[redacted]

Form
Project proposal

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

1 General information

1.1 Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	
1.2 Provide the name of the licenced establishment.	Biomedical Primate Research Centre
1.3 Provide the title of the project.	Brain and Blood PK assessment of VNAR antibody-based blood brain barrier shuttle

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 or routine production
Research into environmental protection in the interest of human or animal
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 2.1.

Malignant tumors of the brain and central nervous system (CNS) are relatively rare, representing approximately 3% of new cancer cases estimated worldwide. However, they cause significant morbidity and have a very poor prognosis. The average survival rate in adults with a primary malignant brain tumor

is only 34.7%. Glioblastoma is the most common and aggressive malignant brain tumor, and the treatment options are very limited (1). The current five-year survival rate is only 4% in patients aged 55-64. In children and adolescents, primary brain tumors are the leading cause of cancer-related death, surpassing leukemia. Additionally, most cancers that arise in other organs of the body have the ability to metastasize to the brain. An estimated 24-45% of all cancer patients have brain metastases, which is associated with poor survival and high morbidity. Consequently, brain cancer remains an area of urgent and unmet medical need.

Antibodies that specifically target certain antigens on tumor cells have proven to be highly effective in the treatment and/or eradication of a variety of highly malignant forms of cancers. However, many potential antibody therapies are not effective in brain cancer, largely because they are unable to cross the blood-brain barrier (BBB) (2). Recently, the use of single domain variable new antigen receptor domain (VNAR) antibodies as BBB shuttles that can deliver therapeutic antibodies showed brain-specific, best-in-class, robust parenchymal penetration in mice (3). VNAR domains are the smallest naturally occurring antibodies that can be combined with monospecific therapeutic antibodies to develop bispecific agents to be transported over the BBB and target cancer cells. Also other compounds that do not easily penetrate the BBB, such as specific enzymes, growth factors or antisense oligonucleotides can be coupled to VNAR and translocated over the BBB into the brain to potentially treat a variety of disorder. Importantly, there are significant differences in receptor-mediated transport between distantly related species that preclude the direct translation of these mouse studies to humans. Quantitative proteomic studies have shown that there are major differences in the expression of BBB transporters between rodent and human, however the profile is nearly identical between primates (macaque/marmoset/human) (4-7).
Various in vitro screens have been developed to identify antibodies that bind to BBB receptors. However, there is currently no in vitro transcytosis system that can predict the transport over the BBB in vivo or serve as a reliable in vivo model. For instance, many antibodies to the transferrin receptor selected in vitro have adverse effects in vivo because they interfere with function in vivo by either blocking transport of the endogenous ligand or targeting the receptors for lysosomal degradation. In our previous studies (license [redacted]20185885), VNAR phage display libraries were constructed and preselected on transferrin in vitro. Subsequently, these libraries were injected into macaques in order to identify highly potent binders that can cross the BBB without competing with transferrin or disrupting the receptor. After perfusion (and terminal anesthesia) the brain was removed and fractionated to recover the phage particles expressing the specific VNAR from the brain parenchyma. Based on the first results in these studies several promising VNAR candidates that were able pass the BBB without any side-effects were identified. Using novel antibody selection methods, a highly efficient BBB transporter based on VNAR antibody was developed and its brain penetration potential was confirmed in macaques. The antibody named TXP1 (aka H01) showed specificity to human and monkey Transferrin Receptor protein 1 (TfR1) in vitro and no binding to mouse or rat TfR1 was observed. When next tested in macaques (license[redacted]20185885), it showed highly efficient brain delivery reaching 4nM concentration in the brain at 20-hour timepoint when injected at 1.35mg/kg dose (Figure 1). VNAR isotype antibody (G12) was used as negative control and showed brain levels at 0.1-0.2nM concentration what was expected from antibodies lacking BBB shuttling capability. TXP1 brain exposure over negative control (G12) reached up to 35-fold increase in hippocampus and midbrain regions (Figure 2). CSF analysis showed only modest difference of TXP1 over G12 with 2.5-fold increase indicating that the brain delivery was mediated via BBB rather than brain cerebrospinal fluid (CSF) barrier (BCSFB). Importantly, this observation showed that CSF cannot serve as a reliable surrogate for brain exposure, which was a question under [redacted]20185885 in order to replace the need to obtain brain tissue for analysis of penetration. Brain to plasma ratios presented as percentage showed approximately 2.1-2.6% for brain regions and 0.4% for CSF for TXP1 molecule and only up to 0.1% for the negative control (Figure 3). In comparison to BBB shuttles currently undergoing clinical evaluation, TXP1 shows approximately 20-fold dose advantage, which is a major improvement that will be translated to significant increase in efficacy when tested with a therapeutic payload (8).

Further pre-clinical evaluation in NHP to fully characterize TXP1 is needed to determine the selection of the the optimal dosing regimes will be determined. This includes amongst others the determination of optimal administration levels, the subsequent values in blood and the clearance rates, the amounts that can enter the brain and the need and consequences of repeated administration to analyze if steady-state levels can be obtained. These data are essential for the decisions on further development of TXP1 for clinical applications. These data are essential for further development of TXP1. It is expected that the data obtained in this project are sufficiently predictive for the PK of potential future therapeutics based on TXP1.

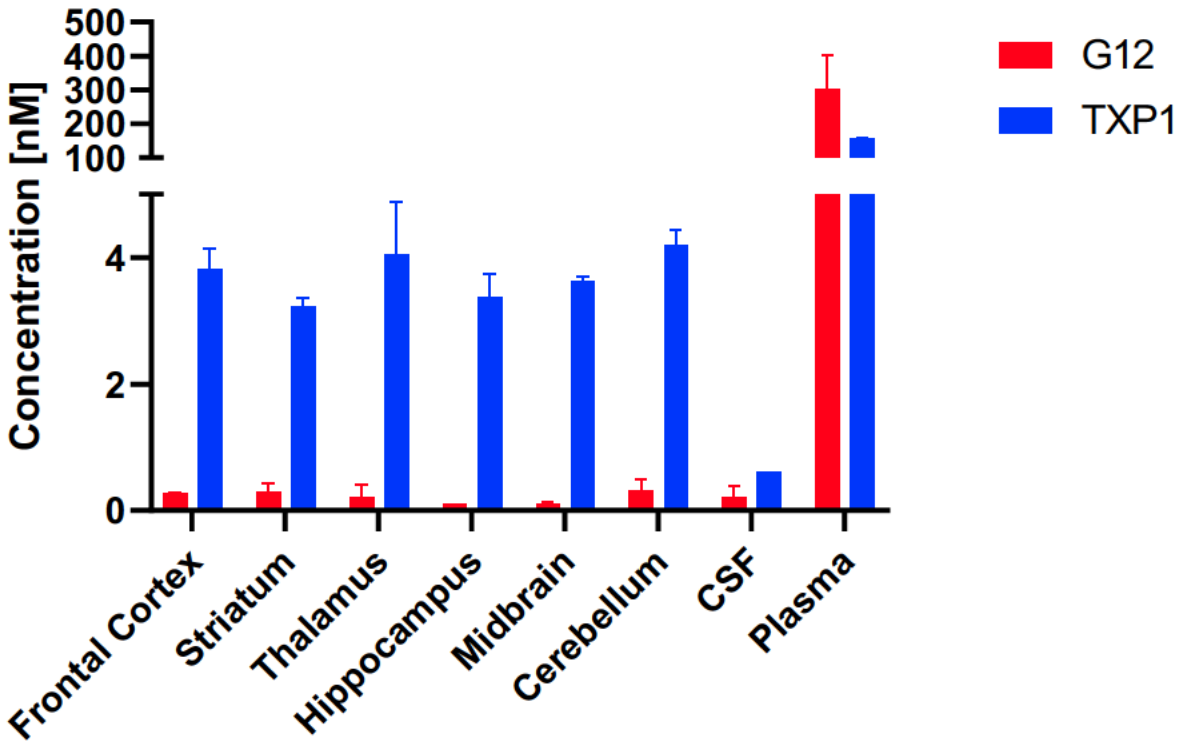

Figure 1. TXP1 and G12 concentrations in NHP brain, CSF and plasma at 20-hour timepoint upon single dose injection at 1.35mg/kg.

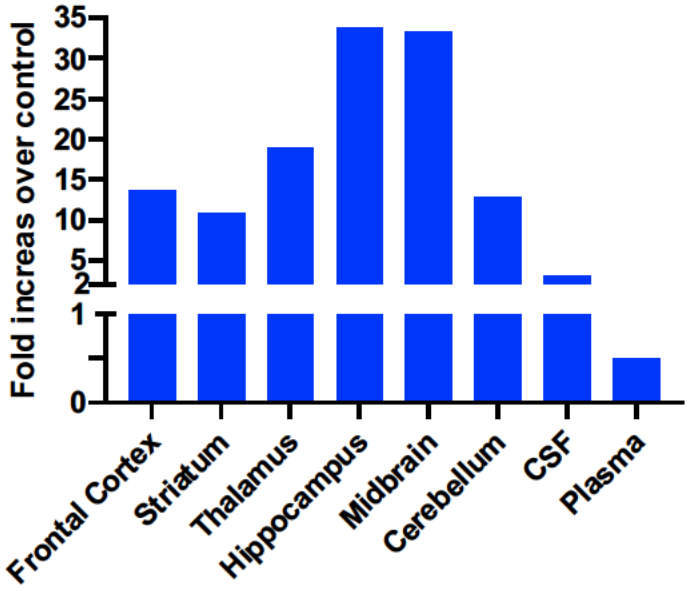

Figure 2. TXP1 fold increase over G12 negative control in brain, CSF and plasma.

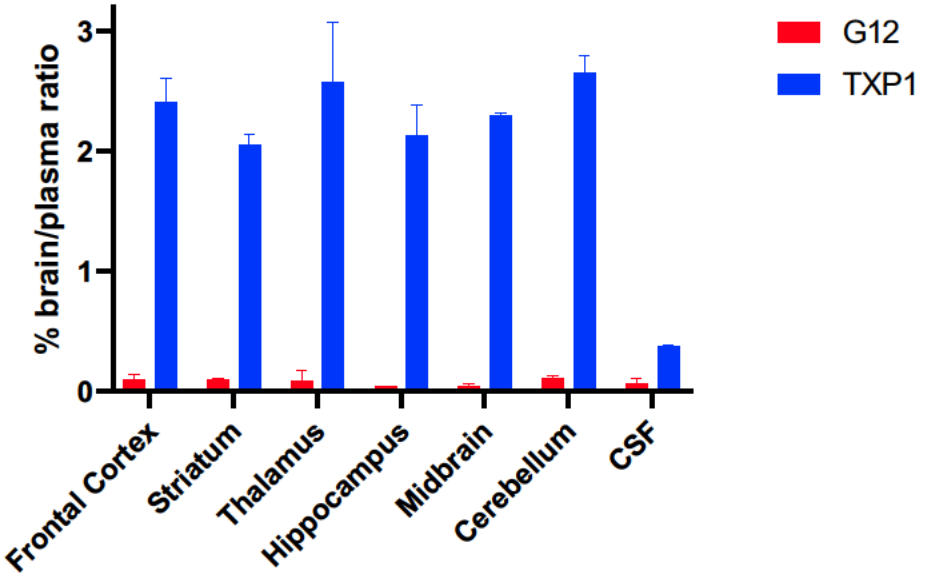

Figure 3. Percentage brain to plasma ratio for TXP1 and G12 negative control.

1. Sastry RA. et al, 2018. The impact of surgery on survival after progression of glioblastoma: a retrospective cohort analysis of the contemporary patient population. J. Clin Neurosci S0967-5868: 31541.
2. Sousa F. et al, 2018. Therapeutic monoclonal antibodies delivery for the glioblastoma treatment. Adv Protein Chem Struct Biol 112:61

3. Stocki, P., et al., Blood-brain barrier transport using a high affinity, brain-selective VNAR antibody targeting transferrin receptor 1. FASEB J, 2021. 35(2): p. e21172.
4. Uchida Y. et al. 2011, Quantitative targeted absolute proteomics of human blood-brain barrier transporters and receptors. J Neurochem 117:333
5. Hoshi Y et al. 2013. Quantitative atlas of blood-brain barrier transporters, receptors, and tight junction proteins in rats and common marmosets. J Pharm Sci 102:3343.
6. Syvänen S. et al. 2009. Species differences in blood-brain transport of three positron emission tomography radioligands with emphasis on P-glycoprotein transport. Drug Metabolism and Disposition 37:635.
7. Ito K. Et al. 2011. Quantitative membrane protein expression at the blood-brain barrier of adult and younger cynomolgus monkeys. J Pharm Sci 100:3939.
8. Kariolis, M.S., et al., Brain delivery of therapeutic proteins using an Fc fragment blood-brain barrier transport vehicle in mice and monkeys. Sci Transl Med, 2020. 12(545).

3.2 Purpose

3.2.1 Describe the project's immediate and ultimate goals. Describe to which extent achieving the project's immediate goal will contribute to achieving the ultimate goal.

- If applicable, describe all subobjectives

The primary objective of this project is to characterise pharmacokinetic (PK) profile of TXP1 in blood and brain in a series of experiments. It is critically important to understand the PK profile for further clinical translation, so the most optimal dosing regimen can be proposed.

The ultimate goal of this project is to develop new effective therapies against brain cancer using TXP-1 conjugated to therapeutic antibodies

3.2.2 Provide a justification for the project's feasibility.

3.2.3 Are, for conducting this project, other laws and regulations applicable that may affect the welfare of the animals and/or the feasibility of the project?

x No

Yes > Describe which laws and regulations apply en describe the effects on the welfare of the animals and the feasibility of the project.

3.3 Relevance

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

This project addresses currently unmet clinical need of combating brain diseases, e.g. brain tumours. There are currently limited treatment possibilities for these devastating cancers and new treatment possibilities are therefore of major social importance. This project will be instrumental to characterise brain and blood PK of TXP1 antibody for further clinical translation. TXP1 BBB transporter can be used as a universal brain delivery shuttle for numerous payloads such as monoclonal antibodies, enzymes, growth factors, antisense oligonucleotides, toxins and small molecules. The therapeutic potential spans from oncology to multiple CNS disorders, which currently remain untreatable.

3.3.2 Who are the project's stakeholders? Describe their specific interests.

The stakeholders from this project can be subdivided into four groups (I) the patients (II) the establishment license holder, (III) the experimental animals and (IV) the industry.

The patients (I) will benefit from the novel therapies that could be developed based on the outcomes of the present project.
The establishment licence holder (II) will benefit by acquiring new knowledge and insights allowing scientists to use this new knowledge and insights for scientific and educational purposes. Additionally, the newly acquired knowledge may be extrapolated by scientists towards other scientific fields of expertise to make groundbreaking discoveries.
The animals (III) are stakeholders as they are used for this research plan. Animals have the right to be protected from discomfort caused by this research Due to this, it is of great importance that the total number of animals during this research is as low as possible with the least possible discomfort without jeopardizing the scientific foundation of this research plan.
The industry will gain due to the possibility to market new therapies when the compounds are successful.

3.4 Strategy

3.4.1 Provide an overview of the overall design of the project (strategy). If applicable, describe the different phases in the project, the coherence, the milestones, selection points and decision criteria.

To assess the applicability of this compound for further development for new therapies against brain cancer, PK studies in blood (appendix 1) and brain (appendix 2) will be performed in parallel. Non-terminal experiments will be used for blood PK assessment, whereas brain PK assessment would require terminal experiments. The PK of TXP1 will be evaluated against an isotype control antibody (G12) (see also 3.4.2, justification).

The minimal dose that maximizes brain exposure will be established. In single-dose escalation study for blood PK the dose with minimal target mediated drug disposition (TMDD; binding of the compound to its receptor) effect over extended time is determined. No TMDD effect would be indicative of the receptor saturation point. Dosing above receptor saturation point would assure extended blood presence, thus providing enough TXP1 in the circulation for sustained brain penetration. That dosing strategy would assure maximal brain exposure over extended time, which is critical for therapeutic delivery and maximizing efficacy.

To correctly establish dose dependent blood PK from both distribution and elimination phases an extended sampling for up to 4 weeks is required. For a single-dose escalation blood PK study maximal 3 doses will be tested (selection based on our earlier results, e.g. 1.35, 6.75 and 13.5mg/kg). Two doses, the highest and lowest, will be tested first. Based upon these data, an intermediate dose might be added retrospectively. This will only be done if necessary to generate a robust data set (Go/No Go).

Brain PK/PD studies could be performed simultaneously with blood PK studies in the same animal but this would result in an increase in the total number of animals in terminal experiments. The proposed experimental scheme is designed to reduce the number of terminal experiments. To establish brain penetration and distribution in the brain it is assumed that this can be done with a single dose and two or three timepoints within a period of within 1 week (in contrast to blood PK, see above). Therefore, the brain PK study will be done independently of the single dose blood PK to limit the number of animals that need to be euthanized. Animals will be injected with an elevated dose of e.g. 6.75mg/kg that is 5-fold higher to the original experiment (Figure 1) in order to increase the saturation point of the transporter receptor and maximise the brain exposure. Based upon our initial data it is not expected that the compounds will induce any side-effects. Initially, two terminal timepoints will be studied, day 1 and e.g. day 5. The data from day 1 will be used to model receptor saturation point upon dose increase together with the data generated in our previous study. The data from both days will be used to estimate brain half-life ($T_{1/2}$ - brain) and brain AUC as a measure of brain exposure. These data will be used to estimate brain half-life ($T_{1/2}$ - brain) and brain AUC as a measure of brain exposure. Also additional blood samples might be obtained to analyse blood PK during this initial time-period and dose and to compare the results with the blood PK data set obtained in the blood PK study.

Go/No Go: Depending on the results of the first brain PK study, an additional timepoint (depending on first data between 2-14 days might be added for further investigation of brain PK. This will only when this is necessary to obtain a robust data set and upon revision of the initial data set.

To analyse tissue biodistribution of TXP1 versus isotype control (G12) also other organs as well as blood and CSF will be collected from animals that have been sacrificed (brain PK study). Such analysis will answer the organ specificity of TXP1 and also maximally utilise the animals sacrificed in the study.

Based upon the evaluation of single-dose blood PK and the brain PK data it can be envisioned that the most optimal dosing strategy for maximal, sustained brain exposure (expressed as AUC for brain) might require a subsequent dose to maintain the targeted receptor saturation for maximal brain transport. Depending on the blood clearance data obtained in the single dose study, the timepoint for this second dose is expected to be between 1 and 7 days after a first dose. This study will only be conducted after evaluation of the single-dose blood PK and brain PK data iand when these data indicate that this two-dose regime will be required for maximal brain transport and targeted receptor saturation (Go/No Go).

3.4.2 Provide a justification for the strategy described above.

To establish the kinetics of the compounds and determine the optimal delivery into the brain, both blood PK and brain PK data are needed. Therefore, the data from blood and brain PK will be analyzed in tandem in order to provide a comprehensive PK data set. Both blood and brain PK studies are needed to establish different parameters e.g. blood half-life ($T_{1/2}$ - blood) for distribution and elimination phase, dose- and time-dependent clearance, dose-dependent plasma exposure (AUC - blood),and dose-dependent median residence time (MRT) for blood and brain half-life ($T_{1/2}$ - brain), brain saturation profile (Time to C_{max}). Because a full blood profile must be obtained, blood and brain PK can not be studied in the same animals

The control antibody, G12 is a non-binding antibody of similar structure and molecular weight to TXP1, which blood PK and clearance is not affected by a target engagement, thus TMDD. Consequently, G12 blood PK profile is used as a reference for TXP1 dose dependent clearance rate, TMDD and receptor saturation point. For terminal brain PK studies, the use of G12 will be limited to essential experiments only. However, it is critical to have a reference antibody to conclusively show the advantage of BBB shuttles such as TXP1. In addition to absolute quantification, the data will be presented as relative increase in the brain of TXP1 over negative control G12. The advantage of relative comparison is that it cannot be distorted by quantification method itself, therefore it is critical for the evaluation of TXP1.

Various go/no decision points are included in order to minimize the number of animals needed i.e. for blood PK and for brain PK first two doses are analysed and only when needed for a robust data set a third dose is added.

This study aims at establishing the minimal dose that would maximize brain exposure. In the dose escalation study for blood PK we expect to determine the dose with minimal target mediated drug disposition (TMDD) effect over extended time. No TMDD effect would be indicative of the receptor saturation point. Dosing above receptor saturation point would assure extended blood presence, thus providing enough TXP1 in the circulation for sustained brain penetration. That dosing strategy will assure maximal brain exposure over extended time, which is critical for therapeutic delivery and maximizing efficacy. Blood PK cannot fully replace brain PK because levels of TXP1 in the brain also depend on penetration rate and distribution and elimination in the brain itself.

Ideally blood sampling could be performed in brain PK studies at several timepoints preceding the final termination to establish the whole distribution and elimination phase of the compound. However, to correctly establish dose dependent blood PK from both distribution and elimination phases a sampling for up to 4 weeks is required. Blood sampling after the first week post-dosing is only once weekly for up to 4 weeks in total. To limit the number of animals used in terminal studies, the blood PK (appendix 1) and brain PK (appendix 2) are done separately. Brain PK requires a terminal study which would limit the

samples to be taken for the blood PK. Brain exposure (brain PK) for brain penetrating shuttles such as TXP1 is correlated with the blood levels (blood PK). However, the relationship of brain to blood concentration is unique to each individual shuttle antibody and depends on multiple factors including association rate (ka) to the endogenous receptor. Therefore, plasma levels for TXP1 can be used as a practical surrogate for brain levels during the dose optimization studies. But for optimal validation and determination of brain penetration and distribution also brain PK measurements on a limited number of timepoints are needed. Although not immediately dependent on blood PK data, the blood PK data will be used to guide the dosages used for the brain PK data.

Only when the data obtained from the blood PK and from the brain PK show that for the most optimal dosing to obtain good, sustained brain exposure more than 1 dose is required a study in which 2 subsequent doses are administered will be applied. For this study, blood PK data are sufficient to predict together with the earlier obtained single dose administrations for both blood and brain PK brain levels and it is anticipated that it is not needed to sacrifice additional animals for a multidose brain PK study.

3.4.3 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	Intravenous injection of antibodies for blood PK evaluation
2	Intravenous injection of antibodies for brain PK evaluation
3	
4	
5	
6	
7	
8	
9	
10	