PROJECT FINAL REPORT

Grant Agreement number: HEALTH-F2-2007-201962

Project acronym: TARCC

Project title: TARGETING ALPHA-PARTICLE EMITTING RADIONUCLIDES TO COMBAT CANCER

Funding Scheme: COLLABORATIVE PROJECT (SMALL OR MEDIUM-SCALE FOCUSED RESEARCH PROJECT)

Period covered: **from January 1st 2008 to June 30th 2011**

Name, title and organisation of the scientific representative of the project's coordinator[1]:

Name of the scientific representative: Dr Jacques Barbet

Organisation: National Institute of Health and Medical Research

Tel: 33 (0)2 28 08 0312

Fax: 33 (0) 2 40 35 66 97

E-mail: Jacques.Barbet@univ-nantes.fr /

Project website[2] address: www.tarcc.org

[1] Usually the contact person of the coordinator as specified in Art. 8.1. of the grant agreement

[2] The home page of the website should contain the generic European flag and the FP7 logo which are available in electronic format at the Europa website (logo of the European flag: http://europa.eu/abc/symbols/emblem/index_en.htm ; logo of the 7th FP: http://ec.europa.eu/research/fp7/index_en.cfm?pg=logos). The area of activity of the project should also be mentioned.

1 Final Executive Summary

Cancer is a human health problem with growing concerns in Europe. Today treatments mainly rely on surgery or external beam radiation to remove or destroy bulky tumours. Chemotherapy is given when tumours cannot be removed or when dissemination is suspected. However, these approaches cannot permanently treat all cancer and relapse occurs in up to 50% of the patients' population. Targeted radionuclide therapy (TRT) is an effective treatment for some disseminated and metastatic diseases, but the large range of the emitted electrons reduces their efficacy against very small tumour cell clusters and isolated tumour cells present in residual disease and in many haematological tumours (leukemias, myeloma). Thus, alpha-emitting radionuclides, because they deliver a larger amount of energy in a much smaller volume, seems particularly promising to selectively destroy disseminated cancer cells.

The **TARCC** project addressed several major issues standing in the way of the development of targeted alpha radionuclide therapy. Indeed, the use of alpha-emitting radionuclides has been hindered by a few devastating experiences with very long half-life radionuclides such as radium or thorium and more recently polonium. Such effects will not occur with short half-life alpha-emitting radionuclides, however the very high cytotoxic potential of high linear energy transfer radiation makes them potentially very effective but potentially very toxic if their biodisposition and targeting in not carefully controlled. It was the primary objective of the **TARCC** project to develop and test means to achieve this targeting in preclinical studies. The second potential problem was a lack of these radionuclides.

TARCC proposed a concerted, multidisciplinary, plan of action to develop targeted alpha-radionuclide therapy to:

- Improve the access to the most promising alpha-emitting radionuclides.
- Investigate in appropriate preclinical models new methods for radiolabelling biological vectors to target the radionuclides to cancer cells in vivo.
- Compare the results obtained with the different vectors (antibodies, peptides, amino-acids or nanocolloids) in different preclinical tumour models.
- Evaluate dosimetry and short-term and long-term treatment toxicity at both macroscopic and microscopic levels.

It was expected that joining the efforts of some internationally recognized European teams, which have already undertaken research in this field, will make it possible to select candidates for future preclinical and clinical developments and to define the most promising settings for targeted alpha-radionuclide therapy in terms of vector properties and modes of administration.

TARCC concentrated on the use actinium-225/bismuth-213 generators, production and use of astatine-211 and development and use of lead-212/bismuth-212 in situ generators. Actinium-225/bismuth-213 generators are made available to the scientific community by the Institute for TransUranium elements. Improved labelling methods for peptides and antibodies have been developed that increase radiolabelling yields and specific activity. New techniques for astatine-211 production and purification have been set and transferred to Arronax, which will start producing astatine-211 in the next few months, with the possibility of producing clinically relevant activities.

Many tumour models have been established for preclinical studies. With these models, different approaches and vectors for targeted alpha-radionuclide have been assessed. Proofs of principle have been established and, for each disease, candidate radiopharmaceuticals for further development have been selected.

Dosimetric methods were developed and assessed at the cellular, tissue and animal levels that will be transferred to the clinical situation. Potential toxic side effects, both short term and long term, have been explored. The acquired knowledge about the relationships between these toxic effects with the nature of the radiopharmaceutical and absorbed doses will make future clinical trials safer.

Overall, the whole TARCC project advanced the use of alpha-emitting radionuclides for cancer treatment. The possibility of using bismuth-213 and astatine-211 in clinical trials has been confirmed for the treatment of various tumours. Improved radiolabelling methods have been designed to this purpose. Systemic and locoregional administration routes are both effective, depending on the vector and the target disease. In all cases, tumour burden must be small, but small molecules (aminoacids, peptides, pretargeted haptens) as well as antibodies must be moved forward to the clinic in carefully selected patient populations. This transfer to the clinic will require dosimetric calculations that have been tested during this project with respect to toxicity and efficacy in several models. Observations made during this project confirm the high relative biological effect of alpha irradiation, compared to that of electron and its absence of sensitivity to hypoxia. Knowledge about alpha-emitter toxicity has been advanced that should greatly improve the safety of future clinical trials.

In conclusion the main **TARCC** objectives have been achieved allowing promising radiopharmaceutical candidates to be proposed to treat various tumours. The advancement to the clinic of astatine-labelled phenylalanine for glioma, of bismuth-213-labelled somatostatin antagonists for neuroendocrine tumours, of pretargeted astatine-labelled haptens for adjuvant treatment of metastatic colorectal cancers and of bismuth-213 or astatine-211-labelled antibodies for the treatment of multiple myeloma by intravenous injection and of gastric and bladder cancer by locoregional administration appears warranted and the most promising candidates have already been tested in patients. Additional studies on astatine, which

could not yet be performed, will be performed and TARCC partners will join with other research teams to promote clinical developments in targeted alpha-radionuclide therapy.

2 Summary description of the work performed and objectives.

2.1 RADIONUCLIDE CHEMISTRY

One important point for the development of the field of targeted alpha therapy is the reliable support with alpha emitting radionuclides and the establishment of robust and fast labelling protocols for labelling of potent antibodies and peptides with these radionuclides. Therefore one important objective was the optimization of the production of alpha-emitting radionuclides (e.g. actinium-225, astatine-211, and bismuth-212) and the development of new methods for radiolabelling biomolecules (e.g. aminoacids, peptides or antibodies) with alpha-emitting radionuclides. The second objective was the evaluation of the stability of radioimmunoconjugates. Therefore the sensitivity of alpha-emitting radiopharmaceuticals to self-radiolysis was determined and ways of reduction of that was investigated. The third objective was the development and evaluation of in situ generators in the respect of applicability in targeted alpha therapy.
Therefore, the main goals were to:

- Improve the availability of a-emitting radionuclides (actinium-225/bismuth-213 generators, thorium-228/lead-212/bismuth-212 generators, astatine-211).
- Improve yields, specific activity, and description of optimized radiolabelling protocols.
- Improve the yield of astatine-211 labelling and stability of astatine-211-labelled products.
- Improve the quality of a-radionuclide radiopharmaceuticals (radiochemical purity, protection from radiolysis).
- Study on longer half-life radionuclides: in situ generators (e.g. lead-212), including encapsulation in nano-colloidal vectors.

2.2 VECTORS

The major objective of this work package was the development and evaluation of new vectors for well defined targets. These targets should be i) relevant, ii) easily accessible iii) specific (or at least highly expressed), iv) highly expressed. In addition, the vectors should have different pharmacokinetics matching the physical half-lives of the different alpha-emitting radionuclides (46 min – 10 days).
Several families of vectors were addressed and developed:

- Peptides:

The targets of radiolabelled small peptides were G-protein coupled receptors which are overexpressed on major human tumours. The main focus were the somatostatin, bombesin, gastrin, NK1 and glucagon-like-peptide 1 receptors. The peptidic ligands had mainly antogonistic properties. The rationale to study radioantagonists was the fact that for most G-protein coupled receptor antagonists were shown to recognise many more receptor binding sites than agonists.
Hybrid peptides were also synthesised.
In addition to these peptides targeting G-protein coupled receptors a peptide termed F3 was conjugated to the chelators DTPA and DOTA and labelling was performed with bismuth-213 and actinium-225. This peptide targets nucleolin and is internalised into the nucleus.

- Amino acids

Increased amino acid transport into tumour cells is important for cell proliferation; overexpression of amino acid transporters is a promising target for targeted diagnosis and radionuclide therapy of different cancers, in particular of brain tumours. This was used for diagnostic purposes with iodinated phenylalanine (Phe) brain tumour patients. Within TARCC a reliable, reproducible protocol for the production of 2-[At-211]-Phe was developed and it was shown that the astatinated amino acids are still good substrates of the amino acid transporter. 4-[At-211]-Phenylalanine was shown to be stable in serum over 21 hours and the superior transporter substrate.

Labelling protocols (along with WP1) were developed with the aim to be translated into the clinic.
In vitro pharmacologic studies such as binding affinities, cell uptake (binding and internalisation) in culture and cell retention (efflux) were performed.
The best performing vectors were studied in available animal models (within and along with WP3).

2.3 ANIMAL MODELS FOR PRECLINICAL ALPHA RADIONUCLIDE THERAPY

Models for human hematologic and solid tumours relevant to the clinical situation have been established for optimization of alpha-radionuclide therapy depending on the characteristics of the vector and the mode of application. These strategies aimed at a high extent of tumour targeting combined with high therapeutic efficacy and low overall toxicity.
Growth of tumours was evaluated by bioluminescence imaging following transfection of cells with firefly luciferase. Tumour targeting was evaluated by biodistribution studies, scintigraphic imaging and autoradiography. The collected data served for dosimetry calculations.
Therapeutic efficacy was investigated by monitoring tumour growth with ultrasound, bioluminescence imaging or MRI and particularly by survival of animals.

Target organ toxicity according to the targeting modality and vectors were defined and long-term toxicity studies were performed (bone marrow, liver, kidneys). Bone marrow toxicity was evaluated by blood cell counts and analysis of chromosomal aberrations in bone marrow cells. Liver toxicity was examined via liver specific enzyme activities. Urea and plasma creatinine levels were used for determination of kidney toxicity. Moreover dose-limiting organs like kidney and liver were examined histopathologicaly.
Therefore, the main goals were to:

- Evaluate targeting, therapeutic efficacy and toxicity following systemic application of peptide or antibody alpha-emitter conjugates in hematologic diseases like multiple myeloma.
- Evaluate targeting, therapeutic efficacy and toxicity following systemic application of phenylalanine peptide or antibody alpha-emitter conjugates in gliomas, prostate cancer and colon cancer.
- Evaluate pretargeting strategies in colorectal tumours.
- Evaluate targeting, therapeutic efficacy and toxicity following intracavitary application of phenylalanine, peptide or antibody alpha-emitter conjugates (intratumoural in gliomas, intraperitoneal in gastric cancer and breast cancer, intravesical in bladder cancer).

2.4 DOSIMETRY AND RADIOBIOLOGY

Important objectives of the project were dosimetry and radiobiology. The absorbed dose (radiation dose) to the tumour and normal tissues are fundamental to be able to understand and to interpret results such as therapeutic effectiveness and toxicity obtained from experimental tumour treatment studies. Much work on dosimetric methods has previously been performed for radionuclides emitting electrons and photons. For alpha particle emitting radionuclides much less effort has been made in establishing methods and models for the relatively short-ranged alpha particles. More knowledge is desired concerning the need of microdosimetric approaches in some tissue types related to the biodistribution of the radionuclide within the tissues. Concerning radiobiology, a higher biological effectiveness of alpha particles compared to electrons and photons is expected, the biological effects and toxicity towards critical organs are studied and the maximum tolerated activities and doses should be defined.
The goals of these studies were:

- To study energy delivery for cell experiments through a microdosimetric approach
- To establish animal dosimetric models adapted to mice and rats
- To establish a detailed tissue dosimetry model
- To study the dose-effect relationship for major risk organs
- To assess the biological effects and relative biological effectiveness for cell and animal experiments
- To investigate the effects of dose-rate and hypoxia in tumour cell experiments
- To determine the maximum tolerated activities and doses by toxicity studies

Ultimately, optimal treatment protocols for future preclinical and clinical studies of targeted alpha-radionuclide therapy will be proposed from the results of the dosimetry and radiobiology studies.

3 TARCC scientific achievements

3.1 RADIONUCLIDE CHEMISTRY

One of the main goals of WP1 was the establishment of the astatine-211 production. Until now the astatine-211 is routinely produced in small amounts and gives a clear perspective for the large scale production. For the separation of astatine-211 the dry distillation (which seems to be more suitable for routine) and wet extraction have been performed. In the frame of the development of novel astatine-211 labelling techniques different reaction ways were evaluated. The radiolabelling of peptides with astatine-211 has been performed with electrophilic and nucleophilic substitution reactions, latter reaction leads to higher yields and fewer by products. The collection of available published data and new experimental developments permitted Partner 7 (CNRS) to determine the Pourbaix Diagram of astatine in non-complexing medium which is the basis of understanding of astatine-211 labelling chemistry.

Astatine-211 is produced using the Bi-209(alpha,2n) reaction. At Arronax, the alpha beam is delivered at a fixed energy. It is then necessary to install a beam energy degrader to decrease the energy from 68 MeV down to 28.3 MeV. This energy has been calculated taking into account the beam energy spreading as well as the production of the astatine-210 which decays mainly to polonium-210, an alpha emitter with long period (138 days), which is a bone seeker. At this energy, an astatine-210/ astatine-211 ratio below 0.01% must be obtained, which is the usual limit for mother-nuclides in eluates from generator-based radionuclides for medical applications. The target is obtained by evaporation under vacuum of high purity bismuth. The support is made of aluminium nitride (AlN), a ceramic which as good thermal properties. A typical target has an area of 14 cm2. Its thickness of 40 micro-m can be obtained within a few hours. The homogeneity of the deposit is checked using profilometry. For the irradiation, the target is tilted at 15° in order to lower the power density on target. At the end of

bombardment, the target is sent to hot cells for treatment through a rabbit system. The first irradiation has not yet been performed but it will start as soon as the energy degrader is installed (September 2011).

The use of astatine in hypervalent state was proposed as an alternative radiolabelling method to SAB. The feasibility of the radiolabelling was first demonstrated by the use of a simple aryltin precursor. The method was then transferred to a bifunctionalized precursor allowing the introduction of hypervalent astatine and the coupling to proteins. These precursors were composed of a tin group on a hexafluorocumyl alcohol allowing the introduction of astatine and the formation of the hypervalent bonds. A maleimide was introduced for the protein coupling with the sulphydryl groups. A two-step procedure was set up to obtain the hypervalent astatinated compounds. In the first step, the tin precursor in methanol/acetic acid was heated at 100°C over 30 min in the presence of astatine-211 and N-chlorosuccinimide to give the monovalent astatinated species in good yield (90-95%). N-bromosuccinimide in chloroform was then added and the hypervalent species were obtained after 30 min at 60°C (95% yield).

The bifunctionalized compound was coupled to bovine serum albumin for in vitro stability evaluation. The radiolabelled BSA was incubated over 12h in human serum and no deastatination was observed (stability >99%).

Manual and automated syntheses of the succinimidyl-astatobenzoate (SAB) prosthetic group from a tin precursor have been improved.

Manual method:

SAB synthesis from dry distillation was obtained easily in 30 min at 60°C. We have demonstrated that a molar ratio of five between the tin precursor and the oxidative species is crucial to obtain a good yield. As a high level of tin contamination can be limiting according to (radio)pharmaceutical criteria, the lowest quantity of tin precursor usable to obtain good radiochemical yield has been determined. However, HPLC purification before coupling to protein is necessary for this tin precursor elimination for the limitation of the tin contamination and also to avoid the coupling of the tin precursor to the proteins as a significant shift of the coupling yield was observed between the purified and non-purified SAB (60% vs. 20-50% coupling yield for astatinated HLL2).

Automated method:

SAB synthesis automation has been also developed with Synthera. The Synthera automated system has been previously developed by IBA for the synthesis of fluorinated compounds such as [Fluorine-18]FDG or [Fluorine-18]FLT. Fluidic displacements are obtained for this automated system using delta of pressure. IBA automated synthesizer is composed of a fixed system including notably the vacuum pump, inert gas arrivals and the heater, and a removal part including reagents vials and one reactor vial. This removal part can also include a purification system such as prepacked chromatographic columns. We have adapted the Synthera module to develop an automated synthesis leading to the delivery of a ready-to-inject solution of SAB for the HPLC purification. This method has been developed exclusively for astatine from dry distillation.

The first part of the work was the determination of the minimal volumes for reagent vials and for the reactor under “cold” conditions. For the reagent vials, the minimal volumes have been estimated to 50 microlitres as this volume remained on the reagents vial during the adding. This result led us to use a pre-mixed solution of tin precursor and NCS in the same vial. “Manual” tests showed that no significant yield modification have been observed when a one-hour-old pre-mixed solution is used for the radiolabelling compared to the two-step procedure (adding of tin precursor then adding of NCS). The minimal volume of astatine has been estimated to 500 microlitres, as this volume is needed to avoid solventless reactions. Using these parameters and previously determined conditions (“manual” method) led to the formation of the SAB precursors easily with good yield (65-70%).

The conditioning step has also been optimized using the Synthera module. After the drying step, we first tested the classical method (200 microlitre of Heptane/AcOEt 80/20). However, 50% of the activity was lost using this method, as the volume was lower compared to the reactor volume. We then choose to use a higher volume of recovery, using a highly volatile solvent and adding an additional evaporation step to obtain the required volume for the HPLC injection. The next step of this work will be the automation of the entire radiolabelling procedure, from the astatine distillation to the radiolabelled protein. Preliminary results have also been obtained by using microfluidics (Nanoteck, Advion). The results are really promising, as first experimentations demonstrated that the SAB can be obtained in 5 s at 120°C (synthesis overall duration: 5 minutes) in good yield. Complementary studies are under investigation.

The encapsulation of lead-212 in nanocolloids (liposomes) was successfully performed at low activity levels and the stability and radiolysis studies confirmed the stability of lead-212-nanocolloids.

Indium–DTPA–tagged liposomes were studied as carriers of in vivo lead-212/bismuth generator to be used in targeted alpha radionuclide therapy. The liposomal uptake of lead-212, into preformed liposomes, was investigated using different lipophilic complexing agents (DCP, 2,3-dimercapto-1-propanol (BAL), sodium acetate, and A23187), as a function of various

parameters (temperature, concentrations of lipids, lead and encapsulated DTPA) with lead-212 as a tracer. Different formulations of liposomes were tested to evaluate the radiolabelling efficiency. No complexing agent was necessary for the passage of Pb2+ through the membrane. It occurs naturally via a partial permeability of the lipid bilayer which increases with the temperature. A complexing agent (DTPA) appears necessary to concentrate lead in the internal compartment of the liposomes. Optimal Conditions were found (T = 65°C, internal DTPA concentration of 0.025 M, pH 7.4,) leading to a high and rapid uptake of lead-212 into liposomes. The protocol thus established provides a novel method for the efficient entrapment of about 2-3 lead atoms per liposome with a yield of 75% under conditions relevant for nuclear medicine.

Stability and radiolysis studies on lead-212 containing nanocolloids were performed in mouse serum of the lead-212 encapsulation in the internal compartment of the liposomes. The lead-212 retention was studied by using the asymmetrical flow field flow fractionation method (AF4). On-line monitoring of lead-212 required the coupling of AF4 system with a gamma rays detector; this coupling, to our knowledge, was never done to date. The gamma rays detector (GabiStar from Raytest) was coupled on-line with the other detectors coupled to the AF4 (MALS, UV, and RI). Owing to the similar flow rate of the AF4 channel and typical flow needs for the gamma detector, the GabiStar detector was connected directly to the RI detector outlet via appropriate PEEK tubing. The gamma signal was recorded in the same time acquisition window than other signals, allowing a comparison between the chromatograms of the different detectors. A fractionation method was set-up allowing the separation between liposomes and the main compounds of the serum (i.e. albumin), which mainly governs lead speciation in human serum. A chromatogram was recorded after a mixing time of 20h at 37°C between liposomes and serum showing the encapsulation of lead into the liposomes. However, the strong adsorption of the liposomes on the AF4 membrane did not allow a quantitative detection of lead-212 encapsulated into the liposomes. A parallel study was done by a dialysis method. In serum medium, 60% of lead-212 is retained in the internal compartment of the liposomes, after 72 h at 37°C. Available activities of lead-212 were not sufficient for radiolysis studies.

For a better understanding and evaluation of the labelling of peptides and antibodies with bismuth-213 a simulation code to determine the maximum specific activity for labelling of antibodies with bismuth-213 was established and verified experimentally. In conclusion e.g. for 555 MBq (15 mCi) [bismuth-213]/mL it is possible to label an antibody (e.g. anti.CD20) with 13 mg [Ab]/mL and 3 chelates per antibody within a reaction time of 7 min with acceptable yield over 90%. This means that 1 of 30 conjugate is labelled and a specific activity of 37 MBq/mg is reachable. Furthermore Successful labelling with bismuth-213 of DOTA-IMP 288 (peptide for pre-targeting) and of DOTA-PP10 was performed. The radiochemical yields were >99% after 5 min at 90°C and the specific activities were for 1.48 MBq (0.4 mCi) [bismuth-213] per 1 nmol DOTA-IMP288.

3.2 VECTORS

3.2.1 Amino acids

3.2.1.1 Astatine-211-labelling of phenylalanine and phenylalanine-analogues

2-[Astatine-211]-L-phenylalanine and 4-[Astatine-211]-L-phenylalanine were prepared from the corresponding iodo and bromo derivatives using the Cu+-assisted nucleophilic exchange. 4-[Astatine]-L-phenylalanine was additionally prepared by destannylation of the BOC-derivatized 4-tributylstannyl-L-phenylalanine. Radiochemical yields of 2-[Asttatine-211]-L-phenylalanine and 4-[Astatine]-L-phenylalanine by nucleophilic exchange were 52–74% and 65–85%. Radiochemical yield of 4-[Astatine-211]-L-phenylalanine by electrophilic destannylation was 35–50%. HPLC sequence analysis showed that 2-[Astatine-211]-L-phenylalanine followed the halogen sequence (F, Cl, Br, I, At) whereas 4-[Astatine-211]-L-phenylalanine eluted between 4-Br-L-phenylalanine and 4-I-L-phenylalanine (F, Cl, Br, At, I), independent on the production pathway. Uptake of 4-[Astatine-211]-L-phenylalanine and 4-[Iiodine-131]-L-phenylalanine in DBTRG-05MG glioma cells was inhibited by L-phenylalanine 7-fold and 6-fold, respectively.

Conclusion: Astatine-labelling by the Cu^{+}-assisted nucleophilic halogen exchange method is highly reproducible and provides adequate yields for experimental and, eventually, clinical applications. 2-, and 4-[Astatine]-Phe are efficiently and specifically transported by the pertinent amino acid transport systems. 4-[Astatine-211]-Phe is the most promising derivative to be further evaluated for intracavitary experimental glioma treatment.

3.2.2 Peptides

3.2.2.1 Somatostatin analogues:

Somatostatin receptors are overexpressed on a variety of human tumours, in particular neuroendocrine tumours. Somatostatin-based agonists are successful in the clinic as imaging and targeted radionuclide agents. Agonists which internalise when bound to their receptors were considered as ideal radiovectors because of the long retention in the tumour. The rationale to study radioantagonists was the fact that most G-protein coupled receptor antagonists recognise many more

receptor binding sites compared to agonists. The idea was to have very high early uptake which matches with the half-lives of short-lived positron- and alpha emitters. It finally and unexpectedly turned out that most of our antagonist studied showed not only higher uptake in the tumour but also longer retention which now allows the use of broad spectrum of half-lives. A variety of peptides with antagonistic properties were developed along with our colleagues from the Salk Institute, La Jolla (Dr. Jean Rivier) and the University of Berne (Prof. Jean Claude Reubi). The synthetic strategy was solid phase peptide synthesis. The unnatural amino acids (D-Aph(Cbm) and Aph(Hor)) were synthesised according to published procedures or peptides containing these amino acids were received from the Salk Institute. As shown below the octapeptides had a change in chirality of the aa-1 and aa-3 compared to the well known octreotide based agonists. The peptides were characterised by ESI-MS and their purity confirmed by HPLC. About 20 new peptides were synthesised and a dozen studied thoroughly in vitro and in 3 mice models.

3.2.2.2 Somatostatin-based antagonists with high hydrophilicity and high affinity

Some of the most promising antagonists are presented in the Table n°1. JR11 and ANT1 were synthesised by partner 2 (UBH), Radiological Chemistry Division, according to an internal GMP protocol, careful analysis followed (residual solvents, TFA-content) and acute toxicity studies were performed. This procedure was accepted by the local ethics committee (Basel and Freiburg) and allowed first clinical studies using indium-111-DOTA-ANT1 (see Table 1). The data although very promising indicated that improved affinity and higher hydrophilicity may help to improve pharmacokinetics even further. We finally came up with the 3 new compounds shown below. They are hydrophilic (compared to [Indium-111]-DOTA-ANT) and show improved somatostatin receptor 2 affinity.

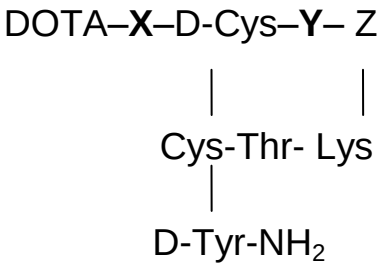


Table 1: Somatostatin-based antagonists

Code	X	Y	Z	IC-50 (nM)	LogD (pH 7.4)
Indium-111-JR10	pNO_2-Phe	Tyr	D-Aph(Cbm)	0.53 ± 0.08	-2.48 ± 0.04
Indium-111-JR11	p-Cl-Phe	Aph(Hor)	D-Aph(Cbm)	0.60 ± 0.06	-2.52 ± 0.18
Indium-111-ANT1	pNO_2-Phe	Tyr	Trp	9.2 ± 1.1	-1.85 ± 0.10
Indium-111-LM3	p-Cl-Phe	Tyr	D-Aph(Cbm)	0.45 ± 0.05	-2.14 ± 0.05

The preclinical data, in particular with Indium-111-DOTA-JR11 (manuscript in preparation) encouraged us to start early human use studies at the university hospital Freiburg (partner 11), department of Nuclear medicine (Prof. W. Weber; Dr D. Wild). The scans of the first patient which was studied with OctreoScan (the only commercially available and registered radiopeptide), Indium-111-DOTA-BASS (ANT1) as well as Indium-111-DOTA-JR11 (ANT2) are shown below (see figure 1). The improvement of DOTA-JR11 was also labelled with lutetium-177 and studied in comparison with Lutetium-177-DOTA-TATE in 3 patients. Tumour uptake was 2-4-fold higher with the antagonist; kidney uptake was also somewhat higher but the therapeutic window is still distinctly wider for the antagonist Indium-111-DOTA-JR11.

The pharmacokinetics of the antagonists is fast; they can be labelled with bismuth-213 and actinium-225 using very similar protocols than those for indium-111, yttrium-90 and lutetium-177. First clinical studies with bismuth-213 were planned along with the department of Nuclear Medicine, University Heidelberg and **partner 8 (JRC-ITU)**. The studies have been delayed due to the lack of GMP-JR11.

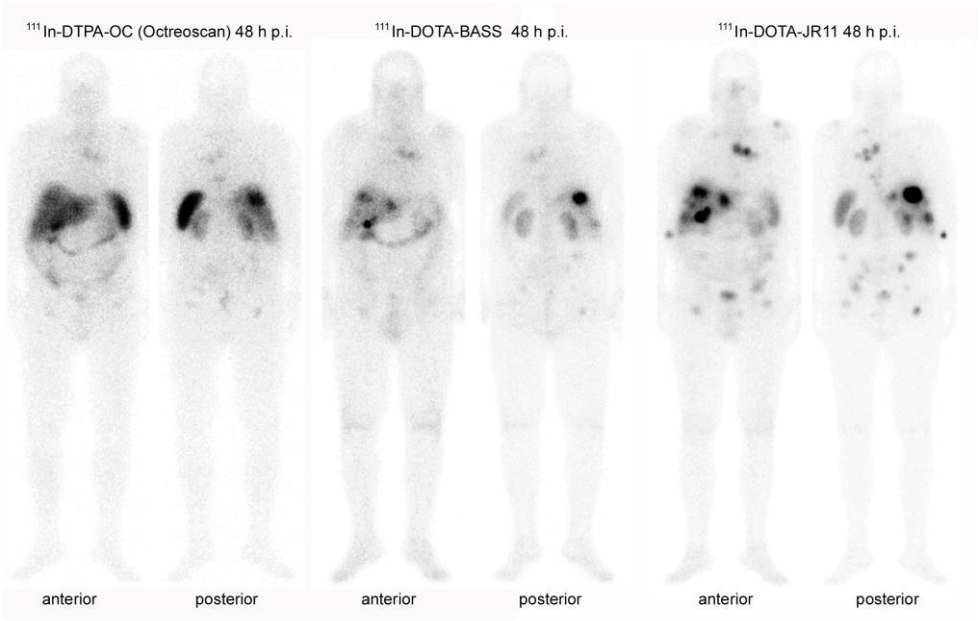
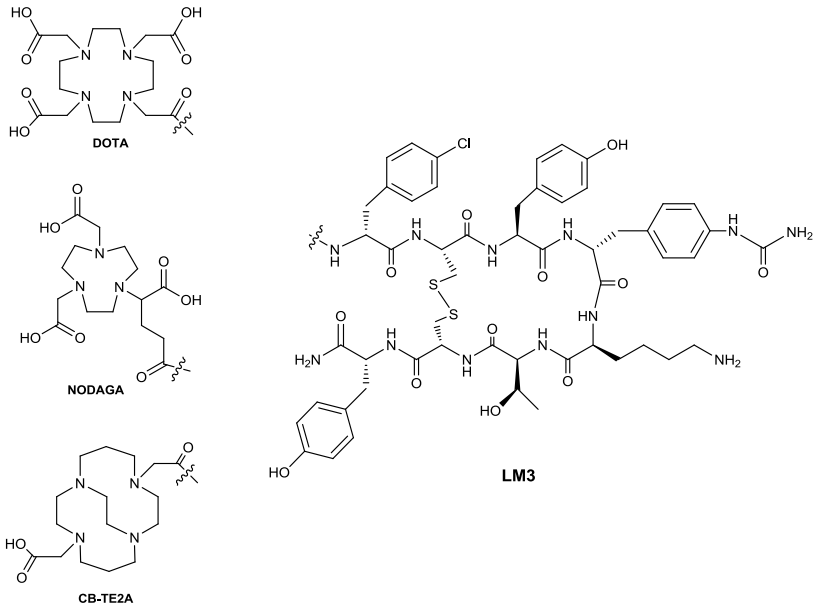


Figure 1: *First in human studies comparing OctreoScan, Indium-111-DOTA-BASS and Indium-111-DOTA-JR11. Indium-111-DOTA-BASS (ANT1) is a low affinity antagonist (IC-50 = 9.2 nM) with relatively low hydrophilicity (log D=-1.85) while Indium-111-DOTA-JR11 (ANT2) has high affinity (IC-50 < 1 nM) and high hydrophilicity (log D = -2.52). There is a difference in the intensity of uptake and the number of metastases localised by Indium-111-JR11. In addition the retention time of the two antagonists is unexpectedly long which may allow treatment with actinium-225.*

Structural formula of the antagonist LM3 conjugates for the labelling with indium-111, lutetium-177, yttrium-90, bismuth-213, actinium-225, gallium-67/68, copper-64/67 (partner 1, 2/11)



These conjugates were labelled with the radiometals mentioned above and biodistribution was studied with the positron emitters copper-64 and gallium-68. (see Fani M et al , "PET of Somatostatin Receptor-Positive Tumours Using Cu-64 and Ga-68-Somatostatin Antagonists: The Chelate Makes the Difference" J. Nucl. Med. 2011;52:1110-1118)

3.2.3 Precursor synthesis for chemical ligation and astatine-211-labelling (Partners 2/11, 4)

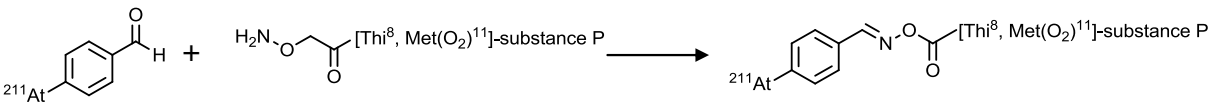
3.2.3.1 Synthesis of precursors for selective astatine-211-labelling of peptides (Substance P)

Substance P is a ligand binding to neurokinin type 1 (NK-1) receptors, which are highly overexpressed in malignant gliomas. A few potential peptide vectors were recently developed. Two of these peptides, DOTAGA-substance P and DOTA-[Thi-8, Met(O2)-11]-substance P were shown to be suitable for diagnostic labelling with indium-111 and therapeutic labelling with yttrium-90, lutetium-177 and bismuth-213. The modification of substance P (introduction of Thi-8 for Phe-8 and Met-11 replaced by the corresponding sulfone) led to improved radiolytic and improved metabolic stability.

The DOTA-conjugated substance-P derivative was successfully studied in the clinic in different brain tumour patients, labelled with bismuth-213 (Eur J Nucl Med Mol Imaging, 2010;37(7):1335-44).

3.2.3.2 Astatination of substance P derivative by selective chemical ligation

The idea of the project is to find a route for routine application of astatine-211-labelled substance P derivatives. The strategy is to conjugate astatine-211-labelled benzaldehyde onto an aminooxy functionalized [Thi-8,Met(O2)-11]-substance P via oxime formation.



The advantage of an aminooxy functionalized peptide is the high reactivity with carbonyl functionalities and the resulting stable oxime formation. In addition, the major advantage is that a carbonyl-moiety can be selectively linked in the presence of an aminooxy group due to pKa differences of the aminooxy functionalized compound (pKa ≈ 5) and the amino functionalized compound (pKa ≈ 11). This means that at a low pH of 3-4, the amino group is fully protected by the proton whereas the aminooxy group is still reactive. The conjugation kinetics was optimised using 4-Fluorine-19-benzaldehyde.

On the other hand the problem with the aminooxy precursor is its high reactivity with all kind of carbonyl compounds such as acetone in solvents and formaldehyde from the air. This all had to be understood and optimized for improved synthesis. Attempts to introduce “weak” protecting groups which are being cleaved during the conjugation step were not successful.

3.2.4 Development of metabolically stabilised GASTRIN derivatives (partners 2/11, 6)

3.2.4.1 Non-ionic and mixed ionic/non-ionic spacer DOTA-gastrin-conjugates as possible precursors for SST/CCKB receptors binding hybrid

To date there is no minigastrin analogue suitable for radiopeptide therapy of medullary thyroid carcinoma and other gastrin receptor expressing tumours such as neuroendocrine tumours and SCLC. The main limiting factor is either their low tumour uptake or/and too high kidney retention which has been related to the N-terminal Glu-s ; deletion of these Glu-s lowered the kidney uptake but the metabolic stability deteriorated. The goal of this work was to develop radiogastrin derivatives with high metabolic stability, high affinity and low kidney uptake. The strategy was to replace the hydrophilic, negatively charged spacers (Glu-s) with hydrophilic but uncharged spacers.

In addition, DOTA-conjugated minigastrin analogues with so called mixed ionic/non-ionic spacers were synthesized manually on solid phase. Gastrin receptor affinities were determined by receptor autoradiography in cooperation with Prof. Reubi, university of Berne. ^{111}In-labelled peptides were evaluated in vitro in AR4-2J cell line and in human serum. The best peptides were studied in rats bearing the AR42J tumour.

We developed DOTA-coupled minigastrin analogues with ionic, non-ionic and mixed ionic/non-ionic spacers with high stability in human serum. All conjugates showed high binding affinity to the gastrin receptor (IC-50-values between 0.5 and 4.8 nM). All radiopeptides showed receptor-specific internalization. The metabolic stability remains high within the series. Feasibility of labelling with bismuth-213 was confirmed. Further biodistribution studies in animal models are needed in order to determine which type of spacer is superior in relation to best tumour-to-kidney ratio.

The most important findings were as follows:

- The metabolic stability in human serum varied by a factor of 500 (PP-F1 vs. PP-F10).

- The best pharmacokinetics was found for PP-F10 and PP-F11.
- The tumour-to-kidney ratios could be improved about 30-fold. Interestingly the (D-Glu)6 spacer (PP-F11) showed a 20-fold lower kidney uptake compared to the (L-Glu)6 spacer.
- Labelling of some of the gastrin analogues was feasible and the biological activity was compared to theindium-111-labeled congeners using an internalisation assay in AR42J cells. There was no significant difference between the 2 radioligands indicating that labelling with the alpha-emitter does not harm the radiopeptide.

3.2.5 Synthesis of hybrid peptides (partner 2/11, 6) and chelated F3 (partner 3)

3.2.5.1 Synthesis of hybrid peptides

The first step of the development of hybrid peptide was the selection of somatostatin and gastrin synthons. TOC as the most widely used somatostatin analogue and PP-F10 as one of the most promising minigastrin analogues were selected. Several attempts to couple somatostatin and CCK2/gastrin synthons were performed.

1. The synthesis strategy is based upon use of orthogonally protected Glu (Fmoc-Glu(O-2-PhiPr)-OH), coupled to either of the synthons (SST or gastrin) but using linkers of different length (PEGn) was not successful. Synthesis attempts were performed both in solution and on the resin.

2. A strategy using "click" chemistry with [3+2] cycloaddition between azides and alkynes using Cu(I) catalyst was successful. The structure of the new hybrid SST-gastrin peptide was confirmed by MALDI analysis (Figure 2). In figure 2, structures of both the precursors (azido-TOC and alkyno-minigastrin analogue) are shown.

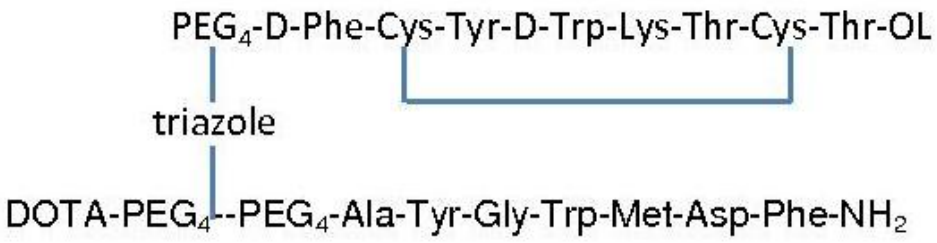


***Figure 2**: Molecular structure of the hybrid SST-gastrin peptide*

3.2.5.2 Synthesis of chelated F3 (Partner TUM): ^{213}Bi-and ^{225}Ac-labeled substance peptide F3

The tumour homing peptide F3 is effectively internalized into the nucleus of tumour cells upon binding to nucleolin and therefore can be used as an appropriate carrier for alpha-particle emitting isotopes in targeted tumour therapy. We have successfully labelled DTPA-chelated F3 peptide with the alpha-emitter bismuth-213 and demonstrated therapeutic efficacy of bismuth-213-DTPA-F3 conjugates in a nude mouse model of peritoneal carcinomatosis. In our effort to expand the study we have now labelled DOTA-chelated F3 with the alpha-emitter actinium-225 and compared cytotoxicity and therapeutic efficacy of both conjugates. ID50 values of bismuth-213-DTPA-F3 (53 kBq/ml) and actinium-225-DOTA-F3 (67 Bq/ml) – as determined via clonogenic assays – differed by a factor of 1,000. Repeated therapy with 6 x 1.85 kBq actinium-225-DOTA-F3 or 6 x 1.85 MBq bismuth-213-DTPA-F3 prolonged median survival to 95 days and 97 days, respectively, with negligible kidney damage (see WP3) (manuscript in preparation).

3.2.6 Antibody production and characterisation

Monoclonal antibodies directed against murine CD138 have been generated by immunization against peptides extracted from the murine CD138 sequence. The peptides were chosen in an area similar to that recognized by the anti-human CD138. Nine hybridomas recognized murine multiple myeloma by flow cytometry. Subcloning was performed and affinity tests are in nM range.

3.2.7 Bivalent hapten synthesis and labelling protocols

3.2.7.1 Bivalent haptens

By definition a hapten is a low molecular weight (generally less than 1000 Da) compound that is recognized by specific antibodies. Usually, haptens are first coupled to carrier proteins, such as serum albumin or keyhole limpet hemocyanin, to

form an immunogenic conjugate capable of triggering an antibody response in animals. Then monoclonal antibodies may be obtained by classical hybridoma technology. The DTPA-indium complex has been used in earlier preclinical and clinical work by partner 1 (INSERM) and collaborating teams. The histamine-succinyl-glycine (HSG) pseudo-peptide has also been proposed as a useful hapten in the context of pretargeting and interest has recently shifted to this hapten because it may be used to derive pretargeting vectors that may be labelled with a variety of radionuclides. Bivalent haptens have been shown to be superior to monovalent ones for in vivo pretargeting of tumours. Small molecular weight molecules comprising two HSG haptens and one or more sites for radioactive labelling have been designed and synthesized.

3.2.7.2 Radiolabelling of bivalent haptens

Another bivalent HSG hapten, provided by the Immunomedics Company (Morris Plains, NJ, USA) was used in the project. It was radiolabelled with radioactive iodine (iodine-125) for biodistribution studies and with lutetium-177 and bismuth-213 for preclinical therapy studies (Griffiths GL, Chang CH, McBride WJ, Rossi EA, Sheerin A, Tejada GR, Karacay H, Sharkey RM, Horak ID, Hansen HJ, Goldenberg DM. Reagents and methods for PET using bispecific antibody pretargeting and 68Ga-radiolabeled bivalent hapten-peptide-chelate conjugates. J Nucl Med. 2004, 45: 30-39).

3.3 ANIMAL MODELS FOR PRECLINICAL ALPHA RADIONUCLIDE THERAPY

3.3.1 Multiple myeloma

Systemic application of alpha-emitter conjugates in hematologic disease a syngeneic murine model of multiple myeloma was established by intravenous transfer of 5T33 myeloma cells that disseminated into the bone marrow. Syndecan-1 (CD138), a heparan sulfate proteoglycan, constantly expressed on tumour cells in multiple myeloma served as surface antigen in targeted therapy. Mice were treated with bismuth-213-labeled anti-mCD138 at 4 different activities (1.85, 3.7, 7.4 and 11.1 MBq). The group treated with 3.7 MBq exhibited a median survival of more than 300 days compared with 45.5 days for the control group. The highest activity (11.1 MBq) turned out toxic while the lowest activity of 1.85 MBq showed no therapeutic effect. 7.4 MBq induced a median survival of 227 days. With activities of 3.7 and 7.4 MBq, surviving mice exhibited a transient haematological toxicity and only the injected activity of 7.4 MBq caused a transient rise of Flt-3L indicative of low myelotoxicity.

This study demonstrates the excellent therapeutic efficacy of the bismuth-213-labeled anti-mCD138 radioimmunotherapy protocol in multiple myeloma. Furthermore, this experiment proved the possibility of targeting multiple myeloma cells with an intact monoclonal antibody by systemic injection.

3.3.2 Prostate cancer

In a human androgen-independent prostate carcinoma model (PC-3-tumour model) therapeutic studies were undertaken using two novel bismuth-213 or lutetium-177 labelled bombesine-based peptides (DOTA-PEG_4-bombesin [DOTA-PESIN] and DO3A-CH_2CO-8-aminooctanoyl-Q-W-A-V-G-H-L-M-NH_2 [AMBA]). For therapy and toxicity studies, mice were injected with lutetium-177-DOTA-PESIN, bismuth-213-DOTA-PESIN or bismuth-213-AMBA, whereas control groups were left untreated or were given nonradioactive lutetium-175-DOTA-PESIN. All radiopeptides showed a rapid blood clearance and tumour uptake. The maximum tolerated dose (MTD) of bismuth-213-DOTA-PESIN and bismuth-213-AMBA was 25 MBq and the MTD of lutetium-177-DOTA-PESIN was 112 MBq, corresponding to kidney doses of 6-11 Gy. At these dose levels, lutetium-177-DOTA-PESIN showed no, bismuth-213-DOTA-PESIN slight, and bismuth-213-AMBA marked kidney damage. At MTD, bismuth-213-DOTA-PESIN and bismuth-213-AMBA were significantly more effective than lutetium-177-DOTA-PESIN. These preclinical data show that alpha-therapy with bismuth-213-DOTA-PESIN or bismuth-213-AMBA is more effective than therapy with beta-particle emitting lutetium-177-DOTA-PESIN. In addition, therapy with bismuth-213-DOTA-PESIN has a better safety profile than treatment with bismuth-213-AMBA and represents an important new approach for treating recurrent prostate cancer.

3.3.3 Colorectal cancer

Subcutaneous colorectal micro-tumours or hepatic micrometastases were established for therapeutic studies using the affinity enhancement system (AES) technology comprising non radioactive bispecific antibodies and a radiolabelled (bismuth-213 or astatine-211) bivalent hapten.

LS174T human colonic carcinoma cells (1 million) were inoculated subcutaneously or via the portal vein (hepatic metastasis model) in NMRI-nu mice. The tumour implantation rate was 100% using aggregated cells and only 26.6% with isolated cells. Photons emitted by 1 million cells were detected by bioluminescence imaging immediately following injection and allowed visual confirmation of hepatic distribution. Therefore, hepatic tumour graft via the portal route and bioluminescence imaging provides a reliable animal model and permits sensitive in vivo detection and follow-up of hepatic metastases. The hepatic

model appears to reproduce more closely colon cancer spread compared to the subcutaneous one. The hepatic model is of particular interest to study radioimmunotherapy.

Pharmacokinetic distribution of the TF2 bispecific anti-Carcino-Embryonic-Antigen (CEA) x anti histamine-succinyl-glycine (HSG) antibody was evaluated. For various antibody / hapten molar ratios (10:1 and 2:1) and pretargeting time intervals (5, 10, 15, 24 h), biodistribution studies were performed in the subcutaneous model with TF2 and Iodine-125-labeled bivalent HSG peptide IMP-288 to optimize tumour uptake and tumour / organ uptake ratios.

Activity blood clearance was very fast. Using an antibody / peptide ratio of 10:1 and a pretargeting time interval between 15 and 24 h, the peak of tumour uptake reached 9.13 ± 4.4 % injected dose per gram (%ID/g) in subcutaneous tumours 2 h after hapten injection. Reducing the time interval between antibody and peptide injections increased tumour uptake, but activity uptake also increased in most tissues, particularly in liver and spleen. Tumour uptake 1h after hapten injection was higher in hepatic metastases (11-26% ID/g) than in subcutaneous tumours (7-12% ID/g). The results of the biodistribution studies and dosimetric calculations revealed that pretargeting must be finely tuned for therapeutic application.

For pretargeted radioimmunotherapy IMP-288 targeting TF2 was labelled with bismuth-213 to a specific activity of 74 MBq/nmol. Seven days after tumour inoculation, groups of 4 animals were selected with matched bioluminescence signals. Four control groups were set: no treatment, TF2 alone, 0.2 or 0.1 nmol of labelled peptide only. Two treatment groups were set: pretargeting with 2 nmol of TF2 followed by 0.2 or 0.1 nmol of bismuth-213-labelled peptide IMP-288 with a pretargeting time interval of 18 h. Haematological toxicity was monitored by weekly blood sampling. Tumour growth was monitored by in vivo bioluminescence imaging (every 4 to 6 days). Animals were sacrificed when a 20% weight loss or cachexia was observed or when the bioluminescence signal reached $20x10^7$ counts. Increase of the bioluminescence signal was delayed in treated animals (TF2 + bismuth-213-labeled IMP-288) with tumour growth starting 10 days after treatment whereas tumours grew continuously in control groups. At day 23 after treatment, the lowest bioluminescence signal was observed in the group treated with 2 nmol TF2 and 0.1 nmol of labelled IMP-288. Animals were euthanized when the bioluminescence signal reached $20x10^7$ photons. The Kaplan-Meier curve showed 0% survival in the untreated group versus 75% in the group treated with 2 nmol TF2 and 0.1 nmol IMP-288. Toxicity was acceptable with a transient decrease of blood cells 6 days after treatment and complete recovery at day 13.

The pretargeting system resulted in a fast tumour accretion and revealed a noticeable anti-tumour effect. However, in accordance with the dosimetry study and a tumour uptake peak two hours after injection, bismuth-213 was not the optimal radionuclide for alpha-therapy in this animal model, due to it is short half live.

3.3.4 Brain tumours

Increased amino acid transport in brain tumours is used for diagnostic purposes. It has been shown that astatine-211-labelled phenylalanine (At-Phe) is taken up by glioblastoma cells in vitro. It was tested, whether systemic intravenous treatment of rats with intracranially implanted glioblastomas with At-Phe would have a beneficial effect. At-Phe was prepared via nucleophilic halogen exchange on L-iodophenylalanine. The rat glioblastoma cell line BT4Ca was implanted into the prefrontal cortex of female BDIX rats by stereotaxic microinjection (10.000 cells/3 microlitre; n=63). At-Phe (2 MBq) or phosphate buffered saline (PBS) were injected intravenously three days after implantation. A third group was treated twice, on days 3 and 10 after implantation. Rat's health condition was assessed each day by using a score system (e.g. weight, pupillary and motor reflexes) and rats were sacrificed when showing a premortal health status. Additionally, rats were sacrificed on days 6, 10, 13 and 17 after transplantation to measure the tumour volume and area of necrosis. Furthermore, the proliferation index was assessed after immunohistological staining for Ki-67.

The mean survival time of the rats who had received two applications of At-Phe was significantly higher compared to control rats ($p<0.05$). The score-analysis showed that At-Phe treated rats had a significant better health condition compared to PBS treated rats ($p<0.05$). Analysis of the tumour volume on days 6, 10, 13 and 17 after transplantation showed no differences between groups, but rats with a premortal health status had a significantly larger tumour than rats in good health condition ($p<0.05$). Overall the proliferation index was not different between groups, but necrosis was marginally larger after At-Phe treatment ($p<0.05$ on day 10).

Intravenous treatment with At-Phe enhanced survival time of rats with intracranial glioblastomas and improved health condition in comparison to controls. These results encourage studies using local treatment of intracranial glioblastoma with At-Phe, either by repeated local injection or by intracavitary application after tumour resection.

3.3.5 Gastric cancer

In a gastric cancer peritoneal carcinomatosis model established by intraperitoneal injection of $1x10^7$ HSC45-M2 gastric cancer cells, expressing mutant d9-E-cadherin therapeutic efficacy and toxicity of bismuth-213-d9MAb (targeting mutant d9-

E-cadherin) were evaluated in comparison to beta-particle emitting lutetium-177-d9MAb conjugates. Biodistribution data showed an uptake of bismuth-213-d9MAb in the tumour nodules up to 40% ID/g at 45 min after injection. In all other tissues the uptake of bismuth-213-d9MAb was below 3.2% ID/g.

For optimization of radioimmunotherapy mice were injected intraperitoneally with 0.37, 1.85, 7.4 or 22.2 MBq of bismuth-213-d9MAb at days 1 or 8 after tumour cell inoculation.

Application of 1.85 MBq of bismuth-213-d9MAb was most efficient in terms of survival. At the end of the observation period almost 90% of the mice were still alive and showed no signs of disease. Bismuth-213-d9MAb activities both higher and lower than 1.85 MBq proved to be less efficient concerning prolongation of survival. Therapy with bismuth-213-d9MAb at day 8 was less efficient than treatment on day 1. Tumour growth as well as regression after therapy could be visualized by bioluminescence imaging.

Toxicity of bismuth-213-d9MAb therapy was negligible at therapeutically effective activities. White blood cell counts were reduced within 2 days after therapy but recovered to pre-treatment values at day 21. Reduction was maximal after application of 22.2 MBq and minimal after 1.85 MBq. Chromosomal aberrations in bone marrow cells could only be observed at day 1 after therapy. Following autopsy none of these mice showed any signs of organ toxicity. Therefore, activities of up to 7.4 MBq of bismuth-213-d9MAb are thought to cause no toxic side effects.

Treatment of mice with 1.85, 7.4 and 14.8 MBq of lutetium-177-d9MAb also significantly prolonged median survival. Therapy with 7.4 MBq of lutetium-177-d9MAb was most effective: more than 50% of the animals survived longer than 250 d. Therapy with 1.85, 7.4 and 14.8 MBq of lutetium-177-d9MAb at day 8 after tumour cell inoculation also resulted in statistically significant improvement of median survival. Intraperitoneal injection of lutetium-177-d9MAb induced a transient reduction in leukocyte counts at activities > 1.85 MBq. Injection of 7.4 MBq and 14.8 MBq of lutetium-177-d9MAb rapidly decreased leukocyte counts to 40% at day 10 and 6% at day 17 of pre-treatment values. Leukocyte numbers did not recover to pre-treatment values after application of 14.8 MBq. Following i.p. injection of mice with 7.4 and 14.8 MBq lutetium-177-d9MAb 3% and 13% of the mice, respectively, developed a massive cervical, axillary and femoral-inguinal lymphadenopathy confirmed as lymphoblastic lymphoma by histological examination. One animal with enlarged lymph nodes had developed a plasmocytoma. Furthermore the spleen of this animal showed a follicular hyperplasia, the kidney a severe proliferative glomerulonephritis and the liver a hepatocarcinoma. Comparison of the results obtained with lutetium-177-d9MAb and bismuth-213-d9MAb demonstrate therapeutic efficacy for both radionuclides. However, lutetium-177-d9MAb turned out to be much more toxic at therapeutically effective doses because of long-lasting circulation of the activity in the blood after resorption from the intraperitoneal cavity.

In order to optimize intraperitoneal radioimmunotherapy, intraperitoneal retention and biodistribution of Fab-fragments, intact IgG and IgM coupled to indium-111 or bismuth-213 were analyzed in tumour free mice after i.p. injection. The results indicate a rapid resorption of Fab-fragments from the peritoneal cavity resulting in a high kidney accumulation. IgG remained in the circulation for more than 72 h after resorption from the peritoneal cavity. IgM, in contrast showed a long-lasting retention in the peritoneal cavity. After resorption IgM is rapidly trapped in the liver resulting in a very low activity in the circulation at all time points. These results could be confirmed by scintigraphic imaging. Therefore high molecular weight IgM antibodies seem to be the preferable radionuclide carriers for intraperitoneal injection.

3.3.6 Breast cancer

F3 is a vascular tumour homing peptide which is internalized into the nucleus of tumour cells upon binding to nucleolin on the cell surface. Therefore, F3 may be an appropriate carrier for alpha-particle emitting isotopes facilitating selective tumour therapies.

A dimer of the vascular tumour homing peptide F3 was chemically coupled to the alpha-emitter bismuth-213 (bismuth-213-DTPA-[F3]2). It was found that bismuth-213-DTPA-[F3]2 accumulate in the nucleus of tumour cells in vitro and in intraperitoneally growing tumours in vivo. To study the anti-tumour effect of bismuth-213-DTPA-[F3]2 mice bearing intraperitoneally growing xenograft tumours were treated with bismuth-213-DTPA-[F3]2. SCID-mice were injected i.p. with 10 million MDA-MB-435 cells expressing firefly luciferase. In the tumour prevention study groups of 8 mice were injected every second day with 1.85 MBq bismuth-213-DTPA-[F3]2, 1.85 MBq bismuth-213-DTPA or 100 microlitre PBS between day 4 and 14 after inoculation of the tumour cells. In the tumour reduction study groups of 8 mice were injected i.p. with 1.85 MBq bismuth-213-DTPA-[F3]2 1.85 MBq bismuth-213-DTPA or 100 microlitre PBS between the days 16 and 26 after inoculation of the tumour cells.

The survival time of the animals was increased from 51 to 93.5 days in the prevention study and from 57 days to 78 days in the tumour reduction study after treatment with bismuth-213-DTPA-[F3]2. No toxicity of the treatment was observed. The serum concentration of creatinine was determined in all tumour bearing mice when they were sacrificed after therapy. No

significant elevation of the serum creatinine level after treatment with bismuth-213-DTPA-[F3]2 at time of sacrifice was observed.

In a second study stable conjugates of the vascular tumour-homing peptide F3 with actinium-225 were generated to accomplish targeted delivery into tumour cells. The aim of this study was to determine the therapeutic window of actinium-225-DOTA-F3 and to compare it with bismuth-213-DTPA-F3.

Anti-tumour activity of bismuth-213-DTPA-F3 and actinium-225-DOTA-F3 was determined in-vitro using clonogenic assays. Activities with equivalent anti-tumour effects *in vitro* were then used for treatment of mice with intra-peritoneal MDA-MB-435 xenograft tumours *in vivo*. Survival of animals was analyzed and therapy monitoring was performed by optical imaging and histological analysis. Nephrotoxic effects were analyzed by histology.

In vitro the ID50 of bismuth-213-DTPA-F3 and actinium-225-DOTA-F3 was determined as 53 kBq/ml and 67 Bq/ml, respectively. *In vivo*, therapy with 6 x 1.85 kBq actinium-225-DOTA-F3 or 6 x 1.85 MBq bismuth-213i-DTPA-F3 or PBS were compared. Median survival in the PBS group was 60 days. Median survival in the group treated with actinium-225-DOTA-F3 was 95 days and 97 days in the group treated with bismuth-213-DTPA-F3. [2] bismuth-213-DTPA-F3 and actinium-225-DOTA-F3 had different effects on tumour growth as determined by optical imaging. bismuth-213-DTPA-F3 reduced the tumour mass at early time points until 30 days after treatment whereas actinium-225-DOTA-F3 has a prolonged anti-tumour effect leading to a reduction of tumour mass until the death of animals. Bismuth-213-DTPA-F3 was more effective in reducing the number of metastases.

3.3.7 Bladder cancer

An orthotopic human bladder carcinoma mouse model using the EGFR-overexpressing luciferase transfected bladder carcinoma cell line EJ28 was successfully established by intravesical instillation of 2 million EJ28 luc cells / 100 microlitre after gentle electrocautery of the murine bladder. Therapeutic efficacy of intravesically instilled bismuth-213-anti-EGFR-MAb was compared with standard mitomycin C chemotherapy. Mice without therapy and those treated with unlabeled anti-EGFR-MAb reached a median survival of 41 d and 89 d, respectively. Mice that underwent therapy with 0.925 MBq of bismuth-213-anti-EGFR-MAb 1 h, 7 d or 14 d after cell instillation survived >300 d in 90%, 80% and 40% of cases, respectively. The results were visualized by bioluminescence imaging. Therapy with 0.37 MBq 1 h or 7 d after tumour cell inoculation resulted in survival >300 d in 90% and 50% of mice, respectively. Mitomycin C treatment after 1 h and 7 d prolonged survival >300 d in 40% and 50%, respectively, however turned out to be nephrotoxic. In contrast, no signs of nephrotoxicity could be observed following bismuth-213-anti-EGFR-MAb treatment. The study suggests that radioimmunotherapy using intravesically instilled bismuth-213-anti-EGFR-MAb is a promising option for treatment of bladder cancer in patients.

In a second study therapeutic efficacy of a fractionated intravesical treatment with bismuth-213-anti-EGFR-MAb in an advanced tumour stage was evaluated. Radioimmunotherapy was initiated between days 14 and 36 after cell instillation when tumours could be clearly detected via bioluminescence imaging. The first group received three intravesical applications of 0.46 MBq of bismuth-213-anti-EGFR-Mab each in a time interval of four days. The second group was instilled with the unlabelled anti-EGFR-MAb and the third group remained untreated. Tumour development was monitored by bioluminescence imaging and survival was observed up to 300 days.

60% of the animals of the group treated three times with 0.46 MBq of bismuth-213-anti-EGFR-Mab in a time interval of 4 days showed a reduction of tumour size while in the untreated group the tumour sizes increased in all animals. The group receiving no treatment or unlabelled anti-EGFR-MAb showed a mean survival of 44 days after tumour cell inoculation while median survival in the bismuth-213 treated group was 88 days. The urothelia of the tumour-free animals treated three times with 0.46 MBq bismuth-213-anti-EGFR-MAb did not show any pathological findings.

Fractionated intravesical therapy with the alpha-emitter bismuth-213 coupled to anti-EGFR-MAb effectively reduces tumour size without radiation damage to the normal urothelium. Thus, fractionated therapy is a very promising approach for successful treatment of bladder carcinoma in an advanced stage.

3.4 DOSIMETRY AND RADIOBIOLOGY

3.4.1 Microdosimetry of alpha particles for cell experiments

Two Monte Carlo (MC) codes, MCNPX and GEANT4, were setup, and after validation used by two of the partners, together with conventional dosimetric methods.

3.4.1.1 Microdosimetric calculations for astatine-211 in a spherical cell and in a non-symmetrical cell

Microdosimetric calculations were performed for astatine-211 using GEANT4. Two cell geometry models were tested: a regular mathematical cell model of a spherical cell with a concentric spherical cell nucleus, and a voxel-based virtual cell model of a non-symmetrical cell.

Astatine-211 was located homogeneously in the nucleus, the cytoplasm, on the cell surface or outside the cell. There were differences between the low energy electromagnetic model (G4-LEM) and the standard electromagnetic model (G4-SEM) in mean specific energy and S values. Compared to published data the mean specific energy demonstrated <2% difference for G4-LEM, <6% difference for G4-SEM for sources in the nucleus, cytoplasm and on the cell surface. For astatine-211 located outside the cell differences of <4% and >10%, respectively, were found. Compared to published S values<6% and <4% differences were found for astatine-211, and <2% and <3% differences for its daughter polonium-211. Results from the non-symmetrical cell reflect the influence of irregular shape and heterogeneous medium.

3.4.1.2 Tumour control probability (TCP) for uniform and non-uniform activity distributions

In a study on cell clusters, we demonstrated how tumour control probability (TCP) depends on the radionuclide distribution in the tumour, both macroscopically and at the sub-cellular level for astatine-211, yttrium-90, lutetium-177, rhodium-103m. The radionuclides were uniformly distributed within the sub-cellular compartment and they were uniformly, normally or log-normally distributed among the cells in the tumour. In summary, the results demonstrate that the radionuclide distribution both within the cells but also within the tumour clearly affect the absorbed dose and the TCP for astatine-211 and also for the low-energy electron emitter rhodium-103m, while this was almost negligible for the other radionuclides studied (lutetium-177, yttrium-90). TCP for astatine-211 and rhodium-103m was affected by the radionuclide distribution to a great extent when the radionuclides were in the cell nucleus and to lower extent when the radionuclides were distributed on the cell membrane or in the cytoplasm. When the radionuclide was distributed in the nucleus, the cumulated activities required for TCP=0.99 increased when the activity distribution became more heterogeneous for astatine-211 and rhodium-103m, with larger increase for normally distributed radionuclides compared to log-normally distributed. When the activity was distributed on the cell membrane, the cumulated activities required for TCP=0.99 were not affected for astatine-211 and rhodium-103m when the activity distribution became more heterogeneous.

3.4.2 Dosimetric models for mouse and rat

3.4.2.1 Mouse dosimetry using MCNPX and GEANT4

A mouse geometric model (Bitar model) was defined based on 3D reconstruction of sequential slices of a frozen 30 g NMRI nude mouse. Calculations were compared with those of the 28 g Digimouse model. Mouse dosimetric models were developed for bismuth-213 and astatine-211. S-values were calculated from absorbed energy distributions obtained with MCNPX and GEANT4, assuming uniform activity distribution in each organ, and contribution of all daughter radionuclides except bismuth-207. Absorbed fractions were high (0.92-0.99), showing that alpha-particles deliver most of their energy in the source organ. For some larger organs, self-dose S-values were similar for both mouse models (difference <6%). For the other organs there were differences up to 2000 %. Cross-dose S-values varied within 3-5000%. These discrepancies can be explained by the variations in organ size, shape, and location between the two mouse models. Comparing GEANT4 and MCNPX, differences in S-values for bismuth-213 were <5% for all organs. When both self-dose and cross-dose were considered, differences of up to 40% were found, but the largest differences were found for very low S-values with low impact on total absorbed dose. For high S-values, relative differences were <15%.

3.4.2.2 Dosimetry of bismuth-213-BSA in mice

Application on biodistribution data: Based on pharmacokinetic data of bismuth-213-BSA in non-tumour-bearing nude mice time activity curves and cumulated activity were derived for each organ. Absorbed dose based on MC S-values were in general similar to those derived from calculations assuming that all emitted energy from particles was absorbed within the organ. The highest differences were found for lungs (8%). Comparisons between the two MC-techniques showed that the highest difference was observed for the kidneys (10% with Bitar model). By comparing the two mouse models the absorbed dose was similar in organs containing high activity (<4% difference), with larger discrepancies in organs containing low activity (up to 110 % difference).

3.4.2.3 Rat dosimetry

No new rat geometrical model was developed because no partner had any need for such a model. Furthermore, since rats are much larger than mice mean absorbed doses can accurately be estimated assuming that all alpha particles deliver their

energy within each organ. Detailed tissue dosimetry, however, might require better knowledge in morphology, and most probably specific models must be defined for each rat type and size/age, as for the mouse models.

3.4.2.4 Biodistribution and dosimetry of free astatide-211, iodide-14 and iodide-131 in rats

Low amounts of astatide-211 are released from the radiopharmaceutical *in vivo*. It is then important to know the biodistribution and the absorbed dose to normal tissues for astatide-211 in order to determine potential risk organs when using astatine-211-labelled radiopharmaceuticals for treatment. Very limited knowledge on biodistribution of astatide-211 is available for rats. Rats were injected simultaneously with iodide-125 and iodide-131, or free astatide-211 and the activities and activity concentrations in organs and tissues were determined, and mean absorbed doses were calculated. The biodistribution of free astatide-211 was different compared to iodide-125- and iodide-131, while it was similar for ^{125}I and iodide-131. The uptake of all three radionuclides was highest in the thyroid (400 %IA/g for iodide-125, 430 %IA/g for iodide-131 and 75 %IA/g for astatide-211 after 18 h). In all other organs and tissues the activity concentration and retention of astatine-211 was in general higher than that of iodide-125 and iodide-131. The thyroid received the highest absorbed dose per injected activity (400 mGy/MBq for iodide-125, and 19000 mGy/MBq for iodide-131 after 7 days, and 17000 mGy/MBq for astatide-211 after 24 h). Otherwise, the highest mean absorbed doses were found in stomach, heart and small intestine for iodide-125 and iodide-131, and in stomach, lungs and spleen for astatine-211. Astatine-211 delivered a much higher absorbed dose per injected activity to all extrathyroidal organs compared to iodide-125 and iodide-131.

3.4.2.5 Translation of data from astatine-211 in rats and mice to dosimetry of astatine-211 in humans

The possibility to translate astatine-211 biodistribution data between different species, especially from rat and mouse to human was studied, since biodistribution and dosimetric data for humans are difficult to obtain. The absorbed dose of astatine-211 and iodine-131 to different tissues in man was estimated based on extrapolation of biodistribution data obtained from rat and mouse. The highest estimated absorbed dose per unit activity administered from both species to man was obtained for the thyroid and stomach together with the pituitary (rat) and the salivary glands (mouse), all expressing NIS. The estimated effective dose to man differed by a factor of two depending on the species. The biodistribution of astatide-211 is different from that of iodide in animals, and the iodide kinetic models (ICRP53) cannot be applied to astatine-211 in man. The iodine-125 values from mice are closer to data in ICRP53 than from rat. Thus, biodistribution and dosimetry data differ between species, with a clear difference between mouse and rat.

3.4.3 Detailed tissue models

The initial plans were to develop geometrical models for tissue scale dosimetry for several tissue types, especially for the critical organs. The thyroid model is finalised, and the work with defining a model of the kidneys is ongoing but will take a longer time than expected due to the high complexity with the need to define the many various parts of the nephron involved in late side effects in radionuclide therapy.

3.4.3.1 Detailed dosimetric model of the thyroid

One of the main risk organs when using astatine-211-labelled radiopharmaceuticals is the thyroid, and therefore much effort was used for this model. The high uptake of free astatide-211 in thyroid (see above) indicates that thyroid-blocking should be used when astatine-211-labelled pharmaceuticals are applied for therapy. A general thyroid follicle was constructed using spherical geometry, with a follicle lumen with a diameter of 10-500 µm, surrounded by a single layer of follicle cells with 4-8 µm (diameter) spherical nuclei. Follicle models were defined for mouse, rat and humans. Calculations were performed for alpha-particles using MCNPX. The target was the follicle cell nucleus, with astatine-211 in the follicle lumen, follicle cells or nuclei. The mean specific energy with astatine-211 within the follicle lumen was 0.17, 1.1 and 2.0 mGy, for human, rat and mouse, respectively, with corresponding single-hit mean specific energy 370, 610 and 1200 mGy. Approximately 45% of the alpha particles in a astatine-211 decay deposit all the kinetic energy within the follicle lumen for the human model, compared to 7% and <0.5% for the rat and mouse model, respectively. The results demonstrate the importance of taking the range of the alpha particles, and the spatial distribution of astatine-211 into account for dosimetric evaluation of thyroid exposure. The dosimetry for ^{211}At in the thyroid cannot readily be translated between the species.

3.4.4 Radiobiological effects in major risk organs

3.4.4.1 Thyroid toxicity of astatine-211 and iodine-131 in mice studied by gene expression analysis

Female BALB/c mice were i.v. injected with astatine-211 or iodine-131, and the absorbed dose to the thyroid was 0.05-32 Gy and 0.85-17 Gy, respectively. Gene expression profiling (Illumina platform and Nexus) was performed using total RNA from thyroids removed 24 h after injection.

Analysis of astatine-211 irradiated thyroids revealed distinct gene expression profiles compared to non-irradiated controls, with more differentially expressed transcripts at lower absorbed doses (1225 and 1636 transcripts for 0.05 and 0.5 Gy, respectively). Down-regulation of gene expression was more frequent at lower and up-regulation at higher absorbed doses, with an intermediate phase around 1.4 Gy. A high amount of differentially expressed transcripts was unique for 0.5 and 1.4 Gy. Less than 7.5% of the transcripts detected at 0.05, 11 and 32 Gy were unique. Of the 130 transcripts differentially expressed in all irradiated groups, 43 transcripts were up-regulated for 1.4 Gy, but down-regulated in all other groups. Biological processes triggered for all absorbed doses were related to transport, metabolism, and muscle contraction. Briefly, high absorbed doses affected genes involved in cytoskeleton reorganization, while low doses produced radiation-induced changes involved in cellular maintenance and metabolism. Interestingly, the type of cellular response to astatine-211 exposure marked a transition phase at 1.4 Gy.

Corresponding results after iodine-131 exposure: Compared with non-irradiated controls, 497 transcripts were altered for 0.85 Gy (more down), 546 transcripts for 8.5 Gy and 90 transcripts for 17 Gy (more up). Of these, 17 transcripts were affected in all irradiated groups. Totally, 36, 31 and 41 biological processes were affected for 0.85, 8.5 and 17 Gy, respectively; only 1 was found in all groups. The cellular response to radiation strongly differs with absorbed dose. For 0.85 Gy, anti-apoptosis, signal transduction and transcription were affected, while 17 Gy affected metabolic functions, immune response and chemotaxis. Inflammatory response was seen for both 8.5 and 17 Gy. Effects on cell cycle arrest, muscle contraction and biogenesis were seen for 8.5 Gy alone. Influence on cell growth, cell adhesion and negative regulation of apoptosis were seen for both 0.85 and 8.5 Gy. Briefly, low absorbed dose activated cellular repair mechanisms, whereas higher doses activated cellular protection mechanisms such as repair and apoptosis.

The response was very different for astatine-211 and iodine-131. In general, irradiation by astatine-211 affected the expression of more genes than by iodine-131. Iodine-131 affected genes involved in cell cycle functions and inflammatory processes, while astatine-211 affected genes involved in transport processes. The dose response relationships were also clearly different between the radionuclides. In conclusion, the results indicate that astatine-211 and iodine-131 irradiation of the thyroid affect the cell signalling pathways differently, with more dramatic effects obtained from astatine-211 than from iodine-131.

3.4.4.2 Radiobiological effects of astatine-211 and iodine-131 on kidney, liver, spleen and lung in mice studied by gene expression analysis

In a similar way as described above BALB/c mice were i.v. injected with astatine-211 or iodine-131. Kidneys, liver, lungs, and spleen were removed after 24 h and total RNA was extracted and analysed. *Results for iodine-131:* A surprisingly strong response was observed despite the low absorbed doses delivered (0.1-9.7 mGy). The number of transcripts affected was lowest for kidney cortex (260) and highest for lung (857). Most affected transcripts were specific for the different absorbed doses and few were found in more than one tissue. For the transcripts affected at all dose levels the response was in general independent of dose, and only a few transcripts showed increasing or decreasing regulation with increasing absorbed dose. Affected biological processes were primarily associated with the normal functions of the each tissue. Immune response only was affected in all tissues, and processes related to metabolism were affected in several tissue types. Evaluation of the *results for astatine-211* is on-going. Briefly, the response was very different for the two radionuclides. In general, astatine-211 affected the expression of more genes than iodine-131 compared to non-exposed mice. Furthermore, the two radionuclides affected expression of different genes involved in different biological processes.

3.4.5 The relative biological effects and effectiveness of alpha emitters

Studies on relative biological effectiveness (RBE) should compare two radiation types while keeping parameters like time of exposure and dose rate constant. Such studies are very difficult to perform with radiopharmaceuticals due to the limited number of radionuclides emitting high and low-LET radiation, radiolabelling techniques and compounds available. Preferably, the same compound should be used and labelled with two radionuclides with similar half-life and labelling technique, while the tumour binding properties are retained. Furthermore, different end-points will give different results.

The focus has been on studies with alpha-emitting radionuclides, and to get data on therapeutic effects and toxicity. Few corresponding studies on reference radionuclides were done, partly due to lack of suitable compounds. In the future, other less strict RBE-like parameters need to be defined for appreciation of the higher biological effects of alpha-emitting radionuclides.

3.4.5.1 Comparison of biological effects of astatine-211, iodine-123, iodine-131 and technetium-99m on thyroid follicle ex vivo

Thyrocytes exposed to astatine-211, iodine-123, iodine-131 and technetitium-99m (all being transported by NIS, the sodium-iodide transporter) *in vitro* had reduced iodide transport and NIS mRNA expression. At the same absorbed dose, the iodide transport was most reduced by astatine-211, followed by iodine-123 and technetitium-99m (equally potent), and iodine-131 was least effective. The onset of NIS down-regulation was early in cells exposed to iodine-123 or astatine-211, but delayed for iodine-131 and ^{99m}Tc. Iodide transport and NIS expression recovered only for astatine-211. ^{123}I gave higher effect than iodine-131 at similar absorbed dose, with an RBE value of about 5. The RBE value for astatine-211 compared to ^{99m}Tc (similar half-life) could not be determined correctly since the time course of the effect obtained was different.

3.4.5.2 Radiobiological effects of bismuth-213-MAb on cancer cells studied by quantification of gamma-H2AX

Cytotoxicity of alpha-particles is particularly based on induction of DNA double-strand breaks (DSBs). Methods detecting DSBs might be useful to estimate absorbed doses in tumour and normal tissue. At sites of DSBs, histones H2AX are phosphorylated to gamma-H2AX that can be detected by immunofluorescence, flow cytometry, and Western blotting. Cultured EJ28 bladder cancer cells were exposed to different activity concentrations of ^{213}Bi-anti-EGFR-MAb for 3 h, and gamma-H2AX was quantified at different times after exposure. The number of gamma-H2AX foci per cell and the number of gamma-H2AX foci positive cells increased with increasing ^{213}Bi activity concentration. Incubation at either 4°C or 37°C unexpectedly gave similar results. The number of gamma-H2AX foci only gradually decreased with time after incubation. Because gamma-H2AX directs repair factors to the sites of DNA-DSBs and rapidly becomes dephosphorylated after repair, the results suggest that DSB repair is impaired in EJ28 cells. Quantification of DSBs via gamma-H2AX foci might allow for estimation of radiation dose and cytotoxicity.

3.4.5.3 Radiobiological effects of bismuth-213-MAb on cancer cells in vitro determined by gene expression analysis

Whole genome gene expression profiling was performed at 6, 24 and 48 h after incubation of HSC45-M2 gastric cancer cells with bismuth-213-d9MAb. Irradiation induced both up- and down-regulation of approximately 1,000 genes at each time point analysed. Eight genes appeared up-regulated and 12 genes down-regulated throughout. Among the genes showing continuous up-regulation, *COL4A2*, *NEDD9* and *C3* have not been associated with cellular response to high LET radiation so far. The same holds true for *WWP2*, *RFX3*, *HIST4H4* and *JADE1* that showed continuous down-regulation. The consistently up-regulated (*ITM2C*, *FLJ11000*, *MSMB*) and down-regulated (*HCG9*, *GAS2L3*, *FLJ21439*) genes have not been associated with any biological process or molecular function so far. Thus, these findings revealed interesting new targets for selective elimination of tumour cells and new insights regarding response of tumour cells to alpha-emitter exposure.

3.4.6 Effects of dose-rate and hypoxia

Hypoxic cells within tumours are radioresistant, but high LET alpha-particles are assumed to damage cells independently of their oxygenation status. Therefore, the cytotoxicity of bismuth-213-anti-EGFR-MAb was compared with that of photon irradiation at both normoxic and hypoxic conditions in squamous epithelium carcinoma_CAL33 cells. The hypoxia marker HIF-1alpha was assayed via Western blotting at different time points after incubation in the hypoxia chamber. CAL33 cells were incubated with bismuth-213-anti-EGFR-MAb (0-1.48 MBq/ml) or irradiated with photons (0-12 Gy) under normoxic or hypoxic conditions. Cell survival, analysed by clonogenic assay and WST colorimetric assay, decreased with increasing activity concentration (bismuth-213) or dose (photons). After photon irradiation survival of hypoxic cells was significantly higher than of normoxic cells. In contrast, similar survival was found in normoxic and hypoxic cells after incubation with ^{213}Bi-anti-EGFR-MAb. Thus, alpha-emitters such as bismuth-213 are effective tools for eradication of hypoxic tumour cells.

3.4.7 Maximum tolerated activities for alpha-emitting vectors

The maximal tolerated activities (MTA) from studies performed within TARCC are summarized in the table below. Toxic effects were obtained in the liver using bismuth-213-labelled antibodies given i.v., while other bismuth-213-labelled antibodies gave bone marrow toxicity after i.p. administration. For smaller peptides toxicity was found for the kidneys and bone marrow, with MTAs of 25 MBq of bismuth-213 and 20-30 kBq of actinium-225. With a time period allowing recovery of side effects more fractions could be given without acute toxicity.

Animal	Radiopharmaceutical	Toxicity (tissue type)	MTA/administration mode
Mouse	Bismuth-213-anti-CD138 MAb	Liver	3.7-11 MBq i.v.

Mouse	Bismuth-213-albumin	Liver	3.7-11 MBq i.v.
Mouse	Indium-111-Exendin-4	Kidney	28 MBq i.v.
Mouse	Bismuth-213-DOTA-PESIN	Bone marrow	25 MBq
Mouse	Bismuth-213-DOTA-PESIN	Bone marrow	3*5 MBq without, and 5*5 MBq with time for recovery
Mouse	Bismuth-213-AMBA	Bone marrow	25 MBq
Mouse	Bismuth-213-AMBA	Bone marrow	3*5 MBq without, and 5*5 MBq with time for recovery
Mouse	Lutetium-177-DOTA-PESIN	Bone marrow	110 MBq
Mouse	Lutetium-177-DOTA-PESIN	Bone marrow	2*28 MBq without, and 4*28 MBq with time for recovery
Mouse	Bismuth-213-d9 MAb	Bone marrow	22 MBq i.p.
Mouse	Lutetium-177-d9 MAb	Bone marrow	7.4 MBq i.p.
Mouse	Bismuth-213 anti-EGFR-MAb	Not reached	>0.9 MBq instillation in urinary bladder
Mouse	Bismuth-213 anti-EGFR-MAb	Not reached	3*0.46 MBq instillation in urinary bladder
Mouse	Bismuth-213-DTPA-[F3] dimer	Not reached	>6*1.8 MBq i.p.
Mouse	Actinium-225-DOTATOC	Kidney	20-30 kBq i.v.
Mouse	Actinium-225-DOTA-[F3] dimer	Kidney	6*1.8 kBq i.p.

3.4.8 Optimal therapy strategies and protocols

Based on the results of the TARCC project some strategies for finding optimal treatment protocols can be drawn. The biodistribution of astatine-211-, bismuth-213- and actinium-225-labelled radiopharmaceuticals studied were very different, and the critical organs should be defined for each radiopharmaceutical. The dosimetry for the critical organs must be further studied, if possible on the cellular level for some organs, and the maximum tolerated activity or tolerance dose should be found. Based on data from the project we can conclude that at least the following critical organs are found for i.v. or i.p. administration: thyroid and bone marrow for astatine-211-labelled compounds, liver and bone marrow for bismuth-213-labelled proteins and peptides, kidneys for actinium-225-labelled proteins, and kidneys for bismuth-213- and actinium-225-labelled peptides. For intracavitary administration other critical organs should also be considered. Thus, techniques blocking thyroid and kidney uptake should be used when appropriate. Furthermore, the promising results showing that alpha particles can eradicate also hypoxic tumour cells indicate that therapy with alpha emitting radiopharmaceuticals should be considered also for patients with reduced tumour perfusion and hypoxia.

4 Potential impacts

4.1 From preclinical to clinical applications

4.1.1 Alpha-emitting radionuclides and radiolabelling techniques

Production of alpha-emitting radionuclides and radiopharmaceuticals for clinical application was considered as a limitation. In this respect the project provided several significant advances.

Bismuth-213 is made available for the whole world scientific and medical community. New and improved techniques to label more efficiently a variety of vector molecules have been developed showing that there is no real obstacle to produce the radiolabelled compounds required for clinical trials. Increasing the amount of actinium-225 to load the bismuth-213 generators will be the necessary next step, but solutions have been developed to produce the parent radionuclide either from nuclear waste or by cyclotron irradiation of radium-226. Bismuth-212, which may be obtained from thorium-228 or radium-224 generators, could also be used instead of bismuth-213 because the physical properties are similar. The Areva company has initiated the development of such generators.

Astatine-211 may be produced by cyclotrons that are capable of accelerating alpha particles up to 28 MeV. Only a few such cyclotrons exist around the world, including one in Hannover and one in Orleans that participated in the project with partners 1 (INSERM), 4 (MHH) and 7 (CNRS). A few other cyclotrons, like the one in Copenhagen, which provided astatine-211 to partner 5 (UGOT) and the cyclotron in North Carolina are actively used to produce astatine-211 for medical research. Although the new Arronax cyclotron could not produce astatine-211 for use during the project, the technology has now been established, thanks to cooperation between 1 (INSERM), 4 (MHH) and 7 (CNRS), and tested at the Orléans cyclotron. Arronax will start producing astatine soon, in quantities sufficient for clinical trials.

In addition this project has advanced our knowledge of the chemistry of astatine-211 and launched a long-term cooperation between chemists and physicists that will continue exploring the chemical properties of astatine, including quantum mechanical approaches. In more practical terms several new routes for the labelling of vector molecules such as amino-acids, peptides and antibodies have been developed showing that high radiolabelling yields may be reproducibly achieved. Attention was paid to the stability of the labelled products, a key factor for clinical and commercial development. Nevertheless problems arising when working with high activities, potentially related to the radiolysis effect, remain to be explored. This will be greatly facilitated by a better knowledge of the basic chemistry of astatine.

4.1.2 Perspectives for clinical alpha radionuclide therapy

The use of alpha-emitting radionuclides has been hindered by some devastating experiences with very long half-life radionuclides such as radium or thorium and more recently polonium. Such effects are not expected with short half-life alpha-emitting radionuclides; however the very high cytotoxic potential of high linear energy transfer radiation makes them potentially very effective but very toxic if their biodisposition and targeting is not carefully controlled. It was the primary objective of the TARCC project to develop and test means to achieve this targeting and to assess the hypothesis that targeting radionuclides active at short range may provide better tumour selectivity. To establish this result, it was considered important to develop dosimetry approaches, adapted to the physical properties of the radionuclides and to assess the anti-tumour efficacy in specific models, as well as toxicity in animals.

Dosimetry calculations at the cell level provided evidence for an extra-nuclear cell sensitivity to alpha irradiation. The microdosimetric calculations based on the developed thyroid follicle model clearly demonstrate the importance of more detailed tissue specific dosimetry. The data also reveal differences between species, and translation of dosimetric results from animals to man proved not straightforward. However the developed methods and software will help further assessment of dosimetry for alpha emitters and dose-effect relationships.

Radiobiological studies gave insights on biological processes and molecular functions at work after alpha-emitter exposure and revealed interesting new targets for selective treatment. The results on normal tissues clearly demonstrate a tissue specific response, different effects from alpha-particles and electrons, and a more complex dose-response relationship. These new information will be taken into account for future developments. This will improve the safety of future clinical trials.

When aiming for clinical studies the following critical organs are found for i.v. or i.p. administration: thyroid and bone marrow for stataine-211-labelled compounds, liver and bone marrow for bismuth-213-labelled proteins, and kidneys for bismuth-213- and actinium-225-labelled peptides. For intracavitary administration other critical organs should also be considered. Thus, techniques blocking thyroid and kidney uptake should be used when appropriate.

Bismuth-213-labelled anti-CD 138-MAb showed high therapeutic efficacy in a mouse multiple myeloma model. Four patients with progressive multiple myeloma were treated with two injections of 555 MBq and 370 MBq of iodine-131-anti-CD138-MAb one week apart at the University of Nantes. Images obtained one hour after the first injection showed high bone marrow uptake, corresponding to the main site of dissemination of the tumour. Even if activity uptake was observed in liver and kidneys, no hepatic and renal toxicity were reported. This study provides a proof of concept for the radioimmunotherapy of multiple myeloma with anti-CD138 antibody. Further RIT studies with a humanized anti-CD138 MAb are planned with alpha-emitters.

Pretargeting strategies also showed potential for therapy of colon cancer metastases in the liver, although pretargeting must be finely tuned for therapeutic application. The major interest of pretargeting in this case is the fast kinetics of tumour uptake that makes it possible to use short half-life radionuclides for antibody targeting of small size solid tumours. Transposition to patients appears feasible. The first clinical trials of pretargeting using a new humanized bispecific antibody (TF2 produced by Immunomedics Inc., New Jersey, USA) have been initiated for imaging with gallium-68-labelled peptides, and therapy, with lutetium-177. These trials will allow us to determine the pharmacokinetic parameters of pretargeting in patients and to prepare the application of astatine-211-labelled peptides for pretargeted alpha radionuclide therapy of cancer.

Intraperitoneal application of bismuth-213-conjugated antibodies and peptides showed high therapeutic efficacy with low systemic toxicity in tumour models of peritoneal dissemination. For intraperitoneal radioimmunotherapy IgG and IgM

antibodies coupled to bismuth-213 have turned out as preferable to antibody fragments or small peptides due to prolonged intraperitoneal retention. Retention increases with increasing molecular weight of the carrier molecule. Therefore high molecular weight IgM antibodies seem to be the optimal radionuclide carriers for use in i.p. radioimmunotherapy.

In the bladder cancer model the urothelia of animals treated 3 times with 0.46 MBq bismuth-213-anti-EGFR-MAb did not show any pathological findings. Moreover no systemic toxicity could be found while therapeutic efficacy in an early and advanced stage of bladder cancer was high. In treatment of bladder carcinoma intravesical retention of alpha-emitter conjugates seems not to be dependent on the molecular weight of the carrier molecule. Therefore, bismuth-213-anti-EGFR-MAb turned out to be optimal for locoregional therapy. Protocols for therapy in patients with bladder cancer in an adjuvant setting after transurethral resection of superficial bladder cancer as well as in advanced tumour stage with bismuth-213-anti-EGFR-MAb are now established at Technische Universität München.

4.2 Conclusions

From the results obtained in the TARCC project, **it could be derived that alpha-radionuclide conjugates are superior to beta-radionuclide conjugates in treatment of minimal disease and disseminated tumour cells**. Many different models have been explored, in both preclinical experiments to demonstrate anti-tumour efficacy and toxicity and in *in vitro* models that explored more fundamental aspects of alpha radionuclide therapy. **Efficacy was clearly established in models of minimal disease, as expected, with minimal toxicity.** It was also confirmed that the cytotoxic effect of alpha particles is independent of hypoxia and that response of the cells to this particular kind of irradiation differs from that observed with low linear energy transfer radiations (photons, electrons). **These results clearly demonstrate that alpha radionuclide therapy has the potential of killing even the last tumour cell, including cancer stem cells, which are known to be resistant to chemotherapy and classical radiotherapy. This aspect will certainly be the objective of future research**.

Future clinical trials could be developed with a maximum safety, despite of the high intrinsic toxicity of alpha particles.

Ultimately, targeted alpha-radionuclide therapy appears as a promising approach that could reduce side-effects resulting from current targeted radionuclide therapy, a treatment option that is generally well tolerated giving patients a fast and clear improvement in quality of life.

5 Overview of participating groups and their field of expertise

N°	Group leader	Beneficiary name	Short name	Country	Expertise
1	Jacques Barbet	Institut National de la Santé et de la Recherche Médicale	INSERM	France	Immunotargeting, radioimmunotherapy
2	Helmut Maecke	University Hospital Basel	UHBS	Switzerland	Peptide radionuclide radiotherapy
3	Reingard Senekowitsch Schmidtke	Technische Universität München	TUM	Germany	Locoregional radionuclide therapy
4	Geerd J. Meyer	Medizinische Hochschule Hannover	MHH	Germany	Radiotherapy of brain tumours
5	Eva Forssell Aronsson	University of Gothenburg	UGOT	Sweden	Radionuclide therapy, dosimetry and radiobiology
6	Tanja Gmeiner Stopar	University Medical Centre Ljubljana	UMCL	Slovenia	Organic synthesis
7	Gilles Montavon	Centre National de la Recherche Scientifique	CNRS	France	Radiochemistry, nuclear physics
8	Frank Bruchertseifer	Commission of the European Communities- Directorate General Joint Research Centre	JRC	Germany	Radiochemistry Transuranium elements
9	Bernard Lambert	Ion Beam Applications SA	IBA	Belgium	Radiopharmaceuticals
10	Patricia Joseph-Mathieu	INSERM Transfert	IT	France	Project management

Coordinator

Pr Jacques Barbet

Centre de Recherche en Cancérologie de Nantes-Angers

Inserm - Université de Nantes, U892

Institut de Recherche Thérapeutique de l'Université de Nantes

8 quai Moncousu, BP 70721, 44007 Nantes cedex 01, FRANCE

Phone: 33 (0)2 28 08 0312

E-mail: jacques.barbet@univ-nantes.fr

Logo:

TARCC website: www.tarcc.org

Email: contact@tarcc.org