1 Publishable summary

***NANOTHER project** (http://www.nanother.eu)*

Cancer is the second cause of mortality in the world only surpassed by the cardiovascular diseases. In 2006 more that 3M cases were diagnosed in Europe and, out of those, more than 1.7M ended in death of the patients. Breast and colorrectal cancer represent 13.5 and 12.9 % of those cases respectively (J. Ferlay et al 2007. Anal. Oncol. 18, 581-592). Although the treatment of those cancers is under study and, in some cases, well established, there is a need for better therapeutics and, in general, a decrease in the secondary effects that those treatments generate.

The NANOTHER project objective is to obtain a novel type of therapy with a better performance in tumour elimination that the actual therapies and also a non-invasive diagnostics tool based on magnetic nanoparticles. The novelty in this project comes with the higher selectivity of the drug using nanoparticle encapsulation and targeting using antibodies or other targeting moieties and the use of new therapeutic drugs. Aplidin® and siRNA, together with already know drugs like doxorubicin and paclitaxel will be used. Besides, a different approach will be carried out based on a property of magnetic nanoparticles. This approach involves the so called effect of hyperthermia which relies on the tumour ablation from inside using magnetic nanoparticles irradiated with electromagnetic (EM) pulses of known frequency. These nanoparticles absorb EM frequencies and increase their temperature, killing tumour cells in their proximity.

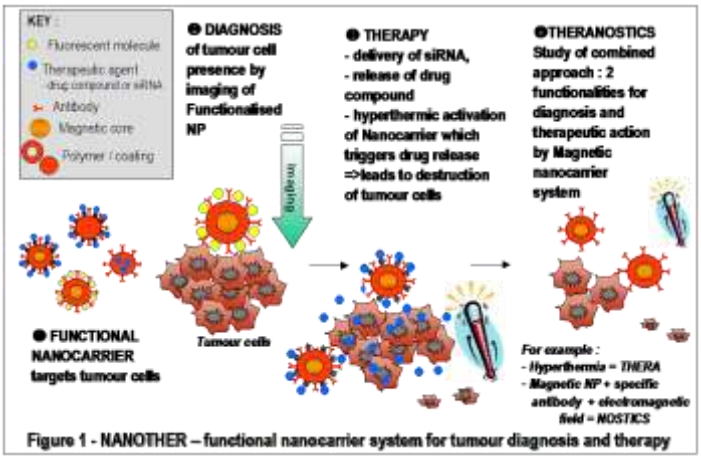

Figure 1 - NANOTHER – functional nanocarrier system for tumour diagnosis and therapy

The project started on September 2008 and will last until September 2012. We are now three quarters into the project duration and have interesting results. The project is divided into 7 main subprojects, all of them interconnected.

In first place, polymer and nanoparticle synthesis and characterisation is crucial for the future development of the therapeutic and diagnostics system. In this respect many polymers have been synthesized and characterised in order to have a battery of nanocarriers that can be used later for encapsulation. Based on properties of those polymers and nanoparticles like known biocompatibility, toxicity, loading capacity and efficiency, antibody attachment, etc, three types of polymers have now been chosen. Overall three types of polymers have been chosen for further development. The physico-chemical characteristics of the polymers selected make them ideal for drug loading and antibody attachment. Also Magnetite and a magnetic nanoparticle denoted as NBrh13 based on Fe ions have been synthesized. At this point of the project, we have a clear view of the possible nanoparticles to be used and a full battery of options in case of problems with the chosen nanoparticles.

The second step in the development of the carrier is the toxicity testing. Although it is known that the toxicity of these polymers is low, testing and determination in our systems as the synthesis of the nanoparticles can have additives and other reagents that may trigger a toxic response. The strategy here is to do an initial in vitro toxicity screening and for those systems that pass, they will be assayed further in vitro and in vivo already with the drugs loaded. The screening results indicate so far that none of the systems is toxic in vitro.

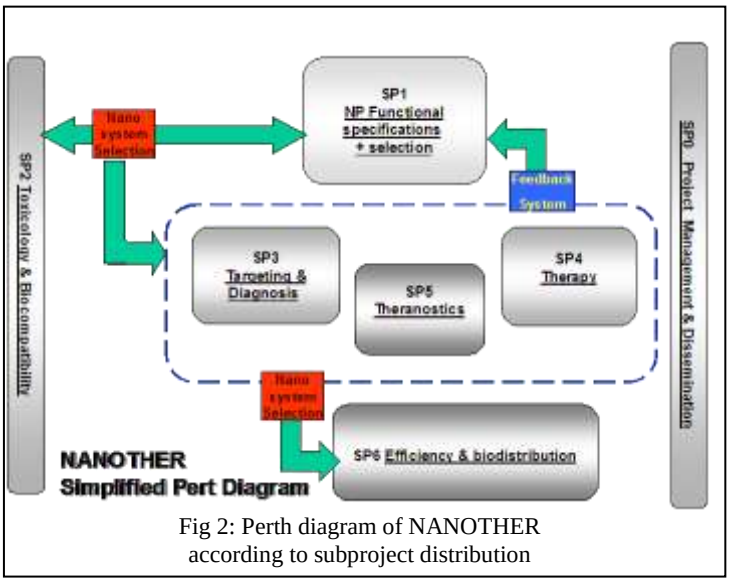

Fig 2: Perth diagram of NANOTHER according to subproject distribution

A third subproject is focused on attaching antibodies or targeting moieties to the systems chosen and imaging of the nanoparticles to use them as diagnostic tool. In this respect, trials have been done with NBrh13 and ^{99}Tc. This radiolabelled nanoparticle has been imaged by PET in mice. Also the fusion proteins for antibody attachment have been generated and we are now ready for antibody attachment to the nanoparticles. Also some polymers have been modified in their chemical groups to make them available for antibody attachment..

The fourth subproject is related to nanoparticle loading functionalisation. In this respect, some nanoparticles were loaded with up to 12 % doxorubicin, and so the results are promising. siRNA and Aplidin have also been loaded. Antibodies targeting EGFR or HER2 are used to functionalize the NPs.

In the fifth subproject, several magnetic nanoparticles have been syntetized and characterised in terms of relaxometry and other parameters. Out of all nanoparticles synthesized, magnetite and NBrh13 have been chosen as they seem to be the most promising for hyperthermic treatment and diagnosis. Other nanoparticles have been discarded. These magnetites and NBrh13 nanoparticles have been functionalised to generate hybrid nanoparticles which will also be assayed in terms of toxicity.

Last, the sixth subproject is related to efficacy of treatment. Assays have been done regarding interaction of the nanoparticles with blood proteins. They show little interaction with proteins in plasma. In addition, preliminary experiments have evaluated the efficacy in vitro and in vivo.

For the next step in the project, selected nanoparticles(loaded and functionalized) will be analyzed. Antibodies are being attached and targeting will be assayed. As tumoral cell lines have been selected for efficacy testing, and these cell lines will also be used to generate tumours in mice, there will be a good correlation in targeting in vitro and in vivo,

Situation three years into the project

SP1

The development of new multicomponent (organic/inorganic) nanosized delivery systems has been established. These versatile systems allow the inclusion of an anticancer drug (e.g. chemotherapy agent, siRNA) together with the grafting of a targeting agent (i.e. monoclonal antibody). Moreover, due to the polymeric structure, imaging agents like fluorescent dyes or magnetic nanoparticles could also be included. At this stage we are able to assemble a library of components enabling the formation of novel theranostic tools, putting us one step closer to innovative anticancer-clinical applications.

SP2

In order to better understand the potential toxicity of the test nanomaterials on in vitro cell culture systems, the physicochemical properties (size, agglomeration and aggregation measured by Dynamic Light Scattering – DLS) of NPs (provided by SP1) in their stock solution and in cell culture media have been examined. Among those investigated, we have identified a group of NPs for further analysis. These NPs systems, in fact, resulted suitable for in vitro and in vivo studies as they did not aggregated/agglomerated and their dispersion protocol was applicable and successful.

In vitro and ex vivo cell culture systems representative of different body compartments such as lung, skin, liver, intestine and kidney, have been used in this study to identify the basal cytotoxicity of NPs. We have performed Colony Forming Efficiency (CFE) assay, MTT and Alamar Blue as in vitro tests to evaluate the potential toxicity and the dose-response relationship exerted by NPs. In addition, we have investigated the pro-oxidant and pro-inflammatory activity of NPs by measuring the secretion of reactive oxidative species (ROS).

Furthermore, the potential toxicity exerted by magnetic NPs, which are suitable for hyperthermic treatments, has been investigated. The aim of the study was identifying the presence of short-lived and long-lived toxic intermediates generated by magnetic NPs after exposure to electric fields.

From the cytotoxicity study we have then identified some promising NPs and their in vitro uptake and intracellular fate is currently under investigation in selected cell lines (activities performed in SP6).

To evaluate the possible interaction of NPs with tissues and organs, in vivo experiments have been performed administering selected NPs to animal models.

SP3

At this stage of the project, two strategies of functionalization of PTMC-b-PGA polymerosomes with anti-Her2 antibodies have been developed and optimized. A pathological model that use BT474 cells has been evaluated and validated, using specific MRI sequences acquisition specially designed for the project. It has also been demonstrated that the real-time biodistribution of NPs can be evaluated using MR imaging sequence. Using these results, it has been shown that the couple BT474/anti Her2-mAb-PTMC-b-PGA can be used to asses specific NPs targeting, which can be tested in vitro using an infusion protocol.
At the same time, a PET system with field of view of 5x5cm has been successfully tested for mice imaging, and a part of the candidates nanoparticles have been successfully TC^{99m} radiolabelled and imaged in-vivo. One of the NP candidates has been fully and successfully validated for nuclear imaging.

SP4

At this stage of NANOTHER project, the formulation of nanoparticles has been optimised on several aspects. In details, five types of therapeutic agents, including three chemotherapeutics (Doxorubicin, Paclitaxel, Aplidin®), one biotherapeutic agent (siRNAs) and one inorganic core (magnetite NPs) aimed both at hyperthermia cancer treatment and as MRI diagnosis tool, were loaded into nanocarriers and investigated in combination with four different targeting models (1. anti-EGFR mAb for colon, 2. anti-Her2mAb for breast, 3. Folate for general cancer targeting, including colon and lung). Additionally, the production under scale-up conditions of polymeric materials, inorganic cores and their assembly into nanoparticles have been accomplished. The in vitro and in vivo characterizations performed until now, point out the high potential of the developed nanovectors especially for the chemical-physical, magnetic and relaxivity properties as well as for the efficacy of the treatment.Studies of targeting and efficacy refinement are in progress

SP5

The situation of SP5 has been improved in the last months. The magnetic, hyperthermic, relaxometric and dielectric characterizations of the systems available have been successfully accomplished and several systems have been selected as suitable potential candidates for theranostic applications. Only few activities are still in progress concerning the radio-labelling of the developed systems and they are expected to be accomplished in the very next months. SPECT imaging protocols have been successfully defined and biodistribution studies in vivo (not-treated mice) on several organic and hybrid systems carried out as well.
Preliminary in vitro trials on hybrid not-targeted NPs under hyperthermia have been carried out; as output results the need to refine and update the experimental protocols in staff came out and it will be done starting from the next meeting.
Recently the study of hybrid targeted (hEGFR) NPs has started and results should be available on scheduled time. In vivo trials are about to start by exploiting the knowledge acquired by in vitro experimental sessions.

SP6

The first task of this SP6 is interaction of nanoparticles with plasma components has been completed at M36. Uptake rate and co-localisation is still not fully finished although the main systems being assayed in vitro and in vivo for efficacy have already been tested. The in vitro efficacy testing has already started. Biodistribution and efficacy in vivo have not yet been started but they will be finished on schedule as a clear and strict planning has been made. Nanosystems to be assayed in vivo are going to be split to the different partners involved so each partner will test no more than two systems.

SP0

The management of the project is efficiently led by the Executive Board, which controls tightly the work programme and results obtained. After the investigation phase of the first 18M, the project has now re-focused on the main priorities identified in the initial phase, and this was reflected in the resources allocation within the project.
As research work progresses, more publications are becoming available and partners are getting a more accurate perspective on the exploitable results they will gain, and how these will be implemented. This is being supervised by the exploitation manager, who ensures that visibility of partners' plans is shared within the consortium and that the exploitation of these results will serve not only the interests of the partners, but also that of the scientific and medical communities, and the patients as a whole.

Short name	Role in NANOTHER
GAIKER	Efficacy of nanocarriers and in vitro toxicity. Efficacy & toxicity of magnetic NP in vitro. Coordinator of NANOTHER.
CICbio	New therapeutic system using nanonucleic carrier. Therapeutic effect of RNAi using the silencing approach
COLORITA	Formulation & production of innovative nanoparticles, both powders and in liquid solutions, & characterisation of particles in relation with therapeutic, diagnostic and theranostic applications.
INSTM	Synthesis and characterisation of NPs, binding of target molecules to synthesised hybrid NP
LEITAT	Efficiency & biodistribution & antibody purification
RMSB	Measurement of the MR relaxivity of developed NPs and selection of the NP usable for imaging
JRC	Measurement of NP toxicity & biocompatibility in vitro
LCPO	Synthesis of biocompatible & biodegradable (co) polymers. NP drug loading and functionalisation
TEIA	Preclinical evaluation & characterisation of new diagnostic / therapeutic NPs
VC	Optimisation of software to efficiently process large volumes of biomedical data, in order to establish better diagnostics, and to carry out medical parameters analysis.
PHARM	Evaluation of in vivo activity of PHARMAMAR therapeutic agents
TAU	Toxicology, efficiency & biodistribution of nanosystems
Hameln	Evaluation in vivo activity of therapeutic agents (not including PHARM agents)
AHAVA	Determination of NP toxicology profiles for skin model experiments. Biological effect and toxicology effect as based on skin surface U.V stress.
Feyecon	FEYCON will focus on the preparation of NPs by supercritical CO_2 technology.
NUOVO PROBE	Electromagnetics for tissues + NP diagnostics and an assessment of treatment efficiency
Alma	EU project management – administrative, financial reporting, consortium animation & support