# Begeleidingsformulier aanvraag dierproef DEC- UM

*Versie 2006*

**Herziene versie**

**DECNR: 2011-007**

**Ontvangen: 09-02-2011**

| DEC datum goedkeuring# | Type aanvraag [2] |
|---|---|
| 02-03-2011 | Nieuw |

| VROM/GGONR[3] | LNV/CBDNR[4] |
|---|---|
| | |

| Hoofdproject | ~~CARIM~~ | ~~NUTRIM~~ | ~~Hersenen en gedrag~~ | GROW | ~~biomaterialen~~ | ~~Ander UM~~ | ~~Geen UM~~ |
|---|---|---|---|---|---|---|---|
| Deelproject | | | | 3. | | | |

| Financieel beheerder | | Budgetnummer | 30943083B |
|---|---|---|---|

Titel van het onderzoek:

Molecular magnetic resonance imaging of the epidermal growth factor receptor in colorectal cancer

startdatum 01/04/2011 einddatum [9] 01/04/2015 *Duur van de proef [10]: 2 maanden*

| | Naam | Tel (+ Tel privé enkel VO, VVO en VM) | E-mailadres | Bevoegdheid[5] | Cap. groep /afdeling |
|---|---|---|---|---|---|
| 1.Verantwoordelijk onderzoeker (VO) | | | | Art.9 | |
| 2. Vervanger VO (VVO) | | | | Art.9 | |
| 3. Verantwoordelijk medewerker (VM) GGO[7] | | | | | |
| 4. overige uitvoerenden | | | | Art.12 | |
| 5. overige uitvoerenden | | | | Art.9 | |

| Diergroep | 1 | 2 | 3 | 4 | 5 | | |
|---|---|---|---|---|---|---|---|
| ctrl/exp/sham | exp | exp | exp | exp | exp | | |
| Diersoort | 01 | 01 | 01 | 01 | 01 | | |
| Stam | NMRI nu/nu | NMRI nu/nu | NMRI nu/nu | NMRI nu/nu | NMRI nu/nu | | |
| Construct / mutatie ? | - | - | - | - | - | | |
| Herkomst (leverancier) * | 02 | 02 | 02 | 02 | 02 | | |
| Aantal | 72 | 96 | 96 | 48 | 66 | | |
| Geslacht | v | v | m/v | m/v | m/v | | |
| Dieren immuuncompetent ? | nee | nee | nee | nee | nee | | |
| Leeftijd/gewicht | 2m/28-30g | 2m/28-30g | 2m/28-30g | 2m/28-30g | 2m/28-30g | | |
| Doel van de proef * | 30 | 30 | 30 | 30 | 30 | | |
| Belang van de proef * | 01 | 01 | 01 | 01 | 01 | | |
| Toxicologisch onderzoek * | 01 | 01 | 01 | 01 | 01 | | |
| Bijzondere technieken * | 01 | 01 | 01 | 01 | 01 | | |
| Anesthesie * | 04 | 04 | 04 | 04 | 04 | | |
| Pijnbestrijding * | 01 | 01 | 01 | 01 | 01 | | |
| Mate ongerief * | 05 | 05 | 05 | 05 | 05 | | |
| Toestand dier einde exp* | 01 | 01 | 01 | 01 | 01 | | |

* VHI-coderingen zie bijlage

1 Verantwoording

*Aanvraag dierproef DEC-UM* **(kaders zijn licht flexibel, maar het geheel is max. 5 pag. versie 2006)**

**Titel:** *Molecular magnetic resonance imaging of the epidermal growth factor receptor (EGFR) in colorectal cancer*

1. Doel van de proef

*Overexpression of the epidermal growth factor receptor (EGFR) is a molecular hallmark of epithelial malignancies, including colorectal cancer. EGFR, being actively involved in the tumor development and progression, is therefore an important diagnostic and therapeutic target in oncology.*

*Aim of this study is to evaluate the utility of EGFR-targeted MR contrast agents for imaging of urgent diagnostic targets in colorectal cancer. Cetuximab, a clinically applied anti-EGFR antibody, served as a targeting ligand for the development of EGFR-specific probes. Considering the relevance of EGFR in oncology, the proposed imaging strategy can serve a wide range of applications. The differentiation between EGFR-positive and -negative tumors is especially desired for planning and monitoring of the anti-EGFR therapy, which is widely applied in patients with colorectal cancer. Moreover, EGFR imaging is a promising approach for the non-invasive assessment of residual disease after (chemo)radiotherapy, which presence is often unrevealed in a routine clinical examination. Interestingly, the proposed Cetuximab-based molecular imaging approach can serve also as a platform for the combined therapeutic and imaging purposes.*

*The specificity and efficacy of the contrast agent (CA) association with EGFR-expressing cancer cells will be tested in vivo with MRI and ex vivo using optical methods. We will use the inherently EGFR-overexpressing and -underexpressing human tumor xenografts. The HT-29 colorectal human carcinoma will serve as an EGFR-positive tumor model, whereas T-47D breast human carcinoma as an EGFR-negative control. Both tumor models will be established in nude mice. The non-specific contrast agent, containing a non-specific protein, will be used as a control for the passive uptake in the tumor. To characterize the circulation kinetics and biodistribution of the contrast agents, we will determine their circulation half-live and perform analysis of dissected organs.*

*Our second aim is to determine the sensitivity of the EGFR-CA-enhanced MRI to changes in the EGFR-expression. For this purpose, we will apply a newly-established tumor model of U373 2l glioblastoma (+/- dox), in which the EGFR expression can be regulated by doxycyclin (dox). This molecular flexibility appears to be an excellent representation of a positive and negative response to the anti-EGFR-therapy. Therefore, it can provide a reliable evaluation of the applicability of the EGFR-specific imaging for the anti-EGFR-therapy monitoring.*

*The visualization of residual disease after neoadjuvant (chemo)radiotherapy is an important potential application of EGFR-CA. To simplify the treatment protocol, we will treat the tumor-bearing mice with radiation only. After radiotherapy, the mice will undergo MRI examination with the use of the EGFR-targeted contrast agent. The readout obtained from this evaluation will be compared to that obtained using the non-targeted equivalent.*

*Moreover, we will evaluate theranostic (therapeutic and diagnostic) properties of the EGFR-targeted probes. This bifunctional approach may provide valuable predictive parameters at the early stage of treatment, and, thus, contribute to the personalization of the anti-cancer therapy.*

**2. Maatschappelijke relevantie en/of wetenschappelijk belang**

*EGFR is a relevant diagnostic and therapeutic target in colorectal cancer. Moreover, it appears to be a relatively universal cancer marker, as its abundantly expressed by various types of malignant cells. The noninvasive visualization of EGFR expression, which is a leading topic of our study, would add an important contribution to the current state of cancer diagnostics. The currently used imaging methods relay mainly on the anatomical and morphological properties of the tissue. However, the functional and molecular status of the disease is important as well. While the functional imaging is already clinically available, the non-invasive MRI assessment of molecular targets is at the early stage of development.*

*Molecular profiling can be extremely valuable in cancer detection, staging and therapy monitoring. The EGFR-specific imaging is particularly desired for the monitoring of EGFR-targeted therapy. Currently, this is done in rather indirect and delayed manner. In contrast, tracing changes in the tumor EGFR expression would provide early and more specific therapeutic readout. Moreover, the specific visualization of molecules involved in cancerous processes, such as EGFR, is a promising strategy for detection of low volume disease, which is one of the biggest challenges of oncologic radiology. Microscopic cancer cell islets, which remain after partial response to (chemo)radiotherapy, are frequent source of tumor regrowth and spread. Therefore, they require timely detection and therapeutic intervention. Their successful visualization would enable better selection of patients for surgery after neoadjuvant therapy and early assessment of metastases.*

*The proposed study integrates therefore both social and scientific objectives. The non-invasive assessment of EGFR expression will provide more complete picture of the disease in cancer patients. This molecular imaging strategy remains, however, a major scientific challenge. The design of our study, including the use of colorectal tumor model and MRI as an evaluation method, is thought to speed up the translation of pre-clinical findings to the patients with colorectal cancer, who are a group of our particular interest.*

**3. Alternatieven**

*The targeting properties of the proposed EGFR-targeted probes are being evaluated in vitro on EGFR-positive and EGFR-negative tumor cells. However, in vitro experiments cannot reproduce the complexity of the in vivo environment. This is particularly the case for the tumor microenvironment, which is a complex structure, composed of neoplastic cells, blood vessels, immune cells and other supporting components. Moreover, the circulation kinetics and biodistribution, which co-contribute to the in vivo properties of the contrast agent, cannot be tested in vitro. Therefore, it is vital to evaluate this novel approach in more complex system than a cell culture.*

*The execution of this study in humans is impossible since the proposed agents are experimental prototypes and, thus, they are not approved for the clinical use.*

**4. Ethische afweging**

*The topics of this study i.e., molecular imaging, molecular-therapy monitoring, detection of the residual disease after chemo(radiotherapy), and combined imaging and therapy, are important and very actual areas of cancer research. Investigators that are involved in this study are convinced about the importance of this project for health and knowledge interests.*

*We believe that the gains from these experiments overweight animal suffering. This study does not include any procedures that cause serious suffering of animals. MRI allows for painless disease evaluation. The duration of MRI examination and interval between the subsequent measurements as well as all other experimental procedures are set in a way to minimize a degree of discomfort for animals.*

*The use of the non-invasive diagnostic method of MRI that is also applicable to humans will assure more efficient translation of the preclinical findings to the clinical setting.*
*In addition to in vivo experiments, ex vivo evaluation of mouse tissues will be preformed. In this way we will maximize the use of animals sacrificed for this study.*

**5. Wetenschappelijke onderbouwing**

*A high expression of EGFR is a molecular fingerprint of the majority of epithelial malignancies, including colorectal, lung and head and neck carcinomas. The EGFR signaling pathway plays a key role in tumor pathogenesis, by promoting the cancer cell proliferation, angiogenesis, invasion, metastasis and inhibition of apoptosis [1]. EGFR is considered therefore one of the most important molecular markers in oncology.*

*Anti-EGFR therapy is frequently used in combination with (chemo)radiotherapy for treatment of patients with EGFR-expressing cancers [2]. Cetuximab, which is an anti-EGFR antibody, serves as a molecular therapeutic that silences the EGFR-dependant molecular processes [3]. In the current study, we aim to exploit a high binding affinity of Cetuximab to EGFR for the diagnostic purposes. There are several objectives for this application related to colorectal cancer, which is the main focus of our research. The ability to non-invasively visualize the EGFR expression would enable better selection of patients for the anti-EGFR therapy as well as more specific therapy-monitoring. The clinically applied methods measure indirect and rather delayed effects of the treatment. The molecular approach would mean the early detection of therapeutic effects, which would translate to more efficient treatment modification, if required. Moreover, the molecular imaging of EGFR is a promising method for detection of microdisease, which is a serious problem in colorectal cancer patients [4,5]. The present lack of imaging tools that are sensitive enough to visualize small tumor islets leads to, on one hand, the patient overtreatment and, on the other hand, the local and distant cancer reoccurrence. Interestingly, Cetuximab-targeted contrast agent, comprising both therapeutic and diagnostic capabilities, is a promising candidate theranostic agent [6,7].*

*Considering a broad application of magnetic resonance imaging (MRI) in cancer diagnostics, and in colorectal cancer particularly, MRI appears to the most desired technique for imaging of EGFR expression. Molecular imaging with MRI requires, however, a powerful contrast agent that binds specifically and efficiently to the target receptor. The most frequently used approach includes the functionalization of a conventional MR contrast agent with a ligand that binds with high affinity to the molecular epitope of interest. Moreover, multi-modal constructs are often designed, which provide more extensive evaluation of the proposed targeting strategy. For example, the inclusion of a fluorescent dye to the formulation enables ex vivo evaluation of tumors with optical methods, such as fluorescence microscopy or FACS analysis, and, thus, the validation of MRI findings.*

*Three types of MRI-detectable probes of different physicochemical and magnetic properties were designed.*

*This strategy of labeling antibodies is applied predominately for molecular PET-imaging probes. However, few examples can be also found in the MRI-related literature [8]. By using this approach we aim to obtain relatively small agent, which will assure sufficient tissue penetration as well as MRI contrast in vivo. Other two EGFR-specific probes are based on nanoparticles, which are currently the most widely applied in the field of molecular MRI [9]. This is due to their high relaxivity per particle, which is very desired for detection of molecular targets, which are present at very low concentrations in vivo. Ultrasmall*

*are used for this purpose. They are functionalised with Cetuximab for active targeting to EGFR. Each of the agent has its non-targeted equivalent.*

*MRI is a leading imaging modality in oncology and a promising technique for molecular imaging. MRI, at the current state of clinical development, can provide valuable information on the*

*anatomical/morphological and functional state of the tumor. In the present study, the following MRI measurements will be performed: $T_2$-weighted, diffusion-weighted, contrast-enhanced $T_1$-weighted imaging and $T_1$ mapping. Both $T_2$-weighted and diffusion-weighted imaging are anatomical/morphological methods, which are sensitive to the presence of neoplastic tissue. Contrast-enhanced MRI ($T_1$-weighted imaging and $T_1$ mapping) will be used to trace the EGFR-targeted and non-targeted probes within the tumor and determine their targeting properties.*

*To closely bridge the clinical questions with the pre-clinical setup, the targeting properties of the newly designed EGFR-targeted contrast agents will be tested in human xenografts of colorectal cancer established in nude mice in order. The HT-29 colorectal human carcinoma will serve as an EGFR-positive tumor model, whereas T-47D breast human carcinoma as an EGFR-negative control. Moreover, U373 2l glioblastoma (+/- dox), in which the EGFR expression can be regulated by doxycyclin (dox), will be used for the evaluation of the sensitivity of EGFR-specific imaging strategies to changes in EGFR expression within the same tumor model.*

*The idea of our EGFR-targeted contrast agents expands further to the relatively new field of teranostics [6,7]. A key attribute of theranostics is an agent comprising both the therapeutic and diagnostic properties. The proposed EGFR-targeted agents, exerting both anti-EGFR activity and magnetic/fluorescent signal, are therefore excellent multifunctional candidates. To evaluate this aspect of EGFR targeting, we will study how the MR contrast produced in the tumor by EGFR-targeted agents relates to the treatment outcome, i.e., EGFR expression and tumor growth delay, and how it affects the signal produced by a second dose of the same probe.*

**6. Wetenschappelijke beoordeling**

*This project has been evaluated and approved by [redacted] from the Department and [redacted] of Maastricht University Medical Center.*

**7. Proefdier keuze**

**7a. Soort, stam / herkomst / eindbestemming**

*We would like to use NMRI-nu (nu/nu) mice, which are needed to establish human tumor xenografts of the HT-29 colorectal human carcinoma and T-47D breast human carcinoma. We chose HT-29 since the colorectal cancer is one of the leading topics of clinical research in our department. By using the preclinical model of this cancer type we will be able more efficiently translate the pre-clinical findings to the clinical setting. T-47D breast human carcinoma will be used as a negative control for the expression of EGFR. Therefore, it can serve an excellent control for the specificity of our imaging agents.*
*Moreover, we would like to use the U373 2l glioblastoma (+/- dox) to study the sensitivity of the EGFR-targeted probes to changes in the EGFR expression = AIM3*
*The mice will be obtained from a registered breeder in* ).
*At the end of the study, the animals will be sacrificed and histological analysis of tumors and normal organs will be performed.*

**7b. Sexe**

*The sex of the animal is relevant in this study. Growth of T-49D breast human carcinoma is estrogen-dependent. Therefore, for Experiments 1 and 2 female mice are require. In the experiments 3-5, which do not include T-49D tumor model either male or female mice will be used.*

**7.c. Aantallen**

*Three EGFR-targeted contract agents and their non-targeted equivalents will be evaluated:*

| | | |
|---|---|---|
| 1. EGFR-targeted-(_ _ _ _ _ _)x | 1. EGFR-targeted- | 1. EGFR-targeted- |
| 2. non-targeted- | 2. non-targeted- | 2. non-targeted- |

**Experiment 1.** *Determination of the circulation kinetics of EGFR-targeted and non-targeted agents in tumor-bearing mice.*

*We set a power* $\pi = 80\%$ *and the significance level* $\alpha = 0.05$. *To measure the group size we used the formula of L. Sachs:*
$n = 2(z_{\alpha/2} - z_{\pi})^2 * (\sigma/\delta)^2$
$2(z_{\alpha/2} - z_{\pi})^2 = F.$
For $\alpha = 0.05$ and $\pi = 80\%$: F=15.7
*We estimated that the minimal effect considered meaningful (effect = contrast agent concentration) around* $\delta = 75\%$ *and standard deviation of* $\sigma = 30\%$.
*Filling the formula of L. Sachs results:* $n = 15.7 * (30/75)^2 = 2.5 = 3$ mice/group

***There is no drop out expected in this experiment.***

***Experiment 2.*** *In vivo imaging of EGFR-expression in the tumor.*

*We estimated that the minimal effect considered meaningful (effect = MRI contrast change) around* $\delta = 30\%$ *and standard deviation* $\sigma = 20\%$.

*Filling the formula of L. Sachs results:* $n = 15.7 * (20/30)^2 = 6.9$ mice

*Uitvalspercentage* ***10% is related to potential difficulties with MRI measurements, such as technical failure and movement artifacts, which can affect the quality of data considerably or make them even unusable.*** *The estimated percentage is based on the previous experience of the investigators. Great care will be taken to limit the technical-related drop out, however it is often operator-independent.*

$(a - 0.1a) = 4.5$ of $0.9a = 6.9$ or $6.9/0.9 = a$ $a = 7.666 = 8$ mice/group

**Experiment 3**. *Sensitivity of EGFR-CA-enhanced MRI to changes in the EGFR expression.*

*We estimated that the minimal effect considered meaningful (effect = MRI contrast change) around $\delta = 30\%$ and standard deviation $\sigma = 20\%$.*
*Filling the formula of L. Sachs results:* $n = 15.7 * (20/30)^2 = 6.9$ mice

*Uitvalspercentage* ***10% is related to potential difficulties with MRI measurements, such as technical failure and movement artifacts, which can affect the quality of data considerably or make them even unusable.*** *. The estimated percentage is based on the previous experience of the investigators. Great care will be taken to limit the technical-related drop out, however it is often operator-independent.*

$(a - 0.1a) = 6.9$ of $0.9a = 6.9$ or $6.9/0.9 = a$ $a = 7.666 = 8$ mice/group

***Experiment 4****. In vivo imaging of the residual disease after radiotherapy using the EGFR-targeted contrast agent*

*We estimated that the minimal effect considered meaningful (effect = MRI contrast change) around $\delta = 30\%$ and standard deviation $\sigma = 20\%$.*
*Filling the formula of L. Sachs results:* $n = 15.7 * (20/30)^2 = 6.9$ mice

*Uitvalspercentage* ***10% is related to potential difficulties with MRI measurements, such as technical failure and movement artifacts, which can affect the quality of data considerably or make them even unusable.*** *The estimated percentage is based on the previous experience of the investigators. Great care will be taken to limit the technical-related drop out, however it is often operator-independent.*

$(a - 0.1a) = 6.9$ of $0.9a = 6.9$ or $6.9/0.9 = a$ $a = 7.666 = 8$ mice/group

***Experiment*** *5. In vivo theranostic (therapeutic and diagnostic) properties of the EGFR-targeted contrast agent*

*We estimated the minimal effect considered meaningful (effect= MRI contrast change) $\delta = 30\%$ and a standard deviation of $\sigma = 20\%$. We set a significance level $\alpha = 0.05$ and a power $\pi = 0.9$. We set the power at 90 because we expect a high variation in the therapeutic response to the EGFR-targeted probe. This can be due to differences in the baseline EGFR expression. Sensitivity of MRI is quite low, we anticipate therefore that when our power is set to 80 we will not be able to see a significant difference in MRI parameters. To measure the group size we used the formula of L.Sachs :*

$$n = 2(z_{\alpha/2} - z_{\pi})^2 * (\sigma/\delta)^2$$

$2(z_{\alpha/2} - z_{\pi})^2 = F$. For *significance level* $\alpha = 0.05$ *and a power* $\pi = 90\%$ $F_{0.90} = 21.02$

$$n = 21.02 * (\sigma/\delta)^2$$
$$n = 9.342 \text{ mice}$$

*Uitvalspercentage* ***10% is related to potential difficulties with MRI measurements, such as technical failure and movement artifacts, which can affect the quality of data considerably or make them even unusable.*** *The estimated percentage is based on the previous experience of the investigators. Great care will be taken to limit the technical-related drop out, however it is often operator-independent.*

$(a - 0.1a) = 9.342$ $0.9a = 9.342$ $9.342 / 0.9 = a$ $a = 10.38 = 11$ mice/group

***Exp1.*** *6 contrast agents, 4 tumor models (EGFR-positive HT-29, negative T-49D, U373 2l glioblastoma (dox +/-)), 3 mice per group → (6x4x3) = 72*

***Exp2.*** *6 contrast agents, 2 tumor models (EGFR-positive HT-29 and negative T-49D), 8 mice per group → (6x2x8) = 96*

***Exp3.*** *6 contrast agents, 1 tumor model (U373 2l glioblastoma), 2 treatments (dox+/-), 8 mice per group → (6x1x2x8) = 96*

***Exp4.*** *6 contrast agents, 1 tumor model (EGFR-positive HT-29), 8 mice per group → (6x1x8) = 48*

***Exp5.*** *6 contrast agents, 1 tumor model (EGFR-positive HT-29), 11 mice per group → (6x1x11) = 66*

***The total number of animals needed: 72+96+96+48+66=378***

9 Dierproef

**8. Experiment**

***All animal experiments and procedures within this research will be performed according to the code of practice related to animal research within the field of cancer [9]. All human endpoints are based on this document.***

***Experiment 1.*** *Determination of the circulation half-live and the biodistribution of the EGFR-targeted and non-targeted probes.*

*Circulation half-lives will be determined for the following contrast agents:*

| | Iron oxide particles | Paramagnetic liposomes |
|---|---|---|
| 1.EGFR-targeted- | 1.EGFR-targeted- | 1.EGFR-targeted |
| 2.non-targeted-( | 2.non-targeted- | 2.non-targeted- |

*Mice will be inoculated subcutaneously with tumor cells (either HT-29, T-47D or U373 2l glioblastoma (+/- dox)) in lateral flanks. The procedure of tumor cells inoculation is described in SOP1. When tumors are established (tumor size: approximately 150-250mm$^3$ measured with a Vernier calliper), the mice will be anaesthetized and the infusion line filled with the contrast agent will be placed in the tail vain (SOP2). Before contrast agent injection 20µl of blood will be collected from the vena saphena (SOP3). Subsequently, the intravenous administration of a buffered contrast agent solution (maximal volume = 0.2ml) will be performed via catheter. 20 µl of blood will be collected from the vena saphena 2 min, 15 min, 30 min, 45 min, 1 hour after contrast agent administration. The animal will be recovered from the anesthesia. At 4, 8, 24, 48, 72, 96, 120 hours after contrast agent injection, the animal will be fixed in a 50 ml falcon tube and 20 µl of blood will be sampled from the vena saphena. After the last blood sampling (120h after contrast agent injection) the animal will be anesthetized with isoflurane (induction: 2.5%) and the animal will be euthanatized by dislocation of cervical vertebrae. Tumors and other tissues (liver, spleen, lung, muscle, kidney, heart, and skin) will be collected for immunohistochemical analyses and Gadolinium (Gd) determination with Inductively Coupled Plasma-Mass Spectrometry (ICP-MS). The Gd concentration in blood samples will be assessed indirectly by MRI measurements and validated by (ICP-MS).*

***Experiment 2.*** *In vivo imaging of EGFR expression in the tumor.*

*The following contrast agents will be evaluated:*

| | Iron oxide particles | Paramagnetic liposomes |
|---|---|---|
| 1.EGFR-targeted- | 1.EGFR-targeted- | 1.EGFR-targeted- |
| 2.non-targeted- | 2.non-targeted- | 2.non-targeted- |

*Mice will be inoculated subcutaneously with tumor cells in lateral flanks. The procedure of tumor cells inoculation is described in SOP1. When tumors are established (tumor size: approximately 150-250mm$^3$ measured with a Vernier calliper), the mice will be anesthetized and the infusion line with the contrast agent will be placed in the tail vein (SOP2). Subsequently, MRI examination (SOP4) will be performed, during which the contrast agent will be administered. The total time of*

*measurements will be around 3 hours. The second MRI scan will be performed at the time point, at which the contrast agent is completely cleared from the blood to avoid the background signal. This time point will be determined from the kinetics experiment (Experiment 1). The circulation of Cetuximab is very long ($t_{1/2}$=100h), however, its conjugation to other molecules/particles may change the kinetics considerably. We estimated that the contrast agent will be cleared from the blood approximately 72h after administration. The animals will be sacrificed immediately after the second MRI examination. Tumors and normal tissues (liver, spleen, lung, muscle, kidney, heart, skin) will be collected for immunohistochemical analyses to validate MRI results. The timeline of this experiment is presented in Figure 1.*

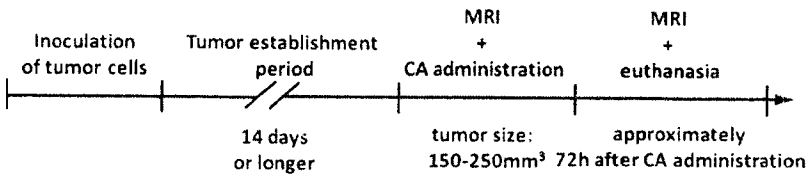


*Figure 1: Timeline of the* ***Experiment*** *2.*

***Experiment*** *3. Sensitivity of EGFR-targeted MRI approach to changes in the EGFR expression.*

*The following contrast agents will be evaluated:*

| | | |
|---|---|---|
| 1.EGFR-targeted- | 1.EGFR-targeted | 1.EGFR-targeted |
| 2.non-targeted· | 2.non-targeted | 2.non-targetec |

*Mice will be inoculated subcutaneously with tumor cells in the lateral flank (SOP1). When tumors are established (tumor size: approximately 150-250mm$^3$ measured with a Vernier calliper), the mice will be assigned to one of two experimental groups, One group will receive doxycyclin in drinking water (2g/l), while the control group will get pure water. 2 days after treatment, the mice will undergo MRI examination (SOP 4), during which one of the investigated contrast agents will be administered (SOP2). Approximately 72h thereafter, the post-contrast MRI will be performed (SOP 4). After these measurements, the mice will be euthanized. Tumors and normal tissues (liver, spleen, lung, muscle, kidney, heart and skin) will be collected for immunohistochemical analyses. The timeline of this experiment is presented in Figure 2.*

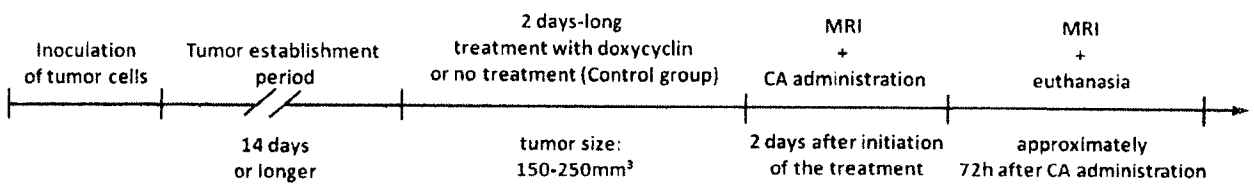


*Figure 2: Timeline of the* ***Experiment*** *3.*

**Experiment 4.** *In vivo imaging of the residual disease after radiotherapy using EGFR-targeted contrast agents*

*The following contrast agents will be evaluated:*

| | | |
|---|---|---|
| 1.EGFR-targeted- | 1.EGFR-targeted- | 1.EGFR-targeted |
| 2.non-targeted- | 2.non-targeted- | 2.non-targeted |

*Mice will be inoculated subcutaneously in the lateral flank with tumor cells, according to SOP1. When tumors are established (tumor size of 150-250mm$^3$ measured with a Vernier calliper), the mice will undergo MRI examination (SOP4) to assess the pre-radiotherapeutic status of the tumor. The total time of this examination will be around 2 hours. The tumors will be irradiated on a subsequent day (SOP5) with a dose of 10Gy. The second MRI will be performed 5 days after radiotherapy. The choice of this readout timepoint is based on the previous experience with this tumor model related and its response to radiotherapy. During the second MRI examination, lasting 3 hours, one of the investigated contrast agents will be injected. The third MRI scan will be performed at the timepoint when the contrast agent is completely cleared from the blood, thus, approximately 72h after administration. After these measurements, the mice will be euthanized. Tumors and normal tissues (liver, spleen, lung, muscle, kidney and heart) will be collected for immunohistochemical analyses. The timeline of this experiment is presented in Figure 3.*

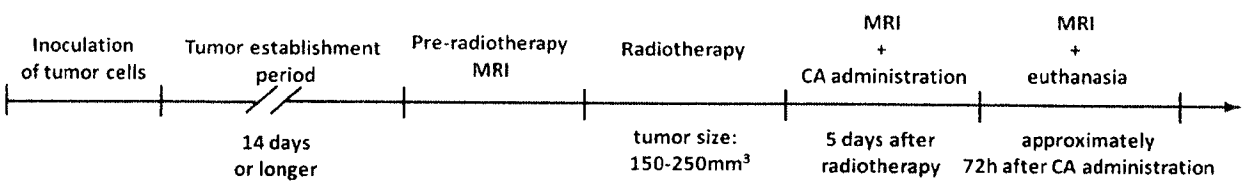


*Figure 3: Timeline of the* ***Experiment 4****.*

**Experiment 5.** *In vivo theranostic (therapeutic and diagnostic) properties of the EGFR-targeted contrast agent*

*The following contrast agents will be evaluated:*

| | | |
|---|---|---|
| 1.EGFR-targeted- | 1.EGFR-targeted- | 1.EGFR-targeted- |
| 2.non-targeted- | 2.non-targeted- | 2.non-targeted- |

*Mice will be inoculated subcutaneously with tumor cells in the lateral flank, according to SOP1. When tumors are established (tumor size: approximately 150-250mm$^3$ measured with a Vernier calliper), the mice will be anaesthetized and an infusion line with one of the contrast agent will be placed in the tail vain (SOP2). Subsequently, first MRI (SOP4) will be performed, during which the contrast agent will be administered. The total measurement time will be around 3 hours. The assessment of the tumor uptake level will be done approximately 72h thereafter. During the second MRI examination, another dose of the contrast agent will be injected. The final MRI will be performed 72h after administration of the second dose. Mice will be sacrificed immediately*

*thereafter. Tumors and normal tissues (liver, spleen, lung, muscle, kidney, heart, and skin) will be collected for immunohistochemical analyses. The timeline of this experiment is presented in Figure 4.*

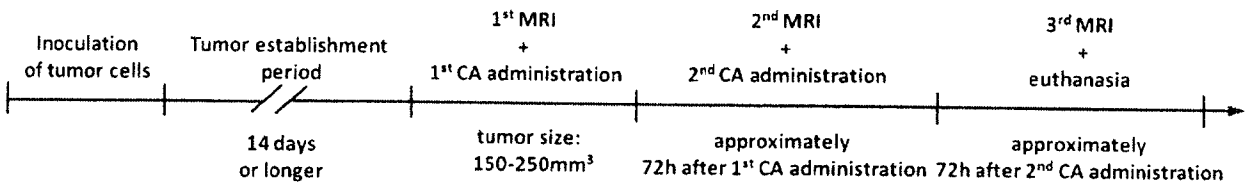


*Figure 4: Timeline of the* ***Experiment*** *5.*

**9. Experimentele condities**

**9a. Anesthesie**

*Placement of an infusion line in the tail vein of a mouse and MRI measurements will be performed under isoflurane anesthesia. The anaesthesia will be induced in the induction chamber with 2.5% of isoflurane and maintained with 1.5-2% of isoflurane delivered via a mouth cap. The medical air will be used as a carrier of isoflurane. The depth of anaesthesia will be controlled with a calibrated vaporizer. During the MRI measurements the body temperature will be kept on the physiological level by the warming plate, on which the mouse is positioned. Respiration will be monitored with a balloon sensor connected to an ECG/respiratory unit.*
*During radiotherapy animals will be sedated with 30-100µl/kg of medetomidine administrated s.c.*

**9b. Pijnbestrijding**

*The subcutaneous injection (tumor inoculation) is expected to cause mild pain. The intravenous injection (drug administration) will be performed under anaesthesia with isoflurane during MRI measurements. Blood sampling from vena saphena until one hour after contrast agent injection will be performed under anaesthesia (anaesthesia needed for i.v. injection of a contrast agent), whereas in later time points without since it is expected to cause mild pain. The radiotherapy will be done on animals sedated with medetomidine administered s.c. No post-treatment pain is expected, as only tumors will be exposed to the radiation. Euthanasia by dislocation of cervical vertebrae will be performed while the animal is under anaesthesia. Mentioned above procedures do not require administration of any analgesics.*

**9c. Euthanasie en Humane eindpunten**

*The animals are euthanized under sedation at the end of experiment by dislocation of the cervical vertebrae. If during the experiment an animal becomes ill (body weight loss of 20%), or suffers heavily (infection, tumour volume>1500mm$^3$), then the animal will be euthanized. When there would exist a doubt over the seriousness of suffering or the possibility of treatment, then the CPV-veterinarian (art.14) will be consulted.*

Zorg

**10a. Ongerief**

***Subcutaneous tumor cell inoculation*** *(discomfort = 03, duration = 2 min)*
*Restraining and subcutaneous injection*

***Tumor growth*** *(discomfort = 01, duration = approximately two weeks from a day of inoculation (HT-29) or longer (T-47D))*
*There are no systemic side effects related to the tumor growth on the flank. The animal discomfort related to the presence of established tumor depends on the tumor size. Degree of discomfort can vary from none or minimal in the case of small tumors to moderate and serious when the tumors reach big size. When there will be threatening of serious discomfort (tumor volume>1500mm$^3$) the animal will be euthanized. However, we do not expect to find such an advance tumor stage during our experiments.*

***Tumor size monitoring*** *(discomfort = 03, duration = the whole duration of the experiment*
*Animal handling and restraining*

***Blood sampling*** *(discomfort = 04, duration = 72h with intervals)*
*Anesthesia with isoflurane (first 1h), i.v. injection, puncture of the vena saphena in 15 min-long intervals, recovery from the anesthesia, animal restraining and puncture of the vena saphena at 4h, 8h, 24h, 48h, 72h, 96h and 120h after CA injection, euthanasia by the cervical vertebrae dislocation.*

***i.v. injection of the contrast agent*** *(discomfort = 04, duration = 15-45 min).*
*Anesthesia with isoflurane, fixing an infusion line in the tail vein, injection of the contrast agent, recovery from the anesthesia. No side effects are expected to be induced by the contrast agent.*

***MRI examination*** *(discomfort = 04, duration ~3 hours, intervals of several days between the subsequent MRI experiments)*
*Anesthesia with isoflurane, placement of an animal in a cradle, recovery from the anesthesia or euthanasia by cervical vertebrae dislocation.*

***Radiotherapy*** *(discomfort = 04, duration ~ 0.5 h).*
*Anesthesia (s.c. injection of medetomidine), radiation applied for a few minutes, recovery from the anesthesia. No systemic effects are associated with the radiation protocol.*

***Degree of discomfort per experimental group:***

| experimental group | experimental procedure | duration | discomfort | frequency | total discomfort |
|---|---|---|---|---|---|
| 1 | Subcutaneous tumor cell inoculation<br>Tumor growth<br>Tumor size monitoring<br>Blood sampling | 2 min<br>two weeks or longer<br>5 min | 03<br>01<br>03<br>04 | 1<br>continuous<br>multiple<br>13 over 120 h | 05 |
| 2 | Subcutaneous tumor cell inoculation<br>Tumor growth<br>Tumor size monitoring<br>MRI examination and i.v. CA injection | 2 min<br>two weeks or longer<br>3 h | 03<br>01<br>03<br>04 | 1<br>continuous<br>multiple<br>2 | 05 |
| 3 | Subcutaneous tumor cell inoculation<br>Tumor growth<br>Tumor size monitoring<br>MRI examination and i.v. CA injection | 2 min<br>two weeks or longer<br>3 h | 03<br>01<br>03<br>04 | 1<br>continuous<br>multiple<br>2 | 05 |
| 4 | Subcutaneous tumor cell inoculation<br>Tumor growth<br>Tumor size monitoring<br>MRI examination and i.v. CA injection<br>Radiotherapy | 2 min<br>two weeks or longer<br>3 h<br>0.5 h | 03<br>01<br>03<br>04<br>04 | 1<br>continuous<br>multiple<br>3<br>1 | 05 |
| 5 | Subcutaneous tumor cell inoculation<br>Tumor growth<br>Tumor size monitoring<br>MRI examination and i.v. CA injection | 2 min<br>two weeks or longer<br>3 h | 03<br>01<br>03<br>04 | 1<br>continuous<br>multiple<br>3 | 05 |

**10b. Welzijnsevaluatie**

*A logbook will be used where daily animals welfare will be evaluated. Tumor volume will be monitored regularly (two times a week or more).*

**11. Verzorging en huisvesting**

*Animals will be housed in animal housing facilities of CPV. Tumor cell inoculation as well as blood sampling will be performed in the animal laboratory of CPV. The MRI measurements will be done on , whereas the euthanasia will be performed in the proefdierruimte located next to the scanner room. These facilities are located on the of azM.*

*During whole experiment animals will be housed socially (3-4 mice per cage). Mice undergoing the same treatment protocol will be housed in the same cage. Standard rodent chow and water will be available ad libitum throughout the study.*

**12. Deskundigheid**

*In the frame of this study the following experimental procedures are planned:*
*-Subcutaneous injection*
*-Induction and maintenance of the anaesthesia*
*-Intravenous injection*
*-Blood sampling from the vena saphena*
*-Monitoring of the life parameters and adjustment of anaesthesia during MRI measurements.*
*-Radiotherapy*
*-Euthanasia by dislocation of the cervical vertebrae*
*They will be performed, as required, by a competent person (with art.9 qualifications). The people involved in this experiment have already experience in this type of investigations and they have the required licence.*

**13. Standard Operation Procedures (SOP)**

*SOP1: Subcutaneous inoculation of tumor cells.*

- *Restraining of an animal*
- *Subcutaneous injection of $1x10^6$ tumor cells (cell suspension in PBS or matrigel, volume of 100µl, needle 26G) in the right flank*
- *The inoculated mice will be marked on the tail with a markerpen*

***SOP2**: Placement of an infusion line in the tail vein*

- *Mice will be transported from CPV to facilities azM in a closed cagekart*
- *Sedation of the animal with isoflurane*
  a) *Induction: 2.5% isoflurane in the induction chamber,*
  b) *Maintenance: the mouse is placed on the warming plate, 1.5% isoflurane delivered via a mouthcap with a flow of isoflurane with medical air as a gas carrier. The depth of anaesthesia will be controlled with a calibrated vaporizer.*
- *The tail will be warmed up with warm "gel dressing" in order to dilate the tail veins and make them more visible.*
- *The needle will be inserted in the vein lumen*
- *An infusion line (26G needle, catheter, and syringe) will be fixed in the tail vein with a glue and a tape. The infusion line will enable the intravenous injection during the MRI experiment (changes in the animal position are avoided).*

***SOP3**: Blood collection from the vena saphena*

- *Sedation of the animal with isoflurane*
  *-Induction: 2.5% isoflurane in the induction chamber,*
  *-Maintenance: the mouse is placed on the warming plate, 1.5% isoflurane delivered via a mouthcap with a flow of isoflurane with medical air as a gas carrier. The depth of anesthesia is controlled with a calibrated vaporizer and by testing the foot-reflex.*
- *The tail will be warmed up with warm "gel dressing" in order to dilate the tail veins and make them more visible.*
- *The needle connected to the infusion line (26G needle, catheter, and syringe)*

*will be inserted in the vein lumen and fixed on the tail with a glue and a tape.*

- *20ul of blood will be sampled from the vena saphena before contrast agent injection*
- *The contrast agent will be infused*
- *20 µl of blood will be collected from the vena saphena 2 min, 15 min, 30 min, 45 min, 1 h after contrast agent administration.*
- *The animal will recover from the anesthesia in the incubator.*
- *4, 24, 48, 72, 96, 120 h after contrast agent injection the animal will be fixed in a 50 ml falcon tube and 20 µl of blood will be sampled from the vena saphena (blood collection from vena saphena will be performed according to the SOP: CPV-3-MR).*
- *After the last blood sampling (120h after contrast agent injection) the animal will be anesthetized with isoflurane. The animal will be euthanatized by dislocation of the cervical vertebrae.*

*SOP4: MRI measurements*

- *Sedation of an animal with isoflurane (induction: 2.5% isoflurane in the induction chamber, maintenance: 1.5% isoflurane delivered via a mouthcap with a flow of isoflurane and medical air as a gas carrier. The depth of anaesthesia is controlled with a calibrated vaporizer.*
- *If the injection of the contrast agent will be performed during MRI, the infusion line is fixed in the tail vein (see SOP2)*
- *Eye ointment is administered on the mouse eyes to prevent from drying. The mouse is placed in a home built cradle, equipped with a mask for anaesthesia gas supply and a warm water pad. The cradle lies in a coil in the MR scanner. The respiration is monitored with a balloon sensor connected to a respiratory unit and the body temperature with a temperature-sensitive rectal probe.*
- *The total time of measurements will not exceed 3 hours. First $T_2$-weighted and diffusion-weighted imaging will be performed.*
- *$T_1$ weighted images will be generated before and after injection of the contrast agent.*
- *After completing MRI measurements mice will be either allowed to recover and placed back in the cage or euthanized by cervical dislocation, while still being under anaesthesia,.*

***SOP5**: Radiation therapy protocol*

- *Mice will be transported from CPV facilities to . in a closed cage*
- *Restraining of an animal*
- *Sedation with medetomidine (30-100 µl/kg; s.c.) administered 10 minutes before the treatment*
- *Mouse is placed on a warm water pad*
- *Eye ointment is applied on the mouse eyes to prevent from drying*
- *The treatment will be performed in a custom-made setup, in which only tumor is exposed to the radiation, whereas the rest of the mouse body is unexposed*
- *Tumors will receive a dose of 10 Gy*
- *The total time of irradiation will be 5 min*
- *After recovery from anaesthesia, mice will be transported back to the animal*

*housing facility.*

**Relevante literatuur**


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*6. Jain KK. "Personalized medicine". Curr Opin Mol Ther. 2002, 4(6):548-58.*

*7. Pene F, Courtine E, Cariou A and Mira JP. "Toward theragnostics". Crit Care Med. 2009, 37(1 Suppl):S50-8.*

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*9. Inspectie W&V, Z.D.H. juli 1999. CODE OF PRACTICE, DIERPROEVEN IN HET KANKERONDERZOEK.*

University Maastricht

Faculty of Health, Medicine and Life Sciences

Dierexperimenten Commissie

DEC

[redacted], voorzitter
p/a Secretariaat DEC-UM
Postbus 616
NL-6200 MD Maastricht
Telefoon:

Aan:

Uw referentie:

Onze referentie :

Maastricht, 02-02-2011

Geachte Onderzoeker,

Uw projectaanvraag: "*Molecular magnetic resonance imaging of the epidermal growth factor receptor in colorectal cancer*", is op de DEC vergadering van 28 januari 2011 besproken.

De DEC heeft een aantal vragen en opmerkingen:

- Op dit protocol is GGO niet van toepassing en daarom kunnen de GGO medewerker en het GGO nummer van het voorblad verwijderd worden.
- De DEC verzoekt op het voorblad de duur van de proef aan te passen in 2 "maanden".
- Bij punt 6 verzoekt de DEC alleen aan te geven, "This project has been evaluated and approved by..."
- De DEC verzoekt bij punt 7c, na de uitvalberekening de groepsgrootte te bepalen (afronden) en daarna deze groepsgrootte te gebruiken voor de berekening van het totaal aantal dieren.
- De DEC vindt de uitvalpercentages erg hoog en wenst een betere motivering hiervoor. De DEC accepteert niet dat het uitvalpercentage zo hoog is door niet goed getrainde onderzoekers (bloedafname moet mogelijk zijn met nagenoeg geen uitval).
- Bij punt 7c- *Exp 2*, hier moeten de aantallen naar boven afgerond worden en dan moet er staan "6x2x9".
- Bij punt 10a verzoekt de DEC in een tabel, de aard, ernst, duur en frequentie beter te definiëren per handeling en groep, en het totale ongerief per groep aan te geven. De DEC is van mening dat het totale ongerief code 05 is.

Conclusie:
Het project wordt aangehouden

Gelieve eventuele vragen te beantwoorden in **een brief en indien noodzakelijk Uw project aan te passen en duidelijk de aanpassingen grijs te markeren.**
Uw project staat bij de DEC geregistreerd onder nummer **2011-007**, gelieve dit nummer in verdere correspondentie te vermelden.

Hoogachtend,

Voorzitter DEC-UM

07.02.2011

Dear DEC members,

Hereby, I address my responses to the comments of DEC on my proposal 2011-007 entitled "*Molecular magnetic resonance imaging of the epidermal growth factor receptor in colorectal cancer*". The answers are given in **bold**.

De DEC heeft een aantal vragen en opmerkingen:

- Op dit protocol is GGO niet van toepassing en daarom kunnen de GGO medewerker en het GGO nummer van het voorblad verwijderd worden.

**According to the suggestion, the number and GGO responsible researcher have been removed.**

- De DEC verzoekt op het voorblad de duur van de proef aan te passen in 2 "maanden".

**The word "manden" was corrected to "maanden".**

- Bij punt 6 verzoekt de DEC alleen aan te geven, "This project has been evaluated and approved by..."

**Point 6 was adjusted as follows: This project has been evaluated and approved by . from the and from of Maastricht University**

- De DEC verzoekt bij punt 7c, na de uitvalberekening de groepsgrootte te bepalen (afronden) en daarna deze groepsgrootte te gebruiken voor de berekening van het totaal aantal dieren.

**The point 7c was corrected. For the calculation of the total number of animals the rounded up number of animals was used.**

- De DEC vindt de uitvalpercentages erg hoog en wenst een betere motivering hiervoor. De DEC accepteert niet dat het uitvalpercentage zo hoog is door niet goed getrainde onderzoekers (bloedafname moet mogelijk zijn met nagenoeg geen uitval).

**The "uitvalpercentage" was decreased to 10% and in the case of blood sampling experiment to 0%. As a result, the number of requested animals decreased. The motivation for the remaining 10% is the following: Uitvalspercentage 10% is**

**related to potential difficulties with MRI measurements, such as technical failure and movement artifacts, which can affect the quality of data considerably or make them even unusable. The estimated percentage is based on the previous experience of the investigators.**

- Bij punt 7c- *Exp 2*, hier moeten de aantallen naar boven afgerond worden en dan moet er staan "6x2x9".

**The point 7c was corrected. For the calculation of the total number of animals the rounded up number of animals was used.**

- Bij punt 10a verzoekt de DEC in een tabel, de aard, ernst, duur en frequentie beter te definiëren per handeling en groep, en het totale ongerief per groep aan te geven. De DEC is van mening dat het totale ongerief code 05 is.

**A table with detailed depiction of experimental procedures, their duration, discomfort and frequency per experimental group was added to the proposal on page 14. According to the suggestion of DEC, the total discomfort of animals was changed from 04 to 05.**

I hope that the current version of my DEC proposal will be positively evaluated.

Kind regards,

Maastricht University

Faculty of Health, Medicine and Life Sciences

Aan:

| *Ons kenmerk* | *Doorkiesnummer* | *Maastricht* |
|---|---|---|
| | | 07-03-2011 |

**Project:** *Molecular magnetic resonance imaging of the epidermal growth factor receptor in colorectal cancer.*

**Verantwoordelijk onderzoeker (VO):**

Hierbij delen wij U mede dat voornoemd project aan de ethische toetsingscriteria voor proefdiergebruik voldoet.
De DEC maakt geen bezwaar tegen uitvoering van dit project zoals aangevraagd en geeft een **positief advies**.

| | |
|---|---|
| **Projectnummer:** | **2011-007** |
| **Diersoort:** | **muis** |
| **Aantal dieren:** | 378 |
| **Einddatum:** | 02-03-2015 |

Uw project staat bij de DEC en CPV geregistreerd onder bovenstaand nummer. Gelieve dieren, die voor dit project bestemd zijn, ook onder dit nummer aan te vragen.

Voorzitter DEC-UM

Vice-Voorzitter DEC-UM

DEC-UM
Voorzitter DEC-UM

p/a secretariaat DEC-UM

***Secretariaat DEC-UM***

**Bezoekadres**

***Postadres***
Postbus 616
6200 MD Maastricht