H2020-ITN THERACAT (765497)

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Deliverable Title	A – Requirement No. 1						
Contractual Delivery Date	28/02/2019	**Nature**	Ethics			**Dissemination Level**	CO
Actual Delivery Date	10/05/2019	**Contributors**	IBEC, TAU, TAG				

Overview/Abstract

- Copies of relevant authorisations (for breeders, suppliers, users, and facilities) for animal experiments must be obtained, kept in the file and submitted to the REA upon request.

- Copy of ethical approvals (covering also the work with genetically-modified animals, if applicable) must be obtained, kept on the file and submitted to the REA upon request.

- If applicable, copies of training certificates/personal licenses of the staff involved in animal experiments must be kept in the file and submitted to the REA upon request.

Explanation for large delay in submitting deliverable

N/A

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1. A – Requirement No. 1

Animal experiments within the THERACAT project will be conducted by Beneficiaries Tel Aviv University (TAU) and Tagworks Pharmaceuticals BV (TAG). Please find below details on the experiments to be performed as well as on the status of the corresponding authorisations and approvals.

TAU: Mice are the standard animal model by which we evaluate tumour progression following full evaluation of the tumour cells in culture (i.e. *in vitro*). In order to monitor the migration of tumour cells in the bloodstream and follow-up their invasion and extravasation from the blood vessels into a new metastatic niche, we label the cells fluorescently and use non-invasive intravital imaging which significantly decrease the number of animals required for each study. However, in order to detect and study the effect of the new compounds on tumour-host interactions that govern processes studied in this project such as angiogenesis, metastases formation and interactions with the supporting microenvironment, animals are required and cannot be replaced by any *in vitro* model.

In this project, we proposed to design and physico-chemico-biological characterisation of new nanomedicines. In order to study the pharmacokinetics of these new nanomedicines synthesized during the project and evaluate and determine their effect on the tumour itself and its supporting stroma including angiogenic blood vessels formed as well as cancer-associated fibroblasts and immune cells that come from the host (i.e. the animal bearing the tumour), it is necessary to use animals that provide the physiological (and pathological) environment of the whole organism.

The specific strains of mice that will be used correlate with the tumour type and source of the cells. For example, for human tumours inoculated in mice, we will use either SCID or nu/nu mice, whereas for murine tumours, we will use C57BL/6 or BALB/c mice depending on the specific tumour type.

Since experiments on animals are indispensable to follow tumour progression and develop strategies to reduce the effects of disease progression, the experimental design and the experimental procedures are customized to avoid or at least to minimize pain, distress and other suffering for the animals' sake. All animal handling, imaging, euthanasia and discarding will be performed according to institutional guidelines (TAU IACUC).

Small animal studies: This study covers basic fundamental biological questions and is also translational ('applied') by nature, and therefore, it requires a large number of mice. In general, the procedures that will be carried out will include the inoculation of a variety of human tumour cells in a total of 700 SCID and athymic nude immunodeficient mice that will be implanted with, and another 700 mice that will be treated by all compounds and vehicles synthesized during this project, at 3 escalating doses in order to determine toxicity and biodistribution in diseased and non-disease-bearing mice. This is an

important point, as we would like to use clinically-relevant tumour models including human xenografts, and thus it is necessary to use this type of immunodeficient mice.

Two hundred BALB/c and 200 C57Bl wild-type mice will be implanted with syngeneic murine tumour models and treated with our novel polymeric nanomedicines. It is paramount to establish a tumour model in immune-competent mice, since we know that the host including mainly the tumour stroma and microenvironment with associated inflammation, have a central role in tumour progression and metastases.

The anticipated impact of these experiments when completed is to shed light on fundamental cancer biology phenomenon, i.e. tumour progression and metastases and how it is affected by different compounds. Our multidisciplinary approach will offer new nanomedicines with a higher therapeutic efficiency and reduced side-effects. An additional potential outcome would be to obtain an alternative preventive therapy for patients with high risk of outbreak of metastases or for those with minimal residual disease.

Practical considerations: The actual group size for the animal studies (n=6 to 10 animals) will depend on the magnitude of the probe signal or drug effect and the inter-animal variability. After completion of *in vivo* imaging and pharmacological experiments, all animals will be euthanized and tissues will be harvested for HPLC analysis (to determine PK), histological and immuno-histochemical analysis. All not-harvested tissues or post-analysis samples will be discarded according to institutional procedures. Research will be carried out with due concern for the environment, in particular the disposal of all chemical waste generated during the course of the program.

In accordance with Directive 2010/63/EU, in particular Article 33 ("Care and accommodation"), we will ensure that, as far as the care and accommodation of animals is concerned (a) all animals will be provided with accommodation, an environment, food, water and care which are appropriate to their health and well-being; (b) any restrictions on the extent to which an animal can satisfy its physiological and ethological needs will be kept to a minimum; (c) the environmental conditions in which animals are bred, kept or used will be checked daily; (d) arrangements will be made to ensure that any defect or avoidable pain, suffering, distress or lasting harm discovered is eliminated as quickly as possible; and (e) animals are transported under appropriate conditions.

All imaging protocols and procedures will be performed under anaesthesia for immobilization and will induce no pain. Animals will be monitored for levels of health and overall well-being. Any signs of discomfort greater than the disease provoked (body weight loss >15%; tumour volume > 20% of body weight) will trigger removal of the animal from the respective session. All animals will be regularly monitored for body weight, changes in behaviour and signs of any discomfort. All animals will be housed in our animal facility which is under the supervision of two qualified veterinarians. Mice will be given *ad libitum* access to food and water. Bedding is replaced regularly on a 3-weekly basis.

All animal handling, imaging, euthanasia and discarding will be performed according to institutional guidelines. Relevant EU legislation and directives to follow are: Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010; Directive 2003/65/EC of the European Parliament and of the Council of 22 July 2003 amending Council; Directive 86/609/EEC on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes; Directive 86/609/EEC of 24 November 1986 on the protection of Animals used for Experimental and other Scientific Purposes.

To comply with the principles of replacement, reduction and refinement, we will follow the rules of humane animal experimentation.

Replacement: Imaging probes and therapeutic molecules will be first evaluated biochemically in the test tube and in cell culture experiments. Following the in vitro experiments, we will test our newly-synthesized compounds in live animals. The use of animal models is an essential step to develop application procedures that will be applied prospectively in humans.

Reduction: Although our research requires a large number of mice, we intend to reduce the number of mice used in each experiment by several ways:

1. Experiments that are performed in parallel will use the same control group.

2. Experiments will be carried out using non-invasive imaging techniques, i.e. animals can be used in longitudinal studies for monitoring disease progression. This will significantly reduce the number of animals required for the study and enhance the statistical robustness of the experiment. These includes fluorescence-probe labelling of injected cells (by infection or transfection with GFP, mCherry or luciferase) and by using fluorescent probes and fluorescently-labelled polymers. The non-invasive imaging by fluorescence and bioluminescence devices (CRI Maestro and Biospace Photon Imager or IVIS SpectrumCT, respectively) will allow us to significantly reduce the number of mice needed for follow-up of disease progression since we are following-up on the same mouse in each group reducing the variability of the experiment. In addition, there is no need for extra mice for each time point- all experiments are terminated at the end point. Similarly, pharmacokinetic and biodistribution studies of the synthesized probes, new compounds and polymers are imaged using spectral analysis which allows for unmixing of different fluorophores. This means that we can image simultaneously at one wavelength ("colour") the pathological site and at another wavelength- the labelled polymer, compound and/or probe. Pharmacokinetics and biodistribution studies are enabled in this way without performing any unnecessary surgical procedure and resecting the mice at the different time points, but rather imaging them non-invasively until the terminal end point.

3. For each *in vivo* experiment, emphasis will be placed on the amount of information that we can receive from the experiment performed. For example, in each experiment, we will obtain as many measurements as possible in order to reduce the number of similar/parallel experiments that need to be performed. This will include measurements

of body weight and follow-up of fluorescence signal to determine disease volume. Moreover, following experiment termination, we will take measurements of necrosis, hypoxia, microvessel density, proliferation and apoptosis, and specific markers for IHC in only one experiment. Standardization of experiments and service of highly skilled staff will further contribute to a significant reduction in the number of animals.

Refinement: Animals will be housed in SPF animal facility and cared by veterinarians and experts in animal welfare ensuring the highest possible standard of treatment and care practices. Animal care unit has a humane end-point policy and any animal displaying signs of suffering will be immediately euthanized. Animals will be euthanized by cervical dislocation at the first cervical vertebra according to IACUC. For imaging studies, mice will be anaesthetized using isoflurane in medical oxygen.

All procedures comply with the European Commission Recommendation of 07/02/2008 on a Code of Conduct for Responsible Nanosciences and Nanotechnologies Research.

In THERACAT, NU/NU mouse xenograft model of human A375 or MDA-MB-231 cancer cell will be used. A375 cells will be inoculated intradermally under ketamine (100 mg/kg) and xylazine (12 mg/kg) anaesthesia. MDA-MB-231 cells will be inoculated intramammary under ketamine (100 mg/kg) and xylazine (12 mg/kg) anaesthesia.

NU/NU mice bearing A375 tumours will be treated when tumour size will reach 50 mm3; MDA-MB-231 tumour-bearing NU/NU mice will be treated when tumour size will reach 50 mm3. Controls or nanomaterials will be administered by tail vein injection or intraperitonealy. Animals will be euthanized according to the protocol either right after imaging procedure, or at defined endpoint.

Alternatively, for syngeneic mouse models of cancer in immunocompetent mice, we will use murine 4T1 mammary carcinoma in Balb/c mice and B16F10 murine melanoma in C57/BL6 mice.

B16F10 cells will be inoculated intradermally under ketamine (100 mg/kg) and xylazine (12 mg/kg) anaesthesia. 4T1 cells will be inoculated intramammary under ketamine (100 mg/kg) and xylazine (12 mg/kg) anaesthesia.

C57/BL6 mice bearing B16F10 tumours will be treated when tumour size will reach 50 mm3; 4T1 tumour-bearing Balb/c mice will be treated when tumour size will reach 50 mm3. Controls or nanomaterials will be administered by tail vein injection or intraperitoneally. Animals will be euthanized according to the protocol either right after imaging procedure, or at defined endpoint.

TAG: The use of animals for research in The Netherlands is regulated by the Dutch Experiments on Animals Act national law “Wet op de Dierproeven (Stb 1977, 76); Revised Experiments on Animals Act (Stb 1997, 003); Revised Experiments on Animals

Act (Stb 2003, 399); http://wetten.overheid.nl/BWBR0003081/geldigheidsdatum_29-07-2010

All animal experiments have to be approved by the Animal Welfare Committee of the Radboud University. This Animal Welfare Committee is acknowledged by Dutch law and has the responsibility to provide permits for animal experiments. The committee will evaluate beforehand if the scientific and societal interests weigh up to the use and discomfort of animals. This (ethical) review is done according to the previously notes Dutch law on animal experiments.

All animals are observed daily for general assessment of health by the Central Animal Facility staff. Sick animals are reported to the institutional veterinarian and responsible researcher. In case of severe discomfort also the Animal Welfare Body will be informed.

Food and water, and environmental parameters are observed daily by the Central Animal Facility staff. Husbandry logs are documented daily.

In THERACAT, Balb/c nude mouse xenograft models of cancer cells listed in the project will be used. Mice will be inoculated s.c. with cells under halothane anaesthesia. Mouse studies (biodistribution, imaging, or therapy) will commence when the tumours reach 50–100 mm3 size. Compounds will typically be administered by tail vein infusion.

We herein confirm that relevant authorisations and ethical approvals from the TAU Institutional Animal Care and Use Committee as well as from the Animal Welfare Committee of the Radboud University for the abovementioned animal experiments have been obtained and are kept in file, and that they will be submitted to the REA if requested.

Concerning training certificates of the staff involved in animal experiments, we herein confirm that:

- **TAU:** animal experiments will be conducted by ESR13 (Mr. Daniel Rodríguez). The fellow will be trained before conducting any animal experimentation and will not be allowed to perform animal experiments until he obtains the corresponding training certificate.
- **TAG:** animal experiments will be conducted by technicians working in the animal facilities, all of them already having the corresponding training certificate to work with animals.

2. References

N/A