdelijke nadelige gevolgen van dit onderzoek voor de dieren, in de vorm van angst, pijn of stress, rechtvaardigt. Aan de eis dat het belang van de experimenten op dient te wegen tegen het ongerief dat de dieren wordt berokkend, is voldaan.

E. Advies

1. Advies aan de CCD

- ☐ De DEC adviseert de vergunning te verlenen

2. Het uitgebrachte advies is gebaseerd op consensus.

7.

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

Stichting Katholieke Universiteit Nijmegen
p/[redacted]
Postbus 9101
6500 HB NIJMEGEN

Centrale Commissie Dierproeven
Postbus 20401
2500 EK Den Haag
www.zbo-ccd.nl
0900 28 000 28 (10 ct/min)

Onze referentie
Aanvraagnummer
AVD103002015209
Bijlagen
2

Datum 11-08-2015
Betreft Ontvangstbevestiging Aanvraag projectvergunning Dierproeven

Geachte heer/mevrouw [redacted]
Wij hebben uw aanvraag voor een projectvergunning dierproeven ontvangen op 8 augustus 2015.
Het aanvraagnummer dat wij aan deze aanvraag hebben toegekend is AVD103002015209. 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. Zodra uw aanvraag compleet is, ontvangt u binnen veertig werkdagen een beslissing op uw aanvraag. 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 wordt uw aanvraag buiten behandeling 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.zbo-ccd.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:	10300
Naam instelling of organisatie:	Stichting Katholieke Universiteit Nijmegen
Naam portefeuillehouder of diens gemachtigde:	[redacted]
KvK-nummer:	41055629
Straat en huisnummer:	Geert Grooteplein 10
Postbus:	9101
Postcode en plaats:	6500 HB NIJMEGEN
IBAN:	NL90ABNA0231209983
Tenaamstelling van het rekeningnummer:	UMC St Radboud

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

Gegevens verantwoordelijke uitvoering proces

Naam:	[redacted]
Functie:	Instantie voor Dierenwelzijn
Afdeling:	[redacted]
Telefoonnummer:	[redacted]
E-mailadres:	instantievoordierenwelzijn@radboudumc.nl

Gegevens gemachtigde

BSN:	[redacted]
Naam:	[redacted]
Postbus:	9101
Postcode en plaats:	6500 HB NIJMEGEN
Wilt u een nieuwe machtiging afgeven?	Ja

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:	8 september 2015
Geplande einddatum:	8 september 2020
Titel project:	Development of new tumor targeting agents for molecular imaging and therapy of cancer
Titel niet-technische samenvatting:	Ontwikkeling van tracers om tumoren zichtbaar te maken en kanker gericht te behandelen
Naam DEC:	RU DEC
Postadres DEC:	Postbus 9101, 6500 HB Nijmegen (627 DEC B4)
E-mailadres DEC:	dec@iwkv.umcn.nl

Betaalgegevens

De leges bedragen:	€ 741,-
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] Melding Machtiging [x] DEC-advies

Ondertekening

Naam:	[redacted]
Functie:	Instantie voor dierenwelzijn
Plaats:	Nijmegen
Datum:	8 augustus 2015

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

Stichting Katholieke Universiteit Nijmegen
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Postbus 20401
2500 EK Den Haag
www.zbo-ccd.nl
0900 28 000 28 (10 ct/min)

Onze referentie
Aanvraagnummer
AVD103002015209

Bijlagen
2

Datum 11-08-2015
Betreft Ontvangstbevestiging Aanvraag projectvergunning Dierproeven

Factuur
Factuurdatum: 11 augustus 2015
Vervaldatum: 10 september 2015
Factuurnummer: 201570209

Omschrijving	**Bedrag**
Betaling leges projectvegrunning dierproeven Betreft aanvraag AVD103002015209	€ 741,00

Wij verzoeken u het totaalbedrag vóór de gestelde vervaldatum over te maken op rekening NL28RBOS 056.99.96.066 onder vermelding van het factuurnummer en aanvraagnummer, ten name van Centrale Commissie Dierproeven, Postbus 20401, 2500 EK te 's Gravenhage.

8.

[redacted]

Van: [redacted]
Verzonden: dinsdag 25 augustus 2015 9:30
Aan: [redacted]
Onderwerp: RE: vraag AVD103002015209

Beste [redacted],

Kanker komt zowel bij mannen als bij vrouwen voor. Het is daarom van belang om ons onderzoek in beide geslachten uit te voeren. Echter binnen een experiment gebruiken wij bij voorkeur een geslacht proefdieren, zodat de spreiding van de resultaten zo klein mogelijk blijft. Hierdoor kunnen we in experimenten met kleinere groepen proefdieren gebruiken (n=5). In een latere fase van het onderzoek dienen we in bepaalde projecten hormonale invloeden uit te sluiten, waarvoor we aldus enkele malen het andere geslacht zullen gebruiken. In het algemeen gebruiken we bij voorkeur vrouwelijke dieren, omdat mannetjes vaker vechten (wat huisvesting bemoeilijkt en uitval tot gevolg heeft). In het geval dat het onderzoek zich richt op tumor typen die alleen bij mannen voorkomen (zoals prostaatkanker, testis kanker, etc), maken we natuurlijk gebruik van mannelijke proefdieren. In de herziene aanvraag, onder de onderdelen 'choice and justification of animals' is een korte beschrijving hiervan opgenomen.

Met vriendelijke groet,

[redacted]
Radboud University Medical Center
[redacted]
P.O Box 9101
6500 HB Nijmegen
[redacted]

PRIME
preclinical imaging centre

Van: Info-zbo [mailto:info@zbo-ccd.nl]
Verzonden: maandag 17 augustus 2015 17:02
Aan: [redacted]
Onderwerp: vraag AVD103002015209

Geachte [redacted]

Uw aanvraag getiteld: Development of new tumor targeting agents for molecular imaging and therapy of cancer met aanvraagnummer AVD103002015209 is bij de CCD aangeboden ter beoordeling. U beschrijft in de verschillende bijlagen dierproeven het gebruik van muizen en u beschrijft het huisvesten van de dieren in groepshuisvesting om

stress te reduceren. Kunt u nader toelichten of u mannelijke of vrouwelijke dieren gebruikt of dieren van beide geslachten.

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 emailadres!)

9.

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'.	10300	
1.2	Provide the name of the licenced establishment.	Stichting Katholieke Universiteit Nijmegen	
1.3	List the different types of animal procedures. Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.	Serial number 1	Type of animal procedure Characterization of in vivo behaviour of new tumor targeting agents in mice

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

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

The aim of the experiments is to determine the in vivo behaviour (e.g. tumor targeting, retention, clearance, etc) of new tumor targeting agents in animal models. For this purpose, the agent will be admistered (e.g. i.v., i.p., etc) to the animal and subsequently the pharmacokinetics and biodistribution of the agent can be studied by means of biodistribution and/or imaging. In addition, the pharmacokinetics can be studied by for example blood sampling or urine collection.
A standard approach to study the in vivo behaviour of a new tumor targeting agent includes the following experiment:
1. Dose optimization: determine which dose of the tumor targeting agent results in the highest tumor uptake with relative low uptake in normal tissue
2. Pharmacokinetics: Determine the pharmacokinetics of the tracer (e.g. how rapidly accumulates the tracer in the tumor and clears from normal tissue and circulation)
3. Imaging of the distribution of the tumor targeting agent in vivo

To quantitatively determine the in vivo behaviour of the tumor targeting agent we will use the following primary end points
- Tumor and normal tissue uptake measured by biodistribution study. In this set up the animal will be euthanized at a certain time point (e.g. 1h, 24h, 72h) after injection of the tumor targeting agent. The animals will be dissected and tumor and normal organs will be collected. The uptake in tissues of interest will be quantified by using for example a gamma counter. Aliquots of the injected tracer will be counted simultaneously.
- Tumor and normal tissue uptake measured by imaging. In this set up, the injection of the radiotracer will be followed by an imaging procedure (e.g. optical, SPECT or PET) under anesthesia. Since it is not necessary to euthanize the animal, this procedure can be repeated at several time points after injection. After the scan, a 3D image will be reconstructed and a region of interest will be drawn around the tumor and tissue of interest to quantitatively determine the activity concentration.
- Pharmacokinetics of a tracer will be determined by taking blood samples (e.g. via cheek puncture, tail cut) or urine samples. The tracer concentration can be measured by for example ELISA or by measuring the radioactive or fluoresent signal (e.g. in a gamma counter).
These outcome parameters have successfully been used in previous studies performed by our research group and by other international research groups. (1-5)

1. [redacted]

4. Tolmachev V, Varasteh Z, Honarvar H, Hosseinimehr SJ, Eriksson O, Jonasson P, et al. Imaging of platelet-derived growth factor receptor beta expression in glioblastoma xenografts using affibody molecule 111In-DOTA-Z09591. J Nucl Med. 2014;55(2):294-300.

5. [redacted]

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

In a standard experiment the first step is to inject or transplant the animals with a tumor. In case the tumor requires growth factors or hormones to grow, a growth factor pellet will be transplanted subcutaneously. When the tumor reaches a size of approximately 0.1 cm3 (on average this takes 2-3 weeks), the animal will be injected with the tumor targeting agent. After injection, blood samples can be collected to measure the pharmacokinetics of the tracer. Depending on how fast the tracer accumulates in the tumor and clears form the circulation, biodistribution and imaging studies will be performed at several time points (e.g. 1h, 24h, and 72h) after injection to quantitatively determine the biodistribution of the tracer. On average, these type of studies will take three weeks. However, in case of slow growing tumors or longitudinal studies, the total time an animal is in the experiment can be longer (maximum 6 months).

In more detail, the following experimental steps can be carried out:
- Injection of tumor cells (one or two sided, i.p., i.v., s.c., i.m., orthotopically, intracardially, etc). If necessary, discomfort will be minimized by performing the procedure under generally anesthesia and administering analgesics (e.g. carprofen, temgesic, etc). Frequency: in most cases only one injection of tumor cells will be performed (one tumor per animal). In certain studies (e.g. imaging studies with a receptor positive and receptor negative tumor in one animal), two types of tumor cells will be injected. Depending on how fast the tumors grow, they will be injected at the same time, or there will be a certain time interval (e.g. 1 week) between the two tumor cell injections. The injection takes less then a minute. Orthotopic injection may last longer, depending on site of injection.
- Transplantation of tumor tissue. This procedure will be carried out under general anesthesia and if necessary analgesics will be administered. Frequency: in most cases only one tumor transplantation will be performed (one tumor per animal). In certain studies (e.g. imaging studies with a receptor positive and receptor negative tumor in one animal), two types of tumors will be transplanted. Depending on how fast the tumors grow, they will be transplanted at the same time, or there will be a certain time interval (e.g. 1 week) between the two tumor cell transplantation. Subcutaneous transplantations will take approximately 10 minutes. Orthotopic transplantations may last longer, depending on site of transplantation.
- Particular tumors require hormones or growth factor in order to grow in mice (for example certain breast cancer cells require estrogen to grow). These will be administered by transplanting a growth factor pellet subcutaneously. This procedure will be performed once and under general anesthesia. It will take approximately 10 min. When the injection/transplantation of the tumor cells also requires anesthesia, the transplanation of the pellet will be performed in the same session.
- Administration of tumor targeting agent (i.p., i.v., orally, s.c., etc.) In general, tumor targeting agents will be injected intraveneously. However, in certain studies we can choose a different administration route. For example, in case of intraperitoneal tumor growth of ovarian cancer, the tracer can be injected intraperitoneally as well. Frequency: In most studies the tumor targeting agent will be injected once. However, in case of longitudinal studies, agents can be injected multiple times (e.g. every other day, weekly, etc.). The maximum number of injections of a tumor targeting agent is six. The duration of injection is approximately 1 minute (depending on the type of injection). The guidelines published in "A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes" will be followed (reference 1).

- Blood sampling (for example via cheek puncture, orbital puncture, tail cut). This can be performed multiple times. The exact method and frequency will depend on the amount of blood that is required to answer the research question. In case the clearance of the tumor targeting agent is studied, 15-20 ul blood will be taken via a tail cut. This can be performed up to 7 times per week (maximum 10 samples). In case blood is collected for further laboratory analysis of for example leucocytes, hemoglobin or creatinin, 100-150 ul blood (max 7.5% of blood volume) will be taken via cheekpuncture. This can be performed once per week, with a maximum of 10 samples in total. The duration depends on the procedure (cheek puncture: 5 min, tail cut: 3 min). The guidelines published in "A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes" will be followed (reference 1).
- Weighing of animals. The frequency depends on the research question and the general condition of the animal (standard 1-2 per week, but if necessary the animals will be weighed daily). The duration is less then one minute.
- Measuring of tumor size using a caliper. In case tumor size is relevant for the research question, it will be measured 2-3 times per week, and if necessary it will be measured daily. The duration is 2 minutes.
- Imaging under general anesthesia. The frequency depends on the research question. However, the maximum number of times an animal is allowed to recover from anesthesia after imaging is 3 times within one week. The duration of the scan depends on the imaging modalities and ranges from 1 second to 3 hours. Most scans can be acquired in less than 1 hour. In case the radiotracer 18F-FDG is used for PET imaging, the animals will be fasted for a maximum of 12 h prior to injection (the maximum number of times fasting will be used is five times).

Reference 1: Karl-Heinz Diehl et al. Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes. Journal of Applied Toxicology 2001:21;15–23

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

In order to minimize the number of animals all tracers will first be evaluated in vitro. Only tracers that show favorable in vitro characteristics (e.g. high affinity, internalization, immunoreactivity) will be selected for in vivo studies.
For each experiment a power calculation will be performed based on data from literature and previous experiments to determine the minimum amount of animals needed. We will also take into account drop out of animals during the experiment due to experimental procedures, for example surgery, tumor growth, etc. If necessary, a biostatistician will be consulted to assist in these power calculations.

B. The animals

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

In most studies we will use immunodeficient athymic mice, because in these models human tumors can be grown without immunological rejection. For some studies, mice tumor cell lines will be used and they will be grown in syngeneic models (e.g. studies with the B16 melanoma cell line derived from C57BL/6, will be performed in C57Bl/6 mice). The most frequently used animal strains are:
Mice: BALB/c nude, BALB/c rj/nu, SCID, C57Bl/6, BALB/c and Swiss, C3H mice, FVB mice, CBA mice, NSG mice
However, other strains can be used if literature research or previous experiments indicate that this is required to answer the research question.

For most studies, animals will be 6-8 weeks old at start of the experiment. However, for specific research questions, e.g. imaging agents that specifically target tumors in young or elderly mice, animals of the appropriate age will be used.
Since cancer is a disease in both men and women, research can be carried out in both sexes. However, within one experiment we will select only one sexe to minimize the variation within the groups and therefore the experiment can be carried out in smaller groups of animals. In general we will use female animals, because male animals more frequently fight. This results in additional stress, and in case of severe fighting this may incidentally cause problems with housing and loss of animals. However, in case research focuses on tumor types that only occur in male patients (e.g. prostate cancer, testis cancer, etc), we will use male animals. Also, for some research questions, hormones (e.g. testosterone/estradiol) play an important role and will influence whether male or female animals will be selected for the study.
Based on animal experiments that were performed by our research group in 2012-2014 we expect to use a maximum of 4,000 mice. This calculation is based on the following:
In order to study the in vivo behaviour of a new tumor targeting agent, and to optimize it for imaging and therapy purposes, several experiments will be carried out.
- First, a dose-optimization study will be performed to determine which tracer dose will result in the highest tumor uptake with relative low uptake in normal tissue. In general we need six groups to optimize this. Depending on the power calculation, the number of animals per group is on average five. This results in approximately 30 animals per dose optimization study.
- Next, the pharmacokinetics will be studied by performing biodistribution studies at different time points. On average we will need eight groups of animals for this, which results in approximately 40 mice per pharmacokinetics study.
- In the third stage imaging will be performed. For this we will need approximately 10 animals.
Based on this, we estimate that we will need approximately 80 mice to fully characterize and optimize a new tumor targeting agent for imaging and therapy.
During the last three years, we have developed tumor targeting agents for several tumor associated antigens, [redacted]
For each target, different types of radiotracers can be designed, such as monoclonal antibodies or antibody fragments, nanobodies, affibody molecules, peptides, and small molecules. These agents differ in several aspects like affinity, size, tumor accumulation, clearance, etc. In addition, monomers, dimers, or heterodimers can be produced in order to increase the affinity and tumor targeting potential of these agents.
We expect to develop ten new radiotracers per year. In order to fully characterize and optimize these agents (dose optimization, pharmacokinetics, imaging) we will need 800 mice per year. In total this will result in 4,000 animals in five years.

Species	Origin	Maximum number of animals	Life stage
mice		4000	6-8 weeks

C. Re-use

Will the animals be re-used?

[X] No, continue with question D.
[] Yes > Explain why re-use is considered acceptable for this animal procedure.

C. Re-use

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:
Any new agent will first be characterized extensively in vitro. Only agents with promising characteristics will be applied in animals to study their in vivo behaviour. The aim of the studies is to determine the in vivo behaviour of tumor targeting agents (e.g. tumor targeting, clearance, etc). This can only be performed in living animals and there are currently no alternative models to get these data without the use of animals.
Reduction:
Before each experiment we will perform a literature search to find all information relevant for the animal experiment. In addition, a power calculation will be performed based on data from literature and previous experiments to determine the minimum amount of animals needed. We will also take into account drop out of animals during the experiment due to experimental procedures, for example surgery, tumor growth, etc. In case necessary, a biostatisticaion will be involved in the power calculation.
Refinement:
The experiments will be carried out in mice and rats because there are no alternatives to study the in vivo behaviour of tumor targeting agents in lower animal species. Most experimental procedures will only cause moderate discomfort or less. The use of anesthesia and analgesics is in general not required and may cause even more discomfort that the experimental procedures it self. However, if necessary (e.g. after orthotopic or intracardial injection of tumor cells), anesthesia and analgesics will be applied. During imaging procedures and surgical procedures (when the animal is anesthesized), they will be placed on a warmth mattress. to prevent heat loss. To reduce anxiety, mice will be housed in groups and not individually. The general health of the animals will be monitored daily by the researcher and/or the biotechnician. The methods that we use are well establised and the experiments will be performed by experienced researches. Previous research [redacted] and others has shown the translational value of this type of studies. Several tumor targeting agents that have been developed in animal models have been translated successfully into the clinic (1-6)

[redacted]

[redacted]

4. Baum RP, Prasad V, Muller D, Schuchardt C, Orlova A, Wennborg A, et al. Molecular imaging of HER2-expressing malignant tumors in breast cancer patients using synthetic 111In- or 68Ga-labeled affibody molecules. J Nucl Med. 2010;51(6):892-7.

[redacted]

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

In order to prevent unneccesary discomfort or stress the general health of the animals will be checked daily by the researches or the biotechnicians.
In case animals reach a humane end point the responsible researcher will be contacted.
To reduce axiety, mice will be housed in groups and not individually.
If necessary, anesthesia and analgesics wil be applied to reduce discomfort.
For imaging and biodistribution, only a very low dose (tracer dose) of the tumor targeting agent is required. Therefore, we do not expect that this will cause discomfort or side effects.

Environmental effects:
During and after the experiments, radioactive or biohazardous waste will be collected seperately and removed according to the university guidelines.

Repetition and Duplication

E. Repetition

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

Not applicable

Accommodation and care

F. Accommodation and care

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

[X] 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?

[X] 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 I.
[X] Yes > Will anaesthesia, analgesiaor other pain relieving methods be used?

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

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

I. Other aspects compromising the welfare of the animals

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

The most frequently occuring types of discomfort are:
- Stress
- Discomfort due to tumor growth
- Injections or surgical procedures causing pain and stress
- Recovery from anesthesia
- Euthanasia

The tumor targeting agents themself will not cause side effects or discomfort, since the injected dose is very low (tracer dose)

Explain why these effects may emerge.

Stress can be caused by the handling of the animals, injections, and other experimental procedures. In addition, the animals can suffer from the recovery from anesthesia after the imaging procedure.

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

To reduce stress, animals will be housed in groups and not indvidually. In addition, before the experiments starts the animals will be housed for a week in order to acclimatise to their new environment.

The tumor growth can cause discomfort, especially in case of large tumors, ulcerating tumors or invasive tumors. In most studies, the experiment will start when the tumor is only 0.1 cm3 and therefore the tumor not likely to cause discomfort due to size, ulceration or invasive growth. In general, animals will be taken out of the experiment before tumorscause severe discomfort. However, in case tumors unexpectedly cause severe ulceration or invasive growth, the animal may experience severe discomfort and will be taken out of the experiment. We expect that this will happen in less than 1% of the animals.

Recovery from anesthesia is causing discomfort to the animal. However, this is required in case of imaging of a living animal. In order to minimize

this discomfort, the maximum number of times an animal recovers from anesthesia is three times a week and after the last scan, the animal will be euthanized before it recovers from anesthesia. During the anesthesia the animal will be placed on a warmth mattress to prevent heat loss.

Based on extensive experiments with many different types of radiotracers, we do not expect that these tracers themselves will cause discomfort to the animals.
The experiments will be carried out by experienced researchers who have been trained to perform these type of studies.

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.
[X] Yes > Describe the criteria that will be used to identify the humane endpoints.

Animals will be taken out of the experiments and humanely euthanized if one the following humane endpoints is reached:
- Tumor size > 2 cm3
- Ulceration or invasive tumor growth causing discomfort to the animal
- Abnormal motility due to tumor growth (severely reduced motility)
- Other signs of clinical discomfort (for example, dehydration, 15% weight loss in less than 2 days, or a decrease of 20% compared to the weight at start of the experiment).

Indicate the likely incidence.

In most imaging and biodistribution studies the experiments will start when the tumor is only 0.1 cm3 and thus it is very unlikely that the humane end points are reached. Based on previous experience we expect this to be less than 1%.

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).

We expect that 95% of the animals will experience moderate discomfort, because either tumor growth is induced or the animals will have to recover from anesthesia which is required for the imaging procedures. The remaining 5% of the animals will experience mild discomfort, since we will not induce a tumor and perform imaging. In the exceptional situation that a tumor causes unexpected severe discomfort due to for example ulceration or invasive growth, the animal may experience severe discomfort. This will happen in less than 1% of the animals.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

[] No > Continue with Section 3: 'Signatures'.
[X] Yes > Explain why it is necessary to kill the animals during or after the procedures.

At the end of the study we will determine the biodistribution of the agent ex vivo. In addition, tumors or normal tissue can be analyzed immunohistochemicaly.

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.

[X] 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.
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1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	10300	
1.2	Provide the name of the licenced establishment.	Stichting Katholieke Universiteit Nijmegen	
1.3	List the different types of animal procedures. Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.	Serial number 2	Type of animal procedure Assessment of therapeutic efficacy of new tumor targeting agents in mice

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

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

The aim of the experiments is to determine the therapeutic efficacy of new tumor targeting agents in animal models for the treatment of cancer. Tumor targeting agents can be for example antibodies, peptides or small molecules. They can be used 'naked' or be labeled with beta- or alpha-emitting radionuclides (e.g. Lu-177, Y-90 or Bi-213) or cytotoxic drugs. In these experiments, the therapeutic efficacy of these agents will be compared with either the unlabeled compound or conventional anti-cancer treatment (e.g. chemotherapy, irradiation, surgical resection of the tumor, or cell-based therapies)

The primary outcome measurements of these experiments are:
1) Toxicity
Toxicity will be assessed by measuring body weight and by analyzing blood and/or urine for hematological toxicity (hemoglobin, leucocytes, thrombocytes) and renal toxicity (creatinine). Body weight is a reliable indicator of the general health of the animal. In addition, chemotherapy or targeted radiotherapy can cause bonemarrow and kidney toxicity. This can be measured by analyzing blood samples for several hematological and renal parameters. Asssessing the toxicity can not be performed in animal procedure 1, since in these studies only a tracer amount of the tumor targeting agent is administered, while the therapeutic dose is much higher (up to a 100 fold). (1-5)
2) Tumor growth
Tumor growth can be measured by caliper measurements (in case of a subcutaneous tumor) or by non-invasive imaging techniques such as PET, SPECT or MRI (in case of an orthotopic tumor model such as prostate cancer, intraperitoneal tumors, liver metastases, etc). Both methods have shown to reliably monitor tumor growth. (1-5)
3) Survival
Next to tumor growth, the survival is an important primary outcome measure to proof the therapeutic efficacy. Certain therapeutic agents may cause severe toxicity and therefore only measuring tumor growth is not sufficient. For example, tumor growth can be significantly inhibited during the first three weeks, but when the treatment causes severe hematological or renal toxicity, this may result in death of the animals. Thus, the overall effect of the treatment is not beneficial for the animal. In order to study survival, we will longitudinally monitor the animals untill they die or have to be sacrificed based on the humane endpoint (e.g. tumor > 2 cm3, weight loss more than 25% compared to baseline, etc). (1-5)
4) Assessment of molecular processes induced by treatment
Finally, the tumor targeting agent may induce molecular processess in the tumor that can be measured by non-invasive imaging or by analyzing the tumors by western blot or immunohistochemical stainings. An example of such a molecular process is a change in tumor cell proliferation that can be measured by 18F-FLT PET, or a change in glucose metabolism that can be measured with 18F-FDG PET. Changes in 18F-FLT or 18F-FDG often proceed the changes in tumor size as can be measured by caliper measurements and can therefore be used to predict response to treatment. (6, 7)
The experiments will be carried out in the following order:
The first step is to determine which dose results in optimal therapeutic efficacy without inducing severe toxicity (e.g. determination of the maximum tolerable dose (MTD)). The toxicity can be assessed by measuring body weight and by taking blood samples to assess hematological (platelets,

leucocytes, thrombocytes) and renal toxicity (creatinine, urea). Next the therapeutic effect is monitored by measuring tumor growth and survival. Tumor growth can be monitored by caliper measurements or non-invasive imaging techniques (e.g. CT, MRI, PET, SPECT, optical, ultrasound). Finally, we can also study which molecular processes occur in tumors that were treated. Processes of interest are for example proliferation, apoptosis, angiogenesis, expression of growth factor receptor, activation of intracellular signaling pathways. These processes can be measured by non-invasive imaging techniques, or by collecting tumor samples for ex vivo analysis (immunohistochemistry, western blot, etc) at the end of the study.

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

In order to determine the therapeutic effect of tumor targeting agents, the following procedures can be carried out. The study will start with inoculation of a tumor. When the tumor reaches a size of approximately 0.1 cm3 (2-3 weeks) the therapy will be started. Tumor size will be monitored by caliper measurements or non-invasive imaging techniques. Toxicity will be assessed by measuring body weight and by taking blood samples to analyze hematological and renal toxicity. Finally, the molecular processess in tumor (e.g. proliferation, apoptosis, etc) may be monitored by non-invasive imaging techniques, or by removing the tumor at the end of the study for ex vivo analysis (immunohistochemistry, westerblot, etc). The maximum time an animal is in an experiment is 12 months.

In more detail the following procedures will be carried out:
- Injection of tumor cells (one or two sided, i.p., i.v., s.c., i.m., orthotopically, intracardially, etc). If necessary, discomfort will be minimized by performing the procedure under generally anesthesia and administering analgesics (e.g. carprofen, temgesic, etc). Frequency: in most cases only one injection of tumor cells will be performed (one tumor per animal). In certain studies (e.g. imaging studies with a receptor positive and receptor negative tumor in one animal), two types of tumor cells will be injected. Depending on how fast the tumors grow, they will be injected at the same

time, or there will be a certain time interval (e.g. 1 week) between the two tumor cell injections. The injection takes less then a minute. Orthotopic injection may last longer, depending on site of injection.
- Transplantation of tumor tissue. This procedure will be carried out under general anesthesia and if necessary analgesics will be administered. Frequency: in most cases only one tumor transplantation will be performed (one tumor per animal). In certain studies (e.g. imaging studies with a receptor positive and receptor negative tumor in one animal), two types of tumors will be transplanted. Depending on how fast the tumors grow, they will be transplanted at the same time, or there will be a certain time interval (e.g. 1 week) between the two tumor cell transplantation. Subcutaneous transplantations will take approximately 10 minutes. Orthotopic transplantations may last longer, depending on site of transplantation.
- Particular tumors require hormones or growth factor in order to grow in mice (for example certain breast cancer cells require estrogen to grow). These will be administered by transplanting a growth factor pellet subcutaneously. This procedure will be performed once and under general anesthesia. It will take approximately 10 min. When the injection/transplantation of the tumor cells also requires anesthesia, the transplanation of the pellet will be performed in the same session
- Administration of tumor targeting agent (i.p., i.v., orally, s.c., etc.) In general, tumor targeting agents will be injected intraveneously. However, in certain studies we can choose a different administration route. For example, in case of intraperitoneal tumor growth of ovarian cancer, the tracer can be injected intraperitoneally as well. Frequency: In most studies the tumor targeting agent will be injected once. However, in case of longitudinal studies, agents can be injected multiple times (e.g. every other day, weekly, etc). The maximum number of injections in six. The duration of injection is approximately 1 minute (depending on the type of injection). The guidelines published in "A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes" will be followed (reference 1).

- Administration of conventional anti-cancer therapy such as chemotherapy and antibody-based therapy (i.p., i.v., etc). Conventional anti-cancer therapy is generally injected intraperitoneally. However, certain agents can also be administered i.v., s.c., orally, etc. For example, small-molecule inhibitors like BRAF inhibitors require oral administration. Frequency: depends on the duration of the study. Therapy can be injected once, or in case of longitudinal studies, agents can be injected multiple times (e.g. every other day, weekly, etc). The duration of injection is approximately 1 minute (depending on the type of injection).The guidelines published in "A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes" will be followed (reference 1).
- Irradiation: Mice will undergo irradiation under general anesthesia (2 Gy - 10 Gy). Frequency: in general one dose of 10 Gy will be administered or radiation is administered fractionated (e.g. 3x3Gy). The duration of one irradiation session is approximately 10 minutes. Irradiation can be whole body (2 Gy) or only on the tumor (2 - 10 Gy).
- Blood sampling (for example via cheek puncture, orbital puncture, tail cut). This will be performed multiple times. The exact method and frequency will depend on the amount of blood that is required to answer the research question. In case the clearance of the tumor targeting agent is studied, 15-20 ul blood will be taken via a tail cut. This can be performed up to 7 times per week (maximum 10 samples). In case blood is collected for further laboratory analysis of for example leucocytes, hemoglobin or creatinin, 100-150 ul blood (max 7.5% of blood volume) will be taken via cheekpuncture. This can be performed once per week, with a maximum of 10 samples in total.The duration depends on the procedure (cheek puncture: 5 min, tail cut: 3 min). The guidelines published in "A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes" will be followed (reference 1).
- Weighing of animals. The frequency depends on the research question and the general condition of the animal. However, standard bodyweight is measured 2-3 times a week, and if the general condition of the animal is getting worse the animals will be weighed daily. The duration is less then one minute.
- Measuring of tumor size. In case tumor size is relevant for the research question, it will be measured 2-3 times per week, with a maximum of 7 times per week. The duration is 2 minutes.

- Imaging under general anesthesia. The frequency depends on the research question. However, the maximum number of times an animal is allowed to recover from anesthesia after imaging is 3 times within one week. The duration of the scan depends on the imaging modalities and ranges from 1 second to 2 hours. Most scans are acquired in less than 1 hour. In case the radiotracer 18F-FDG is used for PET imaging, the animals will be fasted for a maximum of 12 h prior to injection. (the maximum number of times fasting will be used is five times).

Reference 1: Karl-Heinz Diehl et al. Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes. Journal of Applied Toxicology 2001:21;15–23

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

In order to minimize the number of animals all tracers will first be evaluated in vitro. Only tracers that show favorable in vitro characteristics (e.g. high affinity, internalization, immunoreactivity) will be selected for in vivo studies.
For each experiment a power calculation will be performed based on data from literature and previous experiments to determine the minimum amount of animals needed. We will also take into account drop out of animals during the experiment due to experimental procedures, for example surgery, tumor growth, etc. If necessary, a biostatistician will be consulted to assist in these power calculations.

B. The animals

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

In most studies we will use immunodeficient athymic mice, because in these models human tumors can be grown without immunological rejection. For some studies, mice tumor cell lines will be used and they will be grown in syngeneic models (e.g. studies with the B16 melanoma cell line derived from C57BL/6, will be performed in C57Bl/6 mice). The most frequently used animal strains are:
Mice: BALB/c nude, BALB/c rj/nu, SCID, C57Bl/6, BALB/c and Swiss, C3H mice, FVB mice, CBA mice, NSG mice
However, other strains can be used if literature research or previous experiments indicate that this is required to answer the research question.
For most studies, animals will be 6-8 weeks old at start of the experiment. However, for specific research questions, e.g. imaging agents that specifically target tumors in young or elderly mice, animals of the appropriate age will be used.
Since cancer is a disease in both men and women, research can be carried out in both sexes. However, within one experiment we will select only one sexe to minimize the variation within the groups and therefore the experiment can be carried out in smaller groups of animals. In general we will use female animals, because male animals more frequently fight. This results in additional stress, and in case of severe fighting this may incidentally cause problems with housing and loss of animals. However, in case research focuses on tumor types that only occur in male patients (e.g. prostate cancer, testis cancer, etc), we will use male animals. Also, for some research questions, hormones (e.g. testosterone/estradiol) play an important role and will influence whether male or female animals will be selected for the study.

Based on animal experiments that were performed by our research group in 2012-2014 we expect to use a maximum of 1,000 mice. This calculation is based on the following:
In order to study the therapeutic efficacy we will perform the following studies:
- First, a dose-finding study will be performed to determine at what dose level tumor growth can be inhibited without causing severe toxicity. In general we will need five groups to determine this. Depending on the power calculation, the number of animals per group is on average five. This results in approximately 25 animals per dose-finding study.
- Next, the effect of therapy on tumor growth, survival, and toxicity will be assessed. On average we will need five groups of animals for this. Depending on the power calculation, the number of animals per group is on average 10, which results in approximately 50 mice per study.
- In the third stage we can assess the effect of treatment on molecular processess in the tumor (e.g. proliferation, apoptosis, growth factor receptor expression, etc). For this we will need approximately five groups of animals. Depending on the power calculation, the number of animals per group is on average 5, which results in approximately 25 mice per study.
Based on this, we estimate that we will need approximately 100 mice to fully characterize and optimize a new tumor targeting agent for cancer therapy.
During the last three years, we have developed several tumor targeting agents directed against tumor associated antigens like HER2, TROP-2, CEA, and CAIX. In addition, we are developing new tumor targeting agents directed against PSMA.
We expect to develop two new tumor targeting agents per year. In order to study the therapeutic efficacy and toxicity of these agents we will need 200 mice per jaar. In total this will result in 1,000 mice in five years.

Species	Origin	Maximum number of animals	Life stage
mice		1000	6-8 weeks

C. Re-use

Will the animals be re-used?

[X] 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:
Any new agent will first be characterized extensively in vitro. Only agents with promising characteristics will be applied in animals to study their in vivo behaviour. The aim of the studies is to determine the in vivo behaviour of tumor targeting agents (e.g. tumor targeting, clearance, etc). This can only be performed in living animals and there are currently no alternative models to get these data without the use of animals.
Reduction:
Before each experiment we will perform a literature search to find all information relevant for the animal experiment. In addition, a power calculation will be performed based on data from literature and previous experiments to determine the minimum amount of animals needed. We will also take into account drop out of animals during the experiment due to experimental procedures, for example surgery, tumor growth, etc. In case necessary, a biostatisticaion will be involved in the power calculation.
Refinement:
The experiments will be carried out in mice and rats because there are no alternatives to study the in vivo behaviour of tumor targeting agents in lower animal species. Most experimental procedures will only cause moderate discomfort or less. The use of anesthesia and analgesics is in general not required and may cause even more discomfort that the experimental procedures it self. However, if necessary (e.g. after orthotopic or intracardial injection of tumor cells), anesthesia and analgesics will be applied. During imaging procedures and surgical procedures (when the animal is anesthesized), they will be placed on a warmth mattress. to prevent heat loss. To reduce anxiety, mice will be housed in groups and not individually. The general health of the animals will be monitored daily by the researcher and/or the biotechnician. The methods that we use are well establised and the experiments will be performed by experienced researches. Previous research by our group and others has shown the translational value of this type of studies. Several tumor targeting agents that have been developed in animal models have been translated successfully into the clinic (1-6)

[redacted]

4.Baum RP, Prasad V, Muller D, Schuchardt C, Orlova A, Wennborg A, et al. Molecular imaging of HER2-expressing malignant tumors in breast cancer patients using synthetic 111In- or 68Ga-labeled affibody molecules. J Nucl Med. 2010;51(6):892-7.

[redacted]

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

In order to prevent unneccesary discomfort or stress the general health of the animals will be checked daily by the researches or the biotechnicians.
In case animals reach a humane end point the responsible researcher will be contacted.
To reduce axiety, mice will be housed in groups and not individually.
If necessary, anesthesia and analgesics wil be applied to reduce discomfort.

Environmental effects:
During and after the experiments, radioactive or biohazardous waste will be collected seperately and removed according to the university guidelines.

Repetition and Duplication

E. Repetition

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

Not applicable

Accommodation and care

F. Accommodation and care

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

[X] 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?

[X] 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 I.
[X] Yes > Will anaesthesia, analgesiaor other pain relieving methods be used?

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

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

I. Other aspects compromising the welfare of the animals

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

The most frequently occuring types of discomfort are:
- Stress (code 02)

- Discomfort due to tumor growth (code 03)
- Discomfort due to toxicity of treatment (code 03)
- Injections or surgical procedures causing pain and stress (code 03)
- Recovery from anesthesia (code 03)
- Euthanasia (code 02)

Explain why these effects may emerge.

Stress can be caused by the handling of the animals, injections, and other experimental procedures. In addition, the animals can suffer from the recovery from anesthesia after the imaging procedure.

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

To reduce stress, animals will be housed in groups and not indvidually. In addition, before the experiments starts the animals will be housed for a week in order to acclimatise to their new environment.
The tumor growth can cause discomfort, especially in case of large tumors, ulcerating tumors or invasive tumors. In most studies, the experiment will start when the tumor is only 0.1 cm3 and therefore the tumor not likely to cause discomfort due to size, ulceration or invasive growth. However, in some studies, tumor size will be monitored longitudinally, for example when survival is studied. In this case, mice will be checked daily by the researcher or biotechnician and in case the tumors causes severe discomfort, the animal will be taken out of the experiment according to the humane endpoints.

New tumor targeting agents can cause toxicity to the animal and this will be carefully monitored. The animals will be checked daily by the researcher or biotechnician and body weight will be measured up to 7 times a week. In case mice suffer from severe toxicity, the animal will be taken out of the experiment according to the humane endpoints.

Recovery from anesthesia is causing discomfort to the animal. However, this is required in case of imaging of a living animal. In order to minimize this discomfort, the maximum number of times an animal recovers from anesthesia is three times a week and after the last scan, the animal will be euthanized before it recovers from anesthesia.During the anesthesia the animal will be placed on a warmth mattress to prevent heat loss.

The experiments will be carried out by experienced researchers who have been trained to perform these type of studies.

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.
[X] Yes > Describe the criteria that will be used to identify the humane endpoints.

Animals will be taken out of the experiments and humanely euthanized if one the following humane endpoints is reached:

- Tumor size of 2 cm3
- Ulceration or invasive tumor growth causing discomfort to the animal
- Abnormal motility due to tumor growth (severely reduced motility)
- Other signs of clinical discomfort (for example, dehydration, 15% weight loss in less than 2 days, or a decrease of 20% compared to the weight at start of the experiment).

Treatment induced toxicity will cause changes in the humane endpoint as described above. Therefore, no treatment specific endpoints are described.

Indicate the likely incidence.

The most likely primary end point to be met in these studies is tumor size of 2 cm3 or ulceration or invasive tumor growth that causes discomfort to the animal. However, in most cases experiments start when the tumors are approximately 0.1 cm3 and it is unlikely that the tumor reach one of the humane endpoints. However, in some studies the mice will be followed for longer periods (e.g. to measure survival) and they may reach one of these end points. We expect that this will occur in 20% of the animals.

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).

We expect that 85% of the animals will experience moderate discomfort, because either tumor growth is induced or the animals will have to recover from anesthesia which is required for the imaging procedures. The remaining 15% of the animals will experience severe discomfort and will reach the humane end point, for example in therapy experiments due to tumor growth up to 10% of the body weight (2 cm3)

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

[] No > Continue with Section 3: 'Signatures'.
[X] Yes > Explain why it is necessary to kill the animals during or after the procedures.

At the end of the study we will collect tumor tissue to analyze molecular processes in the tumor.

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

[] No > Descripe the method of killing that will be used and provide justifications for this choice.

[X] 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'.	10300	
1.2	Provide the name of the licenced establishment.	Stichting Katholieke Universiteit Nijmegen	
1.3	List the different types of animal procedures. Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.	Serial number 3	Type of animal procedure Characterization of in vivo behaviour of new tumor targeting agents in rats

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

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

The aim of the experiments is to determine the in vivo behaviour (e.g. tumor targeting, retention, clearance, etc) of new tumor targeting agents in animal models. For this purpose, the agent will be admistered (e.g. i.v., i.p., etc) to the animal and subsequently the pharmacokinetics and biodistribution of the agent can be studied by means of biodistribution and/or imaging. In addition, the pharmacokinetics can be studied by for example blood sampling or urine collection.
A standard approach to study the in vivo behaviour of a new tumor targeting agent includes the following experiment:
1. Dose optimization: determine which dose of the tumor targeting agent results in the highest tumor uptake with relative low uptake in normal tissue
2. Pharmacokinetics: Determine the pharmacokinetics of the tracer (e.g. how rapidly accumulates the tracer in the tumor and clears from normal tissue and circulation)
3. Imaging of the distribution of the tumor targeting agent in vivo
To quantitatively determine the in vivo behaviour of the tumor targeting agent we will use the following primary end points
- Tumor and normal tissue uptake measured by biodistribution study. In this set up the animal will be euthanized at a certain time point (e.g. 1h, 24h, 72h) after injection of the tumor targeting agent. The animals will be dissected and tumor and normal organs will be collected. The uptake in tissues of interest will be quantified by using for example a gamma counter. Aliquots of the injected tracer will be counted simultaneously.
- Tumor and normal tissue uptake measured by imaging. In this set up, the injection of the radiotracer will be followed by an imaging procedure (e.g. optical, SPECT or PET) under anesthesia. Since it is not necessary to euthanize the animal, this procedure can be repeated at several time points after injection. After the scan, a 3D image will be reconstructed and a region of interest will be drawn around the tumor and tissue of interest to quantitatively determine the activity concentration.
- Pharmacokinetics of a tracer will be determined by taking blood samples (e.g. via cheek puncture, tail cut) or urine samples. The tracer concentration can be measured by for example ELISA or by measuring the radioactive or fluoresent signal (e.g. in a gamma counter).
These outcome parameters have successfully been used in previous studies performed [redacted]

4.Tolmachev V, Varasteh Z, Honarvar H, Hosseinimehr SJ, Eriksson O, Jonasson P, et al. Imaging of platelet-derived growth factor receptor beta expression in glioblastoma xenografts using affibody molecule 111In-DOTA-Z09591. J Nucl Med. 2014;55(2):294-300.

5.Luo H, Hong H, Slater MR, Graves SA, Shi S, Yang Y, et al. PET of c-Met in cancer with 64Cu-labeled Hepatocyte Growth Factor. J Nucl Med. 2015.

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

In a standard experiment the first step is to inject or transplant the animals with a tumor. In case the tumor requires growth factors or hormones to grow, a growth factor pellet will be transplanted subcutaneously. When the tumor reaches a size of approximately 0.1 cm3 (on average this takes 2-3 weeks), the animal will be injected with the tumor targeting agent. After injection, blood samples can be collected to measure the pharmacokinetics of the tracer. Depending on how fast the tracer accumulates in the tumor and clears form the circulation, biodistribution and imaging studies will be performed at several time points (e.g. 1h, 24h, and 72h) after injection to quantitatively determine the biodistribution of the tracer. On average, these type of studies will take three weeks. However, in case of slow growing tumors or longitudinal studies, the total time an animal is in the experiment can be longer (maximum 6 months).
In more detail, the following procedures can be carried out:
- Injection of tumor cells (one or two sided, i.p., i.v., s.c., i.m., orthotopically, intracardially, etc). If necessary, discomfort will be minimized by performing the procedure under generally anesthesia and administering analgesics (e.g. carprofen, temgesic, etc). Frequency: in most cases only one injection of tumor cells will be performed (one tumor per animal). In certain studies (e.g. imaging studies with a receptor positive and receptor negative tumor in one animal), two types of tumor cells will be injected. Depending on how fast the tumors grow, they will be injected at the same time, or there will be a certain time interval (e.g. 1 week) between the two tumor cell injections. The injection takes less then a minute. Orthotopic injection may last longer, depending on site of injection.
- Transplantation of tumor tissue. This procedure will be carried out under general anesthesia and if necessary analgesics will be administered. Frequency: in most cases only one tumor transplantation will be performed (one tumor per animal). In certain studies (e.g. imaging studies with a receptor positive and receptor negative tumor in one animal), two types of tumors will be transplanted. Depending on how fast the tumors grow, they will be transplanted at the same time, or there will be a certain time interval (e.g. 1 week) between the two tumor cell transplantation. Subcutaneous transplantations will take approximately 10 minutes. Orthotopic transplantations may last longer, depending on site of transplantation.
- Particular tumors require hormones or growth factor in order to grow in mice (for example certain breast cancer cells require estrogen to grow). These will be administered by transplanting a growth factor pellet subcutaneously. This procedure will be performed once and under general anesthesia. It will take approximately 10 min. When the injection/transplantation of the tumor cells also requires anesthesia, the transplanation of the pellet will be performed in the same session.
- Administration of tumor targeting agent (i.p., i.v., orally, s.c., etc.) In general, tumor targeting agents will be injected intraveneously. However, in certain studies we can choose a different administration route. For example, in case of intraperitoneal tumor growth of ovarian cancer, the tracer can be injected intraperitoneally as well. The maximum number of injections is six. Frequency: In most studies the tumor targeting agent will be injected once. However, in case of longitudinal studies, agents can be injected multiple times (e.g. every other day, weekly, etc). The duration of injection is approximately 1 minute (depending on the type of injection). The guidelines published in "A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes" will be followed (reference 1).
- Blood sampling (for example via cheek puncture, orbital puncture, tail cut). This can be performed multiple times. The exact method and frequency will depend on the amount of blood that is required to answer the research question. In case the clearance of the tumor targeting agent is studied, 20-30 ul blood will be taken via a tail cut. This can be performed up to 7 times per week (maximum 10 samples). In case blood is collected for further laboratory analysis of for example leucocytes, hemoglobin or creatinin, 200-250 ul blood (max 7.5% of blood volume) will be taken via

cheekpuncture. This can be performed once per week, with a maximum of 10 samples in total.The duration depends on the procedure (cheek puncture: 5 min, tail cut: 3 min). The guidelines published in "A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes" will be followed (reference 1).
- Weighing of animals. The frequency depends on the research question and the general condition of the animal (standard 1-2 per week, but if necessary the animals will be weighed daily). The duration is less then one minute.
- Measuring of tumor size using a caliper. In case tumor size is relevant for the research question, it will be measured 2-3 times per week, and if necessary it will be measured daily. The duration is 2 minutes.
- Imaging under general anesthesia. The frequency depends on the research question. However, the maximum number of times an animal is allowed to recover from anesthesia after imaging is 3 times within one week. The duration of the scan depends on the imaging modalities and ranges from 1 second to 3 hours. Most scans can be acquired in less than 1 hour. In case the radiotracer 18F-FDG is used for PET imaging, the animals will be fasted for a maximum of 12 h prior to injection (fasting will be performed with a maximum of 5 times).
Reference 1: Karl-Heinz Diehl et al. Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes. Journal of Applied Toxicology 2001:21;15–23

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

In order to minimize the number of animals all tracers will first be evaluated in vitro. Only tracers that show favorable in vitro characteristics (e.g. high affinity, internalization, immunoreactivity) will be selected for in vivo studies.
For each experiment a power calculation will be performed based on data from literature and previous experiments to determine the minimum amount of animals needed. We will also take into account drop out of animals during the experiment due to experimental procedures, for example surgery, tumor growth, etc. If necessary, a biostatistician will be consulted to assist in these power calculations.

B. The animals

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

In general, new tumor targeting agents can be tested in mice. However, sometimes rat models are preferred over mice models because of their larger size. For example, we have developed a reliable rat model for colorectal cancer liver metastases, where the tumor cells are injected in the portal vein. This is a realistic metastatic colorectal cancer model, which is difficult to set up in mice because of the very small size of the portal vein. In most studies we will use immunodeficient animals because in these models human tumors can be grown without immunological rejection. For some studies, rat tumor cell lines will be used and they will be grown in syngeneic models (e.g. studies with the CC531 colon carcinoma cell line derived from Wag/Rij, will be performed in Wag/Rij rats). The most frequently used animal strains are:
Rat: Wistar, Lewig, Wag/Rij, BN, Sprague Dawley
However, other strains can be used if literature research or previous experiments indicate that this is required to answer the research question.
For most studies, animals will be 6-8 weeks old at start of the experiment. However, for specific research questions, e.g. imaging agents that specifically target tumors in young or elderly mice, animals of the appropriate age will be used.

Since cancer is a disease in both men and women, research can be carried out in both sexes. However, within one experiment we will select only one sexe to minimize the variation within the groups and therefore the experiment can be carried out in smaller groups of animals. In general we will use female animals, because male animals more frequently fight. This results in additional stress, and in case of severe fighting this may incidentally cause problems with housing and loss of animals. However, in case research focuses on tumor types that only occur in male patients (e.g. prostate cancer, testis cancer, etc), we will use male animals. Also, for some research questions, hormones (e.g. testosterone/estradiol) play an important role and will influence whether male or female animals will be selected for the study.
Based on animal experiments that were performed by our research group in 2012-2014 we expect to use a maximum of 400 rats. This calculation is based on the following:
In order to study the in vivo behaviour of a new tumor targeting agent, and to optimize it for imaging and therapy purposes, several experiments will be carried out.
- First, a dose-optimization study will be performed to determine which tracer dose will result in the highest tumor uptake with relative low uptake in normal tissue. In general we need six groups to optimize this. Depending on the power calculation, the number of animals per group is on average five. This results in approximately 30 animals per dose optimization study.
- Next, the pharmacokinetics will be studied by performing biodistribution studies at different time points. On average we will need eight groups of animals for this, which results in approximately 40 rats per pharmacokinetics study.
- In the third stage imaging will be performed. For this we will need approximately 10 animals.
Based on this, we estimate that we will need approximately 80 to fully characterize and optimize a new tumor targeting agent for imaging and therapy.
During the last three years, we have developed tumor targeting agents for several tumor associated antigens, eg: HER2, IGF-1R, EGFR, TROP-2, [redacted]
For each target, different types of radiotracers can be designed, such as monoclonal antibodies or antibody fragments, nanobodies, affibody molecules, peptides, and small molecules. These agents differ in several aspects like affinity, size, tumor accumulation, clearance, etc. In addition, monomers, dimers, or heterodimers can be produced in order to increase the affinity and tumor targeting potential of these agents.
We expect to develop one new radiotracers per year that has to be tested in rats. In order to fully characterize and optimize these agents (dose optimization, pharmacokinetics, imaging) we will need 80 rats per year. In total this will result in 400 animals in five years.

Species	Origin	Maximum number of animals	Life stage
rat		400	6-8 weeks

C. Re-use

Will the animals be re-used?

[X] 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:
Any new agent will first be characterized extensively in vitro. Only agents with promising characteristics will be applied in animals to study their in vivo behaviour. The aim of the studies is to determine the in vivo behaviour of tumor targeting agents (e.g. tumor targeting, clearance, etc). This can only be performed in living animals and there are currently no alternative models to get these data without the use of animals.
Reduction:
Before each experiment we will perform a literature search to find all information relevant for the animal experiment. In addition, a power calculation will be performed based on data from literature and previous experiments to determine the minimum amount of animals needed. We will also take into account drop out of animals during the experiment due to experimental procedures, for example surgery, tumor growth, etc. In case necessary, a biostatisticaion will be involved in the power calculation.
Refinement:
The experiments will be carried out in rats because there are no alternatives to study the in vivo behaviour of tumor targeting agents in lower animal species.In general, new tumor targeting agents can be tested in mice. However, sometimes rat models are preferred over mice models because of their larger size. For example, we have developed a reliable rat model for colorectal cancer liver metastases, where the tumor cells are injected in the portal vein. This is a realistic metastatic colorectal cancer model, which is difficult to set up in mice because of the very small size of the portal vein.
Most experimental procedures will only cause moderate discomfort or less. The use of anesthesia and analgesics is in general not required and may cause even more discomfort that the experimental procedures it self. However, if necessary (e.g. after orthotopic or intracardial injection of tumor cells), anesthesia and analgesics will be applied. During imaging procedures and surgical procedures (when the animal is anesthesized), they will be placed on a warmth mattress. to prevent heat loss. To reduce anxiety, mice will be housed in groups and not individually. The general health of the animals will be monitored daily by the researcher and/or the biotechnician. The methods that we use are well establised and the experiments will be performed by experienced researches. Previous research by our group and others has shown the translational value of this type of studies. Several tumor targeting agents that have been developed in animal models have been translated successfully into the clinic (1-6)

4.Baum RP, Prasad V, Muller D, Schuchardt C, Orlova A, Wennborg A, et al. Molecular imaging of HER2-expressing malignant tumors in breast cancer patients using synthetic 111In- or 68Ga-labeled affibody molecules. J Nucl Med. 2010;51(6):892-7.

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

In order to prevent unneccesary discomfort or stress the general health of the animals will be checked daily by the researches or the biotechnicians.
In case animals reach a humane end point the responsible researcher will be contacted.
To reduce axiety, mice will be housed in groups and not individually.
If necessary, anesthesia and analgesics wil be applied to reduce discomfort.
For imaging and biodistribution, only a very low dose (tracer dose) of the tumor targeting agent is required. Therefore, we do not expect that this will cause discomfort or side effects.
Environmental effects:
During and after the experiments, radioactive or biohazardous waste will be collected seperately and removed according to the university guidelines.

Repetition and Duplication

E. Repetition

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

Not applicable

Accommodation and care

F. Accommodation and care

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

[X] 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?

[X] 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 I.
[X] Yes > Will anaesthesia, analgesiaor other pain relieving methods be used?

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

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

I. Other aspects compromising the welfare of the animals

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

The most frequently occuring types of discomfort are:
- Stress
- Discomfort due to tumor growth
- Injections or surgical procedures causing pain and stress
- Recovery from anesthesia
- Euthanasia

The tumor targeting agents themself will not cause side effects or discomfort, since the injected dose is very low (tracer dose)

Explain why these effects may emerge.

Stress can be caused by the handling of the animals, injections, and other experimental procedures. In addition, the animals can suffer from the recovery from anesthesia after the imaging procedure.

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

To reduce stress, animals will be housed in groups and not indvidually. In addition, before the experiments starts the animals will be housed for a week in order to acclimatise to their new environment.The tumor growth can cause discomfort, especially in case of large tumors, ulcerating tumors or invasive tumors. In most studies, the experiment will start when the tumor is only 0.1 cm3 and therefore the tumor not likely to cause discomfort due to size, ulceration or invasive growth. In general, animals will be taken out of the experiment before tumors cause severe discomfort. However, in case tumors unexpectedly cause severe ulceration or invasive growth, the animal may experience severe discomfort and will be taken out of the experiment. We expect that this will happen in less than 1% of the animals.
Recovery from anesthesia is causing discomfort to the animal. However, this is required in case of imaging of a living animal. In order to minimize this discomfort, the maximum number of times an animal recovers from anesthesia is three times a week and after the last scan, the animal will be euthanized before it recovers from anesthesia. During the anesthesia the animal will be placed on a warmth mattress to prevent heat loss.
Based on extensive experiments with many different types of radiotracers, we do not expect that these tracers themselves will cause discomfort to the animals.
The experiments will be carried out by experienced researchers who have been trained to perform these type of studies.

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.
[X] Yes > Describe the criteria that will be used to identify the humane endpoints.

Animals will be taken out of the experiments and humanely euthanized if one the following humane endpoints is reached:
• Tumor size > 2 cm3
• Ulceration or invasive tumor growth causing discomfort to the animal
• Abnormal motility due to tumor growth (severely reduced motility)
• Other signs of clinical discomfort (for example, dehydration, 15% weight loss in less than 2 days, or a decrease of 20% compared to the weight at start of the experiment).

Indicate the likely incidence.

In most imaging and biodistribution studies the experiments will start when the tumor is only 0.1 cm3 and thus it is very unlikely that the humane end points are reached. Based on previous experience we expect this to be less than 1%.

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).

We expect that 95% of the animals will experience moderate discomfort, because either tumor growth is induced or the animals will have to recover from anesthesia which is required for the imaging procedures. The remaining 5% of the animals will experience mild discomfort, since we will not induce a tumor and perform imaging. In the exceptional situation that a tumor causes unexpected severe discomfort due to for example ulceration or invasive growth, the animal may experience severe discomfort. This will happen in less than 1% of the animals.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

L. Method of killing

[] No > Continue with Section 3: 'Signatures'.

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

At the end of the study we will determine the biodistribution of the agent ex vivo. In addition, tumors or normal tissue can be analyzed immunohistochemicaly.

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

[] No > Descripe the method of killing that will be used and provide justifications for this choice.

[X] 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'.	10300	
1.2	Provide the name of the licenced establishment.	Stichting Katholieke Universiteit Nijmegen	
1.3	List the different types of animal procedures. Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.	Serial number 4	Type of animal procedure Assessment of therapeutic efficacy of new tumor targeting agents in rats

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

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

The aim of the experiments is to determine the therapeutic efficacy of new tumor targeting agents in animal models for the treatment of cancer. Tumor targeting agents can be for example antibodies, peptides or small molecules. They can be used 'naked' or be labeled with beta- or alpha-emitting radionuclides (e.g. Lu-177, Y-90 or Bi-213) or cytotoxic drugs. In these experiments, the therapeutic efficacy of these agents will be compared with either the unlabeled compound or conventional anti-cancer treatment (e.g. chemotherapy, irradiation, surgical resection of the tumor, or cell-based therapies)
The primary outcome measurements of these experiments are:
1) Toxicity
Toxicity will be assessed by measuring body weight and by analyzing blood and/or urine for hematological toxicity (hemoglobin, leucocytes, thrombocytes) and renal toxicity (creatinine). Body weight is a reliable indicator of the general health of the animal. In addition, chemotherapy or targeted radiotherapy can cause bonemarrow and kidney toxicity. This can be measured by analyzing blood samples for several hematological and renal parameters. Asssessing the toxicity can not be performed in animal procedure 1, since in these studies only a tracer amount of the tumor targeting agent is administered, while the therapeutic dose is much higher (up to a 100 fold). (1-5)
2) Tumor growth
Tumor growth can be measured by caliper measurements (in case of a subcutaneous tumor) or by non-invasive imaging techniques such as PET, SPECT or MRI (in case of an orthotopic tumor model such as prostate cancer, intraperitoneal tumors, liver metastases, etc). Both methods have shown to reliably monitor tumor growth. (1-5)
3) Survival
Next to tumor growth, the survival is an important primary outcome measure to proof the therapeutic efficacy. Certain therapeutic agents may cause severe toxicity and therefore only measuring tumor growth is not sufficient. For example, tumor growth can be significantly inhibited during the first three weeks, but when the treatment causes severe hematological or renal toxicity, this may result in death of the animals. Thus, the overall effect of the treatment is not beneficial for the animal. In order to study survival, we will longitudinally monitor the animals untill they die or have to be sacrificed based on the humane endpoint (e.g. tumor > 2 cm3, weight loss more than 25% compared to baseline, etc). (1-5)
4) Assessment of molecular processes induced by treatment
Finally, the tumor targeting agent may induce molecular processess in the tumor that can be measured by non-invasive imaging or by analyzing the tumors by western blot or immunohistochemical stainings. An example of such a molecular process is a change in tumor cell proliferation that can be measured by 18F-FLT PET, or a change in glucose metabolism that can be measured with 18F-FDG PET. Changes in 18F-FLT or 18F-FDG often proceed the changes in tumor size as can be measured by caliper measurements and can therefore be used to predict response to treatment. (6, 7)
The experiments will be carried out in the following order:
The first step is to determine which dose results in optimal therapeutic efficacy without inducing severe toxicity (e.g. determination of the maximum tolerable dose (MTD)). The toxicity can be assessed by measuring body weight and by taking blood samples to assess hematological (platelets, leucocytes, thrombocytes) and renal toxicity (creatinine, urea). Next the therapeutic effect is monitored by measuring tumor growth and survival.

Tumor growth can be monitored by caliper measurements or non-invasive imaging techniques (e.g. CT, MRI, PET, SPECT, optical, ultrasound). Finally, we can also study which molecular processes occur in tumors that were treated. Processes of interest are for example proliferation, apoptosis, angiogenesis, expression of growth factor receptor, activation of intracellular signaling pathways. These processes can be measured by non-invasive imaging techniques, or by collecting tumor samples for ex vivo analysis (immunohistochemistry, western blot, etc) at the end of the study.

[redacted]

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

In order to determine the therapeutic effect of tumor targeting agents, the following procedures can be carried out. The study will start with inoculation of a tumor. When the tumor reaches a size of approximately 0.1 cm3 (2-3 weeks) the therapy will be started. Tumor size will be monitored by caliper measurements or non-invasive imaging techniques. Toxicity will be assessed by measuring body weight and by taking blood samples to analyze hematological and renal toxicity. Finally, the molecular processess in tumor (e.g. proliferation, apoptosis, etc) may be monitored by non-invasive imaging techniques, or by removing the tumor at the end of the study for ex vivo analysis (immunohistochemistry, westerblot, etc). The maximum time an animal is in the experiment is 12 months.
In more detail the following procedures will be carried out:
- Injection of tumor cells (one or two sided, i.p., i.v., s.c., i.m., orthotopically, intracardially, etc). If necessary, discomfort will be minimized by performing the procedure under generally anesthesia and administering analgesics (e.g. carprofen, temgesic, etc). Frequency: in most cases only one injection of tumor cells will be performed (one tumor per animal). In certain studies (e.g. imaging studies with a receptor positive and receptor negative tumor in one animal), two types of tumor cells will be injected. Depending on how fast the tumors grow, they will be injected at the same time, or there will be a certain time interval (e.g. 1 week) between the two tumor cell injections. The injection takes less then a minute. Orthotopic injection may last longer, depending on site of injection.

- Transplantation of tumor tissue. This procedure will be carried out under general anesthesia and if necessary analgesics will be administered. Frequency: in most cases only one tumor transplantation will be performed (one tumor per animal). In certain studies (e.g. imaging studies with a receptor positive and receptor negative tumor in one animal), two types of tumors will be transplanted. Depending on how fast the tumors grow, they will be transplanted at the same time, or there will be a certain time interval (e.g. 1 week) between the two tumor cell transplantation. Subcutaneous transplantations will take approximately 10 minutes. Orthotopic transplantations may last longer, depending on site of transplantation.
- Particular tumors require hormones or growth factor in order to grow in mice (for example certain breast cancer cells require estrogen to grow). These will be administered by transplanting a growth factor pellet subcutaneously. This procedure will be performed once and under general anesthesia. It will take approximately 10 min. When the injection/transplantation of the tumor cells also requires anesthesia, the transplanation of the pellet will be performed in the same session - Administration of tumor targeting agent (i.p., i.v., orally, s.c., etc.) In general, tumor targeting agents will be injected intraveneously. However, in certain studies we can choose a different administration route. For example, in case of intraperitoneal tumor growth of ovarian cancer, the tracer can be injected intraperitoneally as well. Frequency: In most studies the tumor targeting agent will be injected once. However, in case of longitudinal studies, agents can be injected multiple times (e.g. every other day, weekly, etc). The maximum number of injections is six. The duration of injection is approximately 1 minute (depending on the type of injection). The guidelines published in "A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes" will be followed (reference 1).

- Administration of conventional anti-cancer therapy such as chemotherapy and antibody-based therapy (i.p., i.v., etc). Conventional anti-cancer therapy is generally injected intraperitoneally. However, certain agents can also be administered i.v., s.c., orally, etc. For example, small-molecule inhibitors like BRAF inhibitors require oral administration. Frequency: depends on the duration of the study. Therapy can be injected once, or in case of longitudinal studies, agents can be injected multiple times (e.g. every other day, weekly, etc). The duration of injection is approximately 1 minute (depending on the type of injection).The guidelines published in "A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes" will be followed (reference 1).
- Irradiation: Mice will undergo irradiation under general anesthesia (2 Gy - 10 Gy). Frequency: in general one dose of 10 Gy will be administered or radiation is administered fractionated (e.g. 3x3Gy). The duration of one irradiation session is approximately 10 minutes. Irradiation can be whole body (2 Gy) or only on the tumor (2 - 10 Gy).
- Blood sampling (for example via cheek puncture, orbital puncture, tail cut). This will be performed multiple times. The exact method and frequency will depend on the amount of blood that is required to answer the research question. In case the clearance of the tumor targeting agent is studied, 20-30 ul blood will be taken via a tail cut. This can be performed up to 7 times per week (maximum 10 samples). In case blood is collected for further laboratory analysis of for example leucocytes, hemoglobin or creatinin, 200-250 ul blood (max 7.5% of blood volume) will be taken via cheekpuncture. This can be performed once per week, with a maximum of 10 samples in total.The duration depends on the procedure (cheek puncture: 5 min, tail cut: 3 min). The guidelines published in "A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes" will be followed (reference 1).
- Weighing of animals. The frequency depends on the research question and the general condition of the animal. However, standard bodyweight is measured 2-3 times a week, and if the general condition of the animal is getting worse the animals will be weighed daily. The duration is less then one minute.
- Measuring of tumor size. In case tumor size is relevant for the research question, it will be measured 2-3 times per week, with a maximum of 7 times per week. The duration is 2 minutes.
- Imaging under general anesthesia. The frequency depends on the research question. However, the maximum number of times an animal is allowed to recover from anesthesia after imaging is 3 times within one week. The duration of the scan depends on the imaging modalities and ranges from 1

second to 2 hours. Most scans are acquired in less than 1 hour. In case the radiotracer 18F-FDG is used for PET imaging, the animals will be fasted for a maximum of 12 h prior to injection. (the maximum number of times fasting will be used is five times).
Reference 1: Karl-Heinz Diehl et al. Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes. Journal of Applied Toxicology 2001:21;15–23

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

In order to minimize the number of animals all tracers will first be evaluated in vitro. Only tracers that show favorable in vitro characteristics (e.g. high affinity, internalization, immunoreactivity) will be selected for in vivo studies.
For each experiment a power calculation will be performed based on data from literature and previous experiments to determine the minimum amount of animals needed. We will also take into account drop out of animals during the experiment due to experimental procedures, for example surgery, tumor growth, etc. If necessary, a biostatistician will be consulted to assist in these power calculations.

B. The animals

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

In general, new tumor targeting agents can be tested in mice. However, sometimes rat models are preferred over mice models because of their larger size. For example, we have developed a reliable rat model for colorectal cancer liver metastases, where the tumor cells are injected in the portal vein. This is a realistic metastatic colorectal cancer model, which is difficult to set up in mice because of the very small size of the portal vein. I n most studies we will use immunodeficient rats, because in these models human tumors can be grown without immunological rejection. For some studies, rat tumor cell lines will be used and they will be grown in syngeneic models (e.g. studies with the CC531 colon carcinoma cell line derived from Wag/Rij, will be performed in Wag/Rij rats). The most frequently used animal strains are:
Rats: Wistar, Lewis, Wag/Rij, BN, Sprague Dewley
However, other strains can be used if literature research or previous experiments indicate that this is required to answer the research question.
For most studies, animals will be 6-8 weeks old at start of the experiment. However, for specific research questions, e.g. imaging agents that specifically target tumors in young or elderly mice, animals of the appropriate age will be used.
Since cancer is a disease in both men and women, research can be carried out in both sexes. However, within one experiment we will select only one sexe to minimize the variation within the groups and therefore the experiment can be carried out in smaller groups of animals. In general we will use female animals, because male animals more frequently fight. This results in additional stress, and in case of severe fighting this may incidentally cause problems with housing and loss of animals. However, in case research focuses on tumor types that only occur in male patients (e.g. prostate cancer, testis cancer, etc), we will use male animals. Also, for some research questions, hormones (e.g. testosterone/estradiol) play an important role and will influence whether male or female animals will be selected for the study.
Based on animal experiments that were performed by our research group in 2012-2014 we expect to use a maximum of 500 rats. This calculation is based on the following:
In order to study the therapeutic efficacy we will perform the following studies:

- First, a dose-finding study will be performed to determine at what dose level tumor growth can be inhibited without causing severe toxicity. In general we will need five groups to determine this. Depending on the power calculation, the number of animals per group is on average five. This results in approximately 25 animals per dose-finding study.
- Next, the effect of therapy on tumor growth, survival, and toxicity will be assessed. On average we will need five groups of animals for this. Depending on the power calculation, the number of animals per group is on average 10, which results in approximately 50 mice per study.
- In the third stage we can assess the effect of treatment on molecular processess in the tumor (e.g. proliferation, apoptosis, growth factor receptor expression, etc). For this we will need approximately five groups of animals. Depending on the power calculation, the number of animals per group is on average 5, which results in approximately 25 rats per study.
Based on this, we estimate that we will need approximately 100 rats to fully characterize and optimize a new tumor targeting agent for cancer therapy.
During the last three years, we have developed several tumor targeting agents directed against tumor associated antigens like [redacted].
We expect to develop one new tumor targeting agents for therapy per year. In order to study the therapeutic efficacy and toxicity of these agents we will need 100 rats per jaar. In total this will result in 500 rats in five years.

Species	Origin	Maximum number of animals	Life stage
Rat		500	6-8 weeks

C. Re-use

Will the animals be re-used?

[X] 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:
Any new agent will first be characterized extensively in vitro. Only agents with promising characteristics will be applied in animals to study their in vivo behaviour. The aim of the studies is to determine the in vivo behaviour of tumor targeting agents (e.g. tumor targeting, clearance, etc). This can only be performed in living animals and there are currently no alternative models to get these data without the use of animals.
Reduction:
Before each experiment we will perform a literature search to find all information relevant for the animal experiment. In addition, a power calculation will be performed based on data from literature and previous experiments to determine the minimum amount of animals needed. We will also take into account drop out of animals during the experiment due to experimental procedures, for example surgery, tumor growth, etc. In case necessary, a biostatisticaion will be involved in the power calculation.
Refinement:
The experiments will be carried out in mice and rats because there are no alternatives to study the in vivo behaviour of tumor targeting agents in lower animal species. Most experimental procedures will only cause moderate discomfort or less. The use of anesthesia and analgesics is in general not required and may cause even more discomfort that the experimental procedures it self. However, if necessary (e.g. after orthotopic or intracardial injection of tumor cells), anesthesia and analgesics will be applied. During imaging procedures and surgical procedures (when the animal is anesthesized), they will be placed on a warmth mattress. to prevent heat loss. To reduce anxiety, mice will be housed in groups and not individually. The general health of the animals will be monitored daily by the researcher and/or the biotechnician. The methods that we use are well estabilised and the experiments will be performed by experienced researches. Previous research by our group and others has shown the translational value of this type of studies. Several tumor targeting agents that have been developed in animal models have been translated successfully into the clinic (1-6)

[redacted]

4.Baum RP, Prasad V, Muller D, Schuchardt C, Orlova A, Wennborg A, et al. Molecular imaging of HER2-expressing malignant tumors in breast cancer patients using synthetic 111In- or 68Ga-labeled affibody molecules. J Nucl Med. 2010;51(6):892-7.

[redacted]

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

In order to prevent unneccesary discomfort or stress the general health of the animals will be checked daily by the researches or the biotechnicians.
In case animals reach a humane end point the responsible researcher will be contacted.
To reduce axiety, mice will be housed in groups and not individually.
If necessary, anesthesia and analgesics wil be applied to reduce discomfort.
Environmental effects:
During and after the experiments, radioactive or biohazardous waste will be collected seperately and removed according to the university guidelines.

Repetition and Duplication

E. Repetition

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

Not applicable

Accommodation and care

F. Accommodation and care

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

[X] 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?

G. Location where the animals procedures are performed

[X] 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 I.
[X] Yes > Will anaesthesia, analgesiaor other pain relieving methods be used?

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

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

I. Other aspects compromising the welfare of the animals

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

The most frequently occuring types of discomfort are:
- Stress (code 02)
- Discomfort due to tumor growth (code 03)
- Discomfort due to toxicity of treatment (code 03)
- Injections or surgical procedures causing pain and stress (code 03)

- Recovery from anesthesia (code 03)
- Euthanasia (code 02)

Explain why these effects may emerge.

Stress can be caused by the handling of the animals, injections, and other experimental procedures. In addition, the animals can suffer from the recovery from anesthesia after the imaging procedure.

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

To reduce stress, animals will be housed in groups and not indvidually. In addition, before the experiments starts the animals will be housed for a week in order to acclimatise to their new environment.
The tumor growth can cause discomfort, especially in case of large tumors, ulcerating tumors or invasive tumors. In most studies, the experiment will start when the tumor is only 0.1 cm3 and therefore the tumor not likely to cause discomfort due to size, ulceration or invasive growth. However, in some studies, tumor size will be monitored longitudinally, for example when survival is studied. In this case, mice will be checked daily by the researcher or biotechnician and in case the tumors causes severe discomfort, the animal will be taken out of the experiment according to the humane endpoints.
New tumor targeting agents can cause toxicity to the animal and this will be carefully monitored. The animals will be checked daily by the researcher or biotechnician and body weight will be measured up to 7 times a week. In case mice suffer from severe toxicity, the animal will be taken out of the experiment according to the humane endpoints.
Recovery from anesthesia is causing discomfort to the animal. However, this is required in case of imaging of a living animal. In order to minimize this discomfort, the maximum number of times an animal recovers from anesthesia is three times a week and after the last scan, the animal will be euthanized before it recovers from anesthesia.During the anesthesia the animal will be placed on a warmth mattress to prevent heat loss.

The experiments will be carried out by experienced researchers who have been trained to perform these type of studies.

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.
[X] Yes > Describe the criteria that will be used to identify the humane endpoints.

Animals will be taken out of the experiments and humanely euthanized if one the following humane endpoints is reached:
• Tumor size of 2 cm3
• Ulceration or invasive tumor growth causing discomfort to the animal
• Abnormal motility due to tumor growth (severely reduced motility)
• Other signs of clinical discomfort (for example, dehydration, 15% weight loss in less than 2 days, or a decrease of 20% compared to the weight at start of the experiment).
Treatment induced toxicity will cause changes in the humane endpoint as described above. Therefore, no treatment specific endpoints are described.

Indicate the likely incidence.

To reduce stress, animals will be housed in groups and not indvidually. In addition, before the experiments starts the animals will be housed for a week in order to acclimatise to their new environment.
The tumor growth can cause discomfort, especially in case of large tumors, ulcerating tumors or invasive tumors. In most studies, the experiment will start when the tumor is only 0.1 cm3 and therefore the tumor not likely to cause discomfort due to size, ulceration or invasive growth. However, in some studies, tumor size will be monitored longitudinally, for example when survival is studied. In this case, mice will be checked daily by the researcher or biotechnician and in case the tumors causes severe discomfort, the animal will be taken out of the experiment according to the humane endpoints.
New tumor targeting agents can cause toxicity to the animal and this will be carefully monitored. The animals will be checked daily by the researcher or biotechnician and body weight will be measured up to 7 times a week. In case mice suffer from severe toxicity, the animal will be taken out of the experiment according to the humane endpoints.
Recovery from anesthesia is causing discomfort to the animal. However, this is required in case of imaging of a living animal. In order to minimize this discomfort, the maximum number of times an animal recovers from anesthesia is three times a week and after the last scan, the animal will be euthanized before it recovers from anesthesia.During the anesthesia the animal will be placed on a warmth mattress to prevent heat loss.

The experiments will be carried out by experienced researchers who have been trained to perform these type of studies.

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).

We expect that 85% of the animals will experience moderate discomfort, because either tumor growth is induced or the animals will have to recover from anesthesia which is required for the imaging procedures. The remaining 15% of the animals will experience

severe discomfort and will reach the humane end point, for example in therapy experiments due to tumor growth up to 10% of the body weight (2 cm3)

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

[] No > Continue with Section 3: 'Signatures'.
[X] Yes > Explain why it is necessary to kill the animals during or after the procedures.

At the end of the study we will collect tumor tissue to analyze molecular processes in the tumor.

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

[] No > Descripe the method of killing that will be used and provide justifications for this choice.

[X] Yes

11.

Centrale Commissie Dierproeven

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T 0900-28 000 28 (10 ct /min)

info@zbo-ccd.nl

Onze referentie
Aanvraagnummer
AVD103002015209

Uw referentie

Bijlagen
2

Datum 2 september 2015
Betreft Beslissing Aanvraag projectvergunning dierproeven

Geachte heer/mevrouw,

Op 8 augustus 2015 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project Development of new targeting agents for molecular imaging therapy of cancer met aanvraagnummer AVD103002015209. Wij hebben uw aanvraag beoordeeld.

Op 26 augustus 2015 heeft u uw aanvraag aangevuld in antwoord op vragen van de CCD.

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 Development of new targeting agents for molecular imaging therapy of cancer starten. De vergunning wordt afgegeven van 08 september 2015 tot en met 08 september 2020

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 RUDEC gevoegd. Dit advies is opgesteld op 7 augustus 2015. Bij de beoordeling van uw aanvraag is dit advies betrokken overeenkomstig artikel 10a lid 3 van de wet.
Wij nemen dit advies van de commissie over, inclusief de daaraan ten grondslag liggende motivering.
Dit advies en de in de bijlage opgenomen beschrijving van de artikelen van de wet- en regelgeving liggen ten grondslag aan dit besluit.

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).

Met vriendelijke groet,

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: Stichting Katholieke Universiteit Nijmegen
Adres: Geert Grooteplein 10
Postcode en woonplaats: 6500 HB Nijmegen
Deelnemersnummer: 10300

deze projectvergunning voor het tijdvak 08 september 2015 tot en met 08 september 2020, voor het project Development of new targeting agents for molecular imaging therapy of cancer met aanvraagnummer AVD103002015209, volgens advies van Dierexperimentencommissie RU-DEC.

De functie van de verantwoordelijk onderzoeker is [redacted] Voor de uitvoering van het project is Instantie voor Dierenwelzijn verantwoordelijk.
De aanvraag omvat de volgende bescheiden:

1. een aanvraagformulier projectvergunning dierproeven, ontvangen op 11 augustus 2015
2. de bij het aanvraagformulier behorende bijlagen:
    a. Projectvoorstel, zoals ontvangen bij digitale indiening> op 08 augustus 2015;
    b. Niet-technische Samenvatting van het project, zoals ontvangen bij digitale indiening op 08 augustus 2015;
    c. Advies van Dierexperimentencommissie, ontvangen op 08 augustus 2015;
    d. De aanvullingen op uw aanvraag; herziening van het projectplan ontvangen op 26 augustus 2015.

Dierproeven

Naam dierproef	Diersoort	Aantal dieren	Ernst	Voorwaarden
characterization of in vivo behaviour of new targetting agents	muizen	4000/ 5jaar	95% matig 5% licht	Inzet beide geslachten
Assessment of therapeutic efficacy of new tumor targeting agents in mice	muizen	1000/ 5 jaar	85% matig 15% ernstig	Inzet beide geslachten
Characterization of in vivo behaviour of new tumor targeting agents in rats	ratten	400/ 5 jaar	95% matig 5% licht	Inzet beide geslachten
Assessment of therapeutic efficacy of new tumor targeting agents in rats	ratten	500/ 5 jaar	85% matig 15% ernstig	Inzet beide geslachten

Na afloop van dit project wordt een beoordeling achteraf uitgevoerd, omdat de ongerief classificatie ernstig is toegekend in een aantal dierproeven. Deze beoordeling zal uiterlijk augustus 2020 plaatsvinden.

Voorwaarden
Op grond van artikel 10a1 lid 2 Wet zijn aan een projectvergunning voorwaarden te stellen
De Instantie voor Dierenwelzijn houdt er toezicht op dat in de dierproeven mannelijke en vrouwelijke dieren in evenredige aantallen worden gebruikt. Tenzij de onderzochte ziekte dit uitsluit doordat deze ziekte alleen in mannen dan wel in vrouwen voorkomt.

Zo doende wordt voorkomen dat surplusdieren in voorraad moeten worden gedood. Het aanpassen van de huisvesting, bijvoorbeeld het solitair huisvesten van mannelijke dieren is inherent aan deze voorwaarde.

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.
deskundig persoon gedaan worden waarbij dieren zo min mogelijk pijn, lijden, angst of blijvende schade ondervinden. Gewonde dieren moeten onderzocht worden en behandeld, tenzij er een wetenschappelijke motivering is om niet te behandelen.

Beoordeling achteraf
Volgens artikel 10a1, lid 1d en lid 3 van de wet worden 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 waarbij die vallen in de categorie ernstig volgens artikel 10b van de wet en wordt daarom voorzien van een beoordeling achteraf.
Deze beoordeling zal uiterlijk augustus 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 lijden van de proevendieren conform de vergunning waren.