CHARLES UNIVERSITY

Faculty of Pharmacy in Hradec Králové

Department of Biophysics and Physical Chemistry

IN VITRO STUDY OF ^{161}Tb-LABELLED HUMAN MONOCLONAL ANTIBODY RAMUCIRUMAB

Diploma thesis

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Hradec Králové, 2024 **Nikta Mehdizadeh**

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In Hradec Králové **Nikta Mehdizadeh**

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Abstract

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Supervisor: Dr. Pavel Bárta

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Title of thesis: In vitro study of ^{161}Tb-labelled human monoclonal antibody ramucirumab

Glioblastoma, characterized by its invasive growth and resistance to conventional treatments, presents a significant therapeutic challenge. Anti-angiogenic therapies, which aim at the inhibition of the formation of new blood vessels that nourish tumours, offer a promising avenue for combating glioblastoma. Ramucirumab, specifically targeting vascular endothelial growth factor receptor 2 (VEGFR-2), disrupts tumour vascularization and impedes tumour growth. The presented thesis delves into the development of a potential radiotherapeutic agent for the treatment of glioblastoma, an aggressive and often fatal form of brain cancer. The study focused on the enhancing ramucirumab's therapeutic efficacy by its labelling with the therapeutic radionuclide terbium-161, creating a potential novel radiopharmaceutical for targeted radionuclide therapy.

The research involved the conjugation of ramucirumab with a bifunctional chelator, DOTA, to enable stable attachment of terbium-161 radionuclide. Subsequently, radiolabelling was performed, and the resulting radiopharmaceutical underwent rigorous quality control and saline/serum assessments to ensure its high radiochemical purity and stability. The *in vitro* binding affinity and internalization of the radiolabelled ramucirumab were evaluated in human glioblastoma astrocytoma cells (U-87 MG) expressing VEGFR-2.

The results demonstrated successful radiolabelling of ramucirumab with terbium-161, achieving high radiochemical purity and stability. *In vitro* studies revealed significant binding

affinity and internalization of [^{161}Tb]Tb-DOTA-ramucirumab in U-87 MG cells, indicating its potential to selectively target and deliver therapeutic radiation to tumour cells. The stability of the radiopharmaceutical in biological media further supports its potential for *in vivo* applications.

These findings highlight the potential of ^{161}Tb-labelled ramucirumab for the targeted radionuclide therapy in glioblastoma, warranting further investigation in preclinical and clinical settings. The ability to selectively deliver therapeutic radiation to VEGFR-2-expressing tumour cells offers a promising approach for enhancing the efficacy of glioblastoma treatment while minimizing damage to healthy tissues. This targeted approach holds the potential to improve patient outcomes and quality of life by providing a more precise and effective treatment option for this devastating disease.

List of Abbreviations

ATCC	American type culture collection
BBB	Blood brain barrier
BCA	Bicinchoninic acid
CNS	Central nervous system
CPM	Counts per minute
CT	Computed tomography
DOTA	S-2-(4-Isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane tetraacetic acid
DTI	diffusion-tensor imaging
DWI	Diffusion-weighted imaging
EPR	Enhanced permeability and retention
FAB	Fragment antigen-binding
FC	Fragment crystallizable
FGF	Fibroblast growth factor
GBM	Glioblastoma
IC50	Inhibitory concentration at 50%
ITLC	Instant thin layer chromatography
KD	Equilibrium dissociation constant
LET	Linear energy transfer
MABs	Monoclonal antibodies
MRI	Magnetic resonance imaging
NSCLC	Non-small cell lung cancer
PBS	Phosphate buffer
PDGF	Platelet-derived growth factor
PET	Positron emission tomography
PWI	Perfusion-weighted imaging
RAM	Ramucirumab
RBE	Relative biological effectiveness
RNT	Radionuclide therapy
SPECT	Single photon emission computed tomography
T1P	T1 permeability imaging

Tb	Terbium
TKI(s)	Tyrosine kinase inhibitor(s)
TRT	Targeted radionuclide therapy
VEGF	Vascular endothelial growth factor
VEGF-A	Vascular endothelial growth factor A
VEGFR2	Vascular endothelial growth factor receptor type 2

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2 Introduction

Cancer is a disease in which some of the body's cells grow uncontrollably and spread to other parts of the body [4]. Cancer is the most common and difficult disease in humans today despite the great efforts employed for prevention and the cure achieved in a significant proportion of cancers by surgery, radiotherapy or chemotherapy, especially when detected early [5]. Glioblastoma is a primary brain cancer that can develop anywhere within the central nervous system (CNS) but is most usually found in the frontal or temporal lobes. Glioblastoma is one of several types of brain tumours known as gliomas, which are thought to derive from glial cells or their precursors and include astrocytomas and oligodendrogliomas [6].

Nuclear medicine is a medical specialty that either use molecular imaging based on labelling a pharmaceutical molecule with a radioactive element in order to either visualize the metabolism of a labelled molecule or image a disease in the human body, or to treat a disease. In general, nuclear medicine imaging techniques are used to diagnose, plan treatment, predict outcomes, and monitor therapeutic effectiveness [7]. Universally, majority of radiopharmaceuticals in nuclear medicine are used particularly in diagnostic imaging and partly in radiotherapy [8]. Theranostics is an emerging field in nuclear medicine allowing for more personalized treatments. The term '**theranostics**' refers to the combined application of a radiopharmaceutical for **thera**py (i.e. radionuclide therapy) and diag**nostics** (i.e. molecular imaging) [9]. Radiopharmacy is an integral part of the multidisciplinary field that provides specialized preparations to use in nuclear medicine. Radiopharmaceuticals are drugs that contain two parts. A drug component for localisation of a specific tissue or organ usually of biological origin and a radionuclide component for diagnostic or therapeutic purposes [10].

In this study used biological molecule ramucirumab (RAM), a fully human IgG1 monoclonal antibody, binds to the human vascular endothelial growth factor (VEGF) receptor type 2 (VEGFR2) with high affinity and potently inhibits binding of receptor ligands responsible for angiogenic process [11]. Cancer angiogenesis is a complex process of a new and abnormal blood vessels network formation that accounts for tumour growth and metastasis [12]. Anti-angiogenic therapy can be beneficial for cancer treatment since it could disrupt the formation of new ones from the pre-existing blood vessels, decreasing oxygen and nutrient supply to cancer cells, and consequently decelerating tumour growth [13]. Therefore, antiangiogenic therapies targeting the VEGF/VEGFR2 axis, including ramucirumab, have been proposed as a promising strategy aimed at preventing tumour growth, invasion, and metastasis [1].

Radioactive isotopes of terbium have emerged as a promising element for nuclear medicine, featuring radionuclides covering all four modalities in the field i.e., terbium-149, terbium-152, terbium-155, and terbium-161. This unique characteristic makes terbium ideal for the "matched-pair" principle of theranostics, allowing the formation of diagnostic and therapeutic radiopharmaceuticals with identical chemical structure and pharmacokinetics [2]. In this study involved terbium-161 has favourable physical properties for therapeutic applications, including its suitable beta and Auger´s electron emission for targeting tumours while minimizing damage to surrounding healthy tissues. Additionally, the half-life of terbium-161 (τ = 6.96 d) aligns well with the pharmacokinetics of monoclonal antibodies, allowing prolonged exposure to cancer cells without the need for frequent dosing [2].

The experimental part of this thesis conducted *in vitro* study to examine the use of terbium-161 for ramucirumab labelling, to assess its potential for a targeted radiotherapy of cancer processes. The extensive analysis of the binding properties of ^{161}Tb-labelled ramucirumab in cancer cells had been conducted, when the found results demonstrated preserved binding properties of the prepared radiopharmaceutical to VEGFR2, indicating its potential use for targeted radiotherapy. These findings lay the groundwork for further exploration in preclinical (*in vivo*) and clinical settings, supporting the potential translational impact of ^{161}Tb-labeled ramucirumab in personalized cancer treatment strategies thanks to its gamma photons concomitant emission [3].

3 Theoretical part

3.1 Glioblastoma

Glioblastoma (GBM) is a type of primary brain cancer that can develop anywhere within the central nervous system (CNS), though it most often occurs in the frontal or temporal lobes. Unlike secondary brain cancers, which are more common and result from metastasis originating from other primary sites such as the lung, breast, or skin, GBM originates within the CNS itself. Glioblastoma is classified under a diverse group of brain tumors known as gliomas, which are believed to arise from glial cells or their precursors, and this group also includes tumors like astrocytomas and oligodendrogliomas [4]. Glioblastoma is a grade 4 astrocytoma and represents the most aggressive form of the tumour arising from astrocytes in a human brain [5]. While the disease can occur in children and adults, median age at diagnosis is 65 years. It is approximately 1.6 times more common in males than females, the reason for which remains uncertain [6]. Most glioblastoma patients do not have any known risk factors for the growth of their tumours. Glioblastoma risk is raised in certain uncommon familiar cancer syndromes, such as Li-Fraumeni syndrome, neurofibromatosis type 1, tuberous sclerosis, and Lynch syndrome [7]. An established history of radiation exposure is the only non-genetic risk. It has been determined that atopic conditions serve as a protective factor, lowering the risk of glioma by about 30% [8].

Patients with glioma frequently experience a variety of symptoms. These symptoms, which frequently have a neurological origin, affect the patient's quality of life [9]. Improving symptom management in order to maintain quality of life has therefore become a major treatment goal [10]. Patients with gliomas may experience symptoms that are brought on by the tumour or as a result of their therapy [11]. Although headaches are the most common early symptom of brain tumours, only 1 in 1000 patients who report headaches to their general practitioner also have a brain tumour [12]. The location, size, and growth rate of tumours can affect the characteristics of headaches, which can range from tension-type to migraines [13]. Cognitive issues and personality changes arise, especially in older people, they are frequently misdiagnosed as mental illnesses or dementia. Usually in larger tumours with significant mass effect, incontinence and imbalance in gait may be present. The location of the tumour is indicated by focal symptoms, which include hemiparesis, sensory loss, and abnormalities in the visual field. Seizures are the presenting manifestation in about 20 to 40 % of patients, and usually a focal onset is reported [14].

Currently, no tumour marker has been found, nor are there any instruments for screening or identifying glioblastoma prior to clinical manifestation. Computed tomography (CT) scans of the brain are typically performed on newly diagnosed glioblastoma patients. A contrast-enhanced magnetic resonance imaging (MRI) is usually ordered once a mass has been found and haemorrhage has been ruled out [15]. Magnetic resonance imaging is considered the gold standard for tomographic imaging techniques used to diagnose glioblastoma. Diffusion-weighted imaging (DWI), perfusion-weighted imaging (PWI or perfusion MR), diffusion-tensor imaging (DTI), dynamic contrast-enhanced T1 permeability imaging (T1P), and MR spectroscopy are some of the more recent methods. Glioblastoma remains as a lethal neoplasm with no effective long-term treatment [16].

The discovery of angiogenesis and VEGF as a potential driver of tumour growth and survival has led to extensive research in this field [17]. The therapeutic management of newly diagnosed glioblastoma typically involves a surgical resection, chemotherapy, and radiotherapy [18]. First line management is maximal surgical resection for local tumour control before adjuvant therapy, thereby reducing the tumour load and establishing a histopathological and molecular diagnosis. Following surgery, adjuvant radiotherapy is given with chemotherapy for maximum of six months [19].

3.2 Angiogenesis mechanisms

Pathological angiogenesis (**Figure 1**) is believed to promote carcinogenesis by improving the delivery of nutrients, oxygen, and other growth factors that are essential for tumour survival. Angiogenesis in glioblastoma, driven by hypoxia-dependent and independent mechanisms, is primarily mediated by vascular endothelial growth factor, and generates blood vessels with distinctive features [20].

Unlike pathological angiogenesis, the physiological angiogenesis (**Figure 1**) is a fundamental process for organ growth and development in prenatal life, and tissue repair or growth, and female reproductive functions in the rest of human life. It is tightly regulated by a balance between pro-angiogenic and anti-angiogenic factors [21].

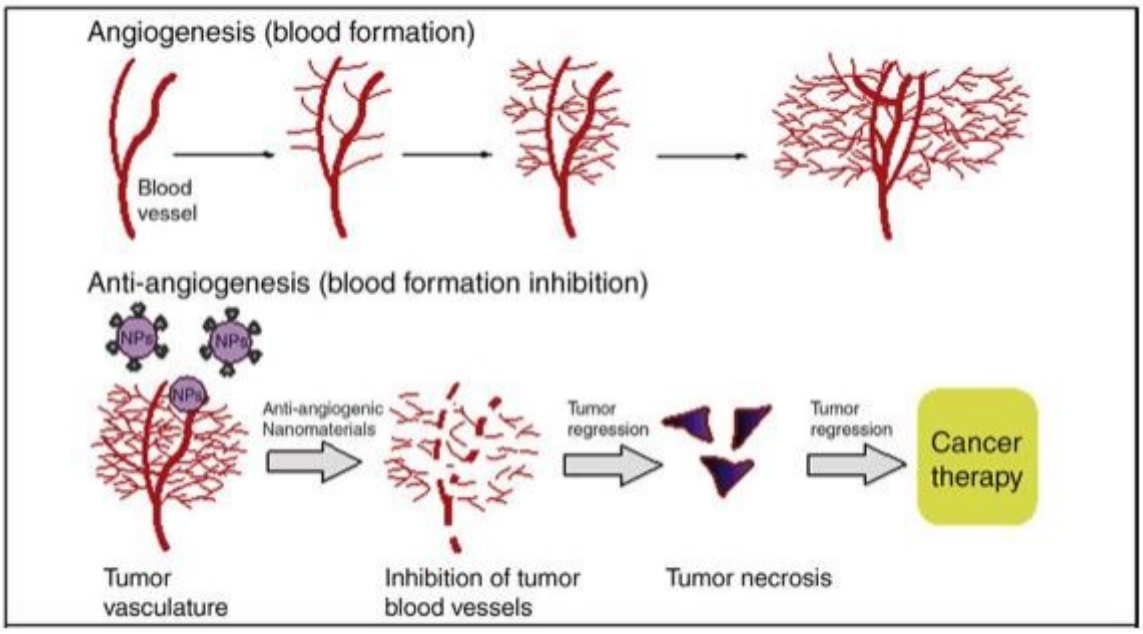

Figure 1 Angiogenesis mechanism [32].

Vascular endothelial growth factors (VEGFs) are a family of glycoproteins that play a critical role in stimulating angiogenesis. VEGFs bind to their specific receptors (VEGFRs) on endothelial cells, initiating a cascade of events that promote blood vessel growth [23]. VEGF-A is the most potent angiogenic factor, promoting endothelial cell proliferation, migration, and survival. VEGF-B is on the other side involved in embryonic blood vessel development and neuroprotection. VEGF-C and VEGF-D are primarily involved in lymphatic vessel development denoted as lymphangiogenesis. Placental growth factor (PlGF), plays a role in placental angiogenesis and may contribute to tumour angiogenesis [24].

As mentioned above, VEGFRs are tyrosine kinase receptors (Figure 2) on the surface of endothelial cells that are targeted by VEGFs and initiate downstream signalling pathways. VEGFR type 1 (VEGFR-1) is expressed on monocytes, macrophages, and endothelial cells, with a complex role in angiogenesis regulation. VEGFR type 2 (VEGFR-2), is the primary receptor for VEGF-A, responsible for most VEGF-induced angiogenic effects. VEGFR type 3 (VEGFR-3) serves as the main receptor for VEGF-C and VEGF-D, essential for lymphangiogenesis [25].

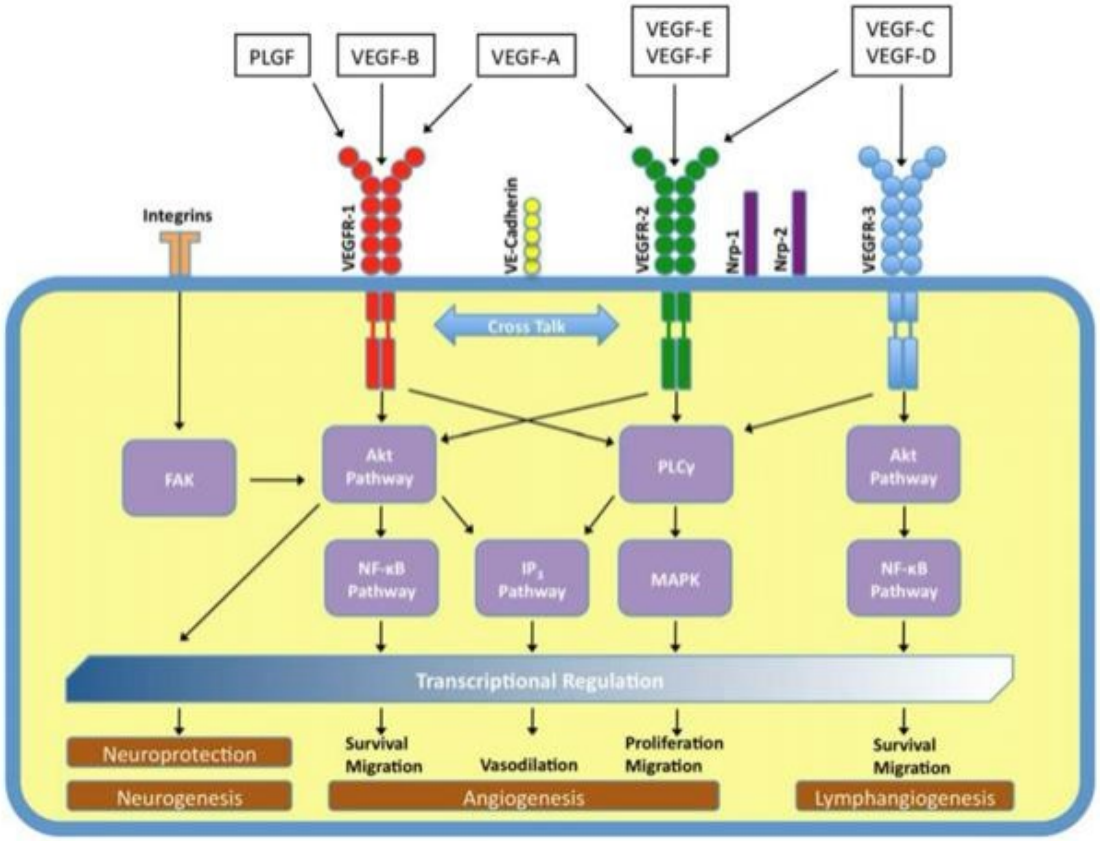

Figure 2 Schematic of pathways activated by the different isoforms of the VEGF-family [26].

Unlike physiological angiogenesis, which is tightly regulated, pathological angiogenesis is the uncontrolled formation of new blood vessels. In cancer, this process is essential for tumour growth and metastasis. Tumours initially grow without a dedicated blood supply, but as they enlarge, they require oxygen and nutrients to continue their expansion. Pathological angiogenesis allows tumours to establish a vascular network, providing a route for nourishment, waste removal, and the spread of cancer cells to distant sites [27]. Tumour angiogenesis occurs through a series of sequential steps that contribute to cancer progression. This process is primarily triggered by the tumour itself, particularly as it reaches a certain size and its cells become hypoxic. In response to hypoxia, the cancer cells release angiogenic molecules such as growth factors, cytokines, bioactive lipids, and matrix-degrading enzymes which bind to receptors on the vascular endothelial cells of nearby blood vessels, thereby starting the development of new blood vessels. [28,29].

3.3 Antiangiogenic process in glioblastoma

Glioblastoma is a highly angiogenic and deadly tumour [30], therefore the antiangiogenic therapy is directed against the tumour supplying blood vessels [31]. It is unclear how antiangiogenic therapy specifically affects GBM. Antiangiogenic therapy may improve perfusion, oxygenation, delivery of chemotherapy agents and peritumoral oedema [23,24]. Although VEGF is the prominent angiogenic factor, glioblastoma tumours frequently express other proangiogenic factors, such as PDGF, and fibroblast growth factor (FGF). The VEGF gene family as mentioned above includes 6 members as follows VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and PlGF [34].

VEGF-A, the best characterized family member, typically localized in adjacent to peri necrotic regions within glioma pseudo palisades, increases its blood concentration with higher glioma grade, and is associated with poor outcome among patients with glioblastoma [26].

Normal vasculature development depends on VEGFR-1 and VEGFR-2, whose inhibition during embryogenesis is fatal [27]. In order to support endothelial cell growth and survival through paracrine signalling pathways, VEGF-A binds to VEGFR-2 on endothelial cells. Tumour angiogenesis including GBM does not seem to be directly impacted by VEGFR-1, although its function is less clear. Via the recruitment of bone marrow-derived cells that can differentiate into cells that support the integrity and structure of new vasculature, VEGFR-1 may, nevertheless, indirectly affect tumour survival [28-30].

3.4 Monoclonal antibodies

Monoclonal antibodies (mAbs) are laboratory-engineered proteins that mimic the natural antibodies produced by our immune system. They possess a distinctive Y-shaped structure, with each arm of the "Y" designed to bind to a specific target called an antigen. This high level of specificity allows them to precisely target and neutralize foreign substances like viruses, bacteria, and even cancer cells [39]. The fundamental structure of an antibody, also known as an immunoglobulin, is that of a Y-shaped protein. It comprises four polypeptide chains: two identical heavy chains and two identical light chains, linked together by disulfide bonds (Figure 3). The heavy chains are larger and contribute to the stem of the "Y," known as the Fc region (Fragment crystallizable). The light chains are smaller and pair with the heavy chains to form the two arms of the "Y," called the Fab regions (Fragment antigen-binding). Each Fab region contains the antigen-binding site, which specifically recognizes and binds to a particular antigen. The Fc region interacts with various immune system components, such as

complement proteins and Fc receptors on immune cells, to trigger immune responses. The variable regions within the Fab regions determine the antibody's specificity for a particular antigen, while the constant regions in both Fab and Fc regions mediate effector functions [40,41].

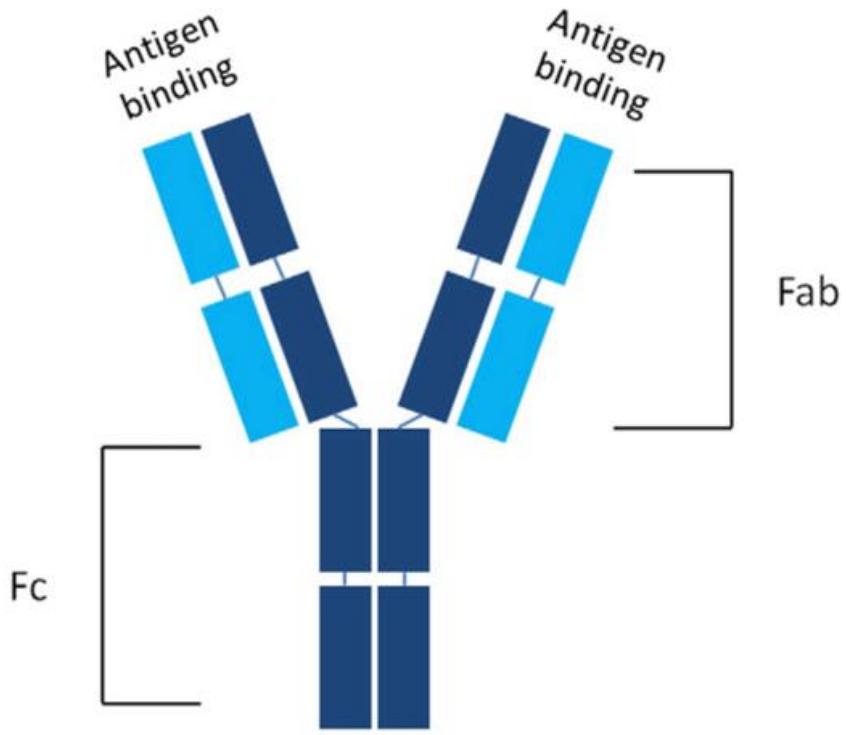

Figure 3 General structure of an IgG antibody showing the heavy and light chains, the Fab, and Fc domains, and antigen binding sites [42].

Advancements in antibody engineering have resulted in various types of mAbs for diverse applications. Murine mAbs, derived entirely from mice, have limited use in human therapy due to potential immune reactions. Chimeric mAbs, combining human and mouse genetic material, offer improved compatibility while retaining target specificity. Humanized mAbs, with predominantly human sequences, further minimize immunogenicity. Fully human mAbs, generated through innovative techniques like transgenic mice or phage display, represent the ideal therapeutic option due to their complete human origin and minimal risk of adverse immune responses.

The choice of mAb type depends on factors such as the intended application, availability, and desired level of immunogenicity [43,44]. The precision targeting

and diverse applications of monoclonal antibodies have revolutionized various medical fields. They have been used to treat numerous diseases, including cancer, autoimmune disorders, and infectious diseases [43,45].

Monoclonal antibodies are produced through a multi-step process that involves immunizing an animal with the target antigen, isolating antibody-producing B-cells, fusing them with myeloma cells to create hybridomas, screening and selecting hybridomas producing the desired antibody, and finally culturing these hybridomas to produce large quantities of the specific mAb, which is then purified and characterized for therapeutic or diagnostic use [45].

Despite the successes of mAb therapy, challenges such as therapeutic resistance persist. Researchers are actively exploring combination therapies with chemotherapy, radiation, targeted drugs, other antibodies, immune checkpoint inhibitors, vaccines, and/or cellular therapies to enhance treatment outcomes. Future research aims to delve deeper into mAb mechanisms of action and identify novel strategies to overcome resistance [46].

3.5 Ramucirumab

Ramucirumab is a human monoclonal antibody that specifically targets and blocks the activation of VEGFR-2 (Figure 4), a receptor essential for the growth of new blood vessels in tumours. Thanks to the occupancy of VEGFR-2, ramucirumab prevents the binding of its ligands (VEGF-A, VEGF-C, and VEGF-D), so that preventing VEGF-stimulated receptor phosphorylation and downstream ligand-induced proliferation, permeability, and migration of human endothelial cells [47]. Its potential for treating rare diseases earned it an 'orphan drug' designation from the US Food and Drug Administration (FDA), encouraging the development of new therapies for less common conditions. As the only FDA-approved anti-angiogenic agent of its kind, ramucirumab is a recombinant human IgG1 monoclonal antibody that binds with high specificity and affinity to the extracellular VEGF-binding domain D2 of VEGFR-2. This prevents VEGF ligands from the activation of the receptor, effectively inhibiting angiogenesis [47,48].

Additionally, by sparing VEGFR-1, ramucirumab allows this decoy receptor to further enhance its anti-angiogenic effect. Furthermore, ramucirumab indirectly impacts tumour growth by suppressing VEGFR-2 on macrophages, leading to a decreased tumour immune infiltration and the release of growth-promoting factors. By disrupting the VEGFR-2 pathway, ramucirumab ultimately prevents endothelial cell proliferation and migration, key processes in tumour angiogenesis. Based on the promising results of both preclinical and early clinical

studies, ramucirumab has been tested in different tumour types either alone or in combination with chemotherapy. It demonstrates efficacy in treating various advanced or metastatic cancers. In non-small cell lung cancer (NSCLC), it is frequently combined with docetaxel or pembrolizumab for second-line treatment. For gastric or gastroesophageal junction adenocarcinoma, ramucirumab is typically administered alongside in the combination with cisplatin and fluoropyrimidine as first line therapy [49] and in combination with paclitaxel as a second-line option [50].

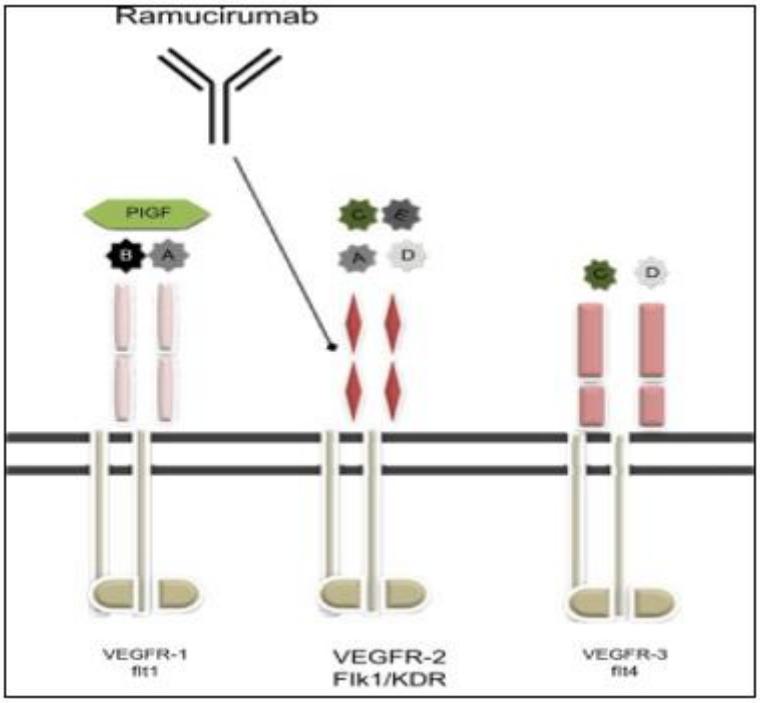

Figure 4 A graphical representation of the effect of ramucirumab when blocking VEGFR-2. VEGF ligands and their receptors are represented. A: VEGF-A, B: VEGF-B, C: VEGF-C, D: VEGF-D, E: VEGF-E [51].

The radiolabeling of ramucirumab represents a nascent yet promising field of research, underscoring the dynamic nature of drug development and offering potential for groundbreaking therapeutic strategies. A recent study investigated the use of ^{89}Zr-labelled ramucirumab for VEGFR-2 targeted imaging of prostate and ovarian cancers. The radiolabeled compound exhibited retained binding affinity *in vitro* and was subsequently evaluated *in vivo* through PET/CT imaging and biodistribution analyses in tumour xenografts [52].

While various techniques exist for assessing VEGFR-2 expression, PET imaging with ^{64}Cu-labelled ramucirumab represents distinct advantages, notably its potential application in the treatment of lung cancer [53].

3.6 Nuclear medicine

The medical field of nuclear medicine uses radiopharmaceuticals, which have been proved to be incredibly helpful in medicine, helping with a wide range of diagnostics and treatments, particularly of cancer [54]. The past fifty years of nuclear medicine history have demonstrated the close relationship between chemical investments and the creation of radionuclides and radiolabelled substances [55]. Nuclear medicine uses radioactive isotopes, also called radionuclides, either attached to specific molecules or being the part of simple inorganic or organic molecules to forming radiopharmaceuticals.

The complex radiopharmaceuticals are made of two components, a radioactive element (radionuclide) for imaging or therapy, and a carrier (drug, biological molecule or a cell) that delivers the radionuclide to a specific tissue or organ. Radiopharmaceuticals can be categorized into two distinct groups based on their containing radionuclide decay period (half-life). First group comprises radionuclides with the half-life of less than 2 hours and the second one with a half-life exceeding 2 hours [56].

Radiopharmaceuticals target and accumulate in specific organs or tissues in the body. By detecting the emitted radiation from localized radiopharmaceuticals, doctors can gain valuable insights into the function and structure of organs, or they can diagnose, control or treat various diseases, particularly cancer. In the imaging modality, radiopharmaceuticals are given orally, intravenously, or by inhalation to see different organs using their radioactive tracers [54]. Radiopharmaceuticals travels distribute through a body and accumulate in the targeted organ or tissue due to its specific chemical properties. For diagnosis, special imaging equipment like single photon emission computed tomography (SPECT) or positron emission tomography (PET) scanners detect the emitted radiation from an accumulated radiopharmaceutical. This creates images that reveal the function and health of the targeted organ or tissue. Areas with higher radioactivity may indicate increased activity, inflammation, or even tumour presence [57,58]. Thanks to targeting properties of radiopharmaceuticals with biological carrier, specific organs or tissues can be aimed. It provides detailed information about their function and further enables early detection of diseases. Moreover, targeted radiation therapy enables the treatment of diseased cells with the minimalization of the risk of the damage to healthy tissues [58].

3.7 Nuclear Medicine Radionuclides

3.7.1 Radionuclides

Radionuclides found in nature, such as uranium and radium, are heavy elements with high toxicity and long half-life (over 1,000 years), so they are not used clinically. Radionuclides used in nuclear medicine are artificially produced by neutron bombardment or nuclear fission [59].

As mentioned above, radiopharmaceuticals generally consist of two components, a radioactive element (radionuclide), which is linked to a non-radioactive element, a tracer. A tracer represents a biologically active molecule, drug or cell (for example red and white blood cells labelled with a radionuclide) that acts as a carrier or ligand, responsible for the delivery of radionuclide to a specific organ [60].

Radionuclides are atoms with an unstable nucleus due to an imbalance in the number of protons and neutrons. This instability causes nuclear transformation which is accompanied by the emission of a high energy radiation in the form of alpha or beta particles, and Auger´s electrons and/or gamma photons/neutrinos as they decay into a more stable state. This unique property of each radionuclide makes them essential for various applications, particularly in nuclear medicine [60].

Nuclear medicine employs radiation to provide insights into organ function and facilitate disease treatment. This information often enables rapid diagnosis, which almost routinely includes the visualization of the bones, brain, heart, kidney, liver, thyroid, and other organs of interest to reveal abnormalities and guide treatment decisions [61]. Therefore, radionuclides are the cornerstone of diagnostic examinations in nuclear medicine. When combined with specialized imaging equipment that captures the emitted gamma rays like in PET/SPECT scanners, these radionuclides allow for the detailed study of various physiological processes within the body. During a diagnostic procedure, a controlled dose of radioactive material is administered to a patient. The localization of this material within specific organs is then visualized as either two-dimensional or three-dimensional images. These gamma or positrons emitting tracers, incorporating short-lived isotopes linked to specific chemical compounds, enable the assessment of particular physiological functions [60].

Radionuclide therapy (RNT) utilizes unsealed sources of radionuclides for the treatment of cancers or other types of pathological conditions [62]. The development of effective agents for radionuclide therapy requires careful selection of the radionuclide. The major criteria for the choice of a radionuclide for therapeutic applications are suitable nuclear

decay characteristics (Figure 5), ease of production and amenable chemistry [63]. In nuclear medicine, particle emitters refer specifically to radioactive isotopes that decay by emitting subatomic particles with specific properties. These emitted particles are crucial for diagnosis and treatment in various procedures. Radionuclides used in radiodiagnosis emit positrons (β^+-decay) or photons (γ-radiation). In contrast, radiotherapy utilizes electrons (β^--decay) or Auger electrons or relatively newly alpha particles (α-decay). Each type of these particles has different effective penetration range in soft tissue as well as different linear energy transfer (LET) and consequently, has various relative biological effectiveness [63]. The type of particle emission required for a given application depends on many factors, such as, the nature of the disease to be treated, the distribution of target cells within the target volume, total volume of the tumour and vascularity in the tumour as well as intramolecular distribution and pharmacokinetics of the tracer [63]

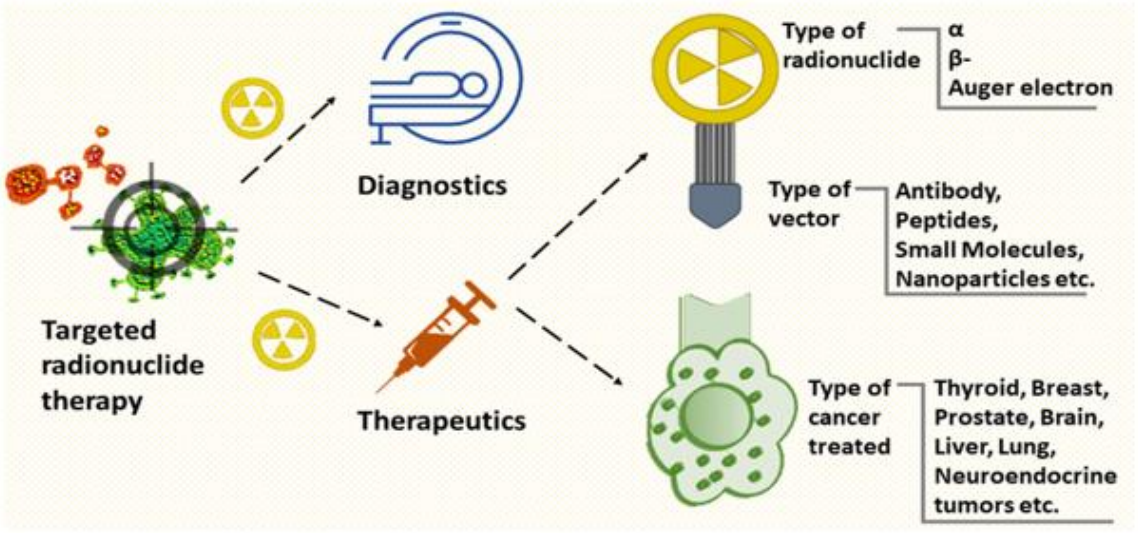

Figure 5 Targeted radionuclide therapy for cancer [64].

Alpha particle therapy is expected to be a more efficacious mode due to the high LET in tissue and the capability to deliver higher radiation dose at the cellular level. The high LET radiation limits the ability of cells to repair damage caused to DNA and is effective in killing cells even in hypoxic conditions [63]. Although more than 100 radionuclides decay by the emission of alpha particles, the vast majority have physical half-lives that are too long to be compatible with radionuclide therapy applications. Therefore, only a limited number of α-particle emitting radionuclides such as terbium-149, bismuth-212, bismuth-213 etc. have been proposed for clinical RNT [63]. Radionuclides emitting β^--particles are most preferred as

of now for radionuclide therapy and several therapeutic radiopharmaceuticals based on β^--emission have found regular clinical applications. The LET value for β^--emitters is adequate for destroying cancer cells. Radionuclides emitting β^--particles thus offer a wide range of choices with respect to energy and hence tissue penetration, physical half-life, availability of gamma rays, production mode etc [63].

The use of several chelating agents, organic molecules, have enabled monoclonal antibodies and peptides radiolabelling with a wide variety of β^--particle emitting radionuclides such as terbium-161, holmium-166, lutetium-177 and rhenium-186, which have been reported as suitable for targeted therapy. Primarily, rhenium-186 and particularly lutetium-177 belong to the commonly used radiotherapeutic nuclides [63].

In conclusion, the selection of a β^--emitting radionuclide used for a particular type of targeted therapy is based on the physical characteristics of the radionuclide, which includes emitted particle energy, physical half-life, nuclide availability and possible abundance of accompanying gamma radiations as well as its production feasibility and chemistry (Figure 6).

Gamma rays (γ) are high-energy, weightless photons emitted during radioactive event. They are similar to visible light but with much higher energy and shorter wavelength. Gamma rays originate from inside the atomic nucleus and have the most energetic form of electromagnetic radiation. They can easily penetrate the barriers that are capable to stop alpha and beta particles. Nevertheless, their LET is very low compared to alpha-particles, therefore γ-photons are not suitable for therapy [65].

However, the Auger electrons are very low-energy electrons, they have the considerable LET. Therapy with Auger electrons is still at its nascent phase and requires much more understanding related to its bio-distribution kinetics at the subcellular level [66].

While radionuclides find applications in various fields of nuclear energy, their most significant impact lies in the realm of medicine. Despite numerous radionuclides being proposed for therapeutic radiopharmaceuticals, only a select few have achieved routine clinical use. Nevertheless, their role in diagnosis and therapy continues to expand globally, underscoring their importance in advancing healthcare worldwide [54].

3.7.2 Terbium isotopes

Terbium has emerged as a highly promising element for theranostic nuclear medicine, a field that combines therapeutic and diagnostic applications. This is due to its unique set of four short-lived radioisotopes (terbium-149, terbium-152, terbium-155 and terbium-161), each with appealing nuclear characteristics (Figure 6). These features make terbium ideal for the "matched-pair" principle of theranostics, allowing the development of diagnostic and therapeutic radiopharmaceuticals with identical chemical structures and pharmacokinetics [66,67]. Terbium-149 decays by low energy α-particles emission and has been proposed as a promising radionuclide for α-therapy. Additionally, the partial positron decay of terbium-149 also allows PET imaging for post-treatment assessments, enabling the visualization of the therapeutic radiopharmaceutical's distribution. Terbium-152 and terbium-155 can be used for PET and SPECT imaging, respectively [67].

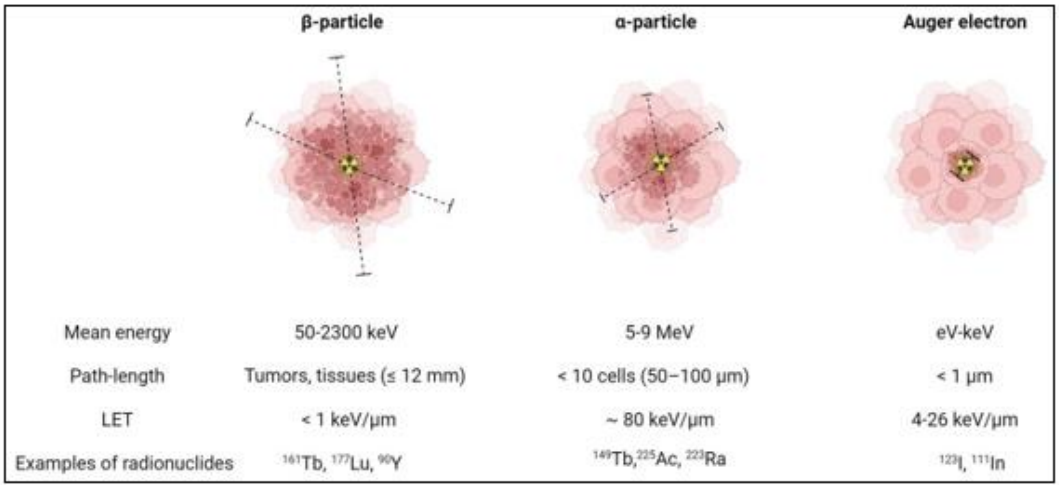

Figure 6 Biophysical properties of beta-particle, alpha-particle and Auger electron [68].

Terbium-161 has interesting decay characteristics that make it a promising radionuclide for treatments in nuclear oncology. It mainly decays by the release of β^--particles, but it also emits Auger electrons. It is believed that high LET of Auger electrons can be effective in reducing the survival capacity of cancer cells. The production of adequate amounts of carrier-free terbium radionuclides constitutes a major challenge for their use in nuclear medicine [66].

Whereas terbium-161 can be produced in nuclear reactors, the other three terbium isotopes can only be produced in cyclotrons or high-energy ion beam facilities [69].

3.7.3 Terbium-161

Choosing the right radioactive materials to make cancer treatments more effective is a complex process. The specific properties of the radioactive element, like how it decays, are crucial in determining its suitability for treatment of a particular type or stage of cancer. It is also important to consider if radionuclide can be easily combined with existing cancer-targeting drugs and whether it can be produced in sufficient quantities for widespread use. Terbium-161 is a radioactive element that has shown promise in recent research and is being considered for clinical use [70]. Terbium-161 decays with a physical half-life of 6.95 days and emits β -particles (E_{mean} = 154 keV) and γ-radiation (of energies 48.9 keV (17.0%), 57.2 keV (2.1 %) and 74.6 keV (10.2%)) similar to the decay properties of lutetium-177 [71]. However, terbium-161 is thought to be better than lutetium-177 because it also releases other particles along with the usual radiation. These extra particles, called conversion and Auger electrons, travel to shorter distances but can effectively destroy tiny cancer deposits that are too small to be seen on PET. These tiny deposits are important because they can lead to cancer coming back or spreading. This idea is based on the calculation that terbium-161 releases a much higher radiation dose to cells and small areas compared to the currently clinically used lutetium-177. Therefore, researchers believe that terbium-161 might be a better choice for treating these tiny cancer deposits in patients [72].

3.7.4 Bifunctional chelating agent DOTA

The DOTA chelator, or 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid, is a macrocyclic ligand that has become the gold standard in both magnetic resonance imaging (MRI) and particularly radiopharmaceutical chemistry. The dominance of DOTA stems from its ability to form exceptionally stable complexes with a variety of metal ions, particularly trivalent ones. The high thermodynamic stability of these complexes ensures that the metal ion remains tightly bound within the DOTA cavity, preventing unwanted release or exchange reactions in biological environments. This is crucial for applications like MRI contrast agents and targeted radiopharmaceuticals, where the metal ion must stay attached to the chelator to reach and interact with the intended target [73].

The characterization of DOTA and its metal complexes involves evaluating their thermodynamic stability, formation kinetics, and dissociation kinetics. Thermodynamic stability is assessed through stability constants, which reflect the strength of the metal-ligand interaction. DOTA complexes exhibit remarkably high stability constants, indicating their resistance to dissociation. Formation kinetics, or how quickly the complexes form, are also

important, especially for radiopharmaceuticals with short-lived isotopes. While DOTA complex formation can be slow at room temperature, it can be accelerated by using elevated temperatures. Dissociation kinetics, which measure how readily the complexes break down, are crucial for *in vivo* applications. DOTA complexes demonstrate exceptional kinetic inertness, meaning they are highly resistant to dissociation even under acidic conditions or in the presence of competing ligands. The combination of high thermodynamic stability and kinetic inertness makes DOTA an ideal chelator for various medical and radiochemical applications [74].

3.8 In vitro receptor binding assays

The **internalization assay** examines the process by which a ligand, such as an antibody, binds to its target receptor on the cell surface and is subsequently taken up into the cell. The assay quantifies the amount of internalized ligand, providing insights into the dynamics of receptor-mediated endocytosis and the intracellular fate of the ligand-receptor complex.

The **saturation binding study** aims to determine the binding affinity represented by the equilibrium dissociation constant (K_D) and the total number of receptors (B_{max}) available for a specific ligand on the cell surface. By incubating cells with increasing concentrations of a radiolabelled ligand, the assay measures the specific binding at equilibrium. The resulting saturation curve allows for the calculation of K_D, which reflects the strength of the ligand-receptor interaction, and B_{max}, which indicates the density of receptors on the cell surface.

The **inhibitory concentration at 50% (IC_{50})** assay evaluates the potency of a substance, such as an antibody or a drug, in inhibiting a specific biological or biochemical process. The IC_{50} value represents the concentration of the substance required to achieve a 50% reduction in the measured activity of a standard ligand binding to the same structure usually receptor. It serves as a valuable parameter for comparing the inhibitory effectiveness of different compounds, which are the subject of a study [75].

4 The aims of the thesis

The presented study was focused on:

a) the conjugation of monoclonal antibody ramucirumab with the chelating agent DOTA
b) the radiolabelling of immunoconjugate DOTA-ramucirumab with terbium-161
c) the radiochemical purity assessment of prepared [^{161}Tb]Tb-DOTA-ramucirumab performed with HPLC analysis with radiometric detection
d) the stability testing of the prepared radiopharmaceutical in saline and mouse plasma
e) the cell culturing of human glioblastoma cell line (U-87 MG)
f) the conduction of *in vitro* binding experiments with [^{161}Tb]Tb-DOTA-ramucirumab in cell line including internalization, saturation, and competition study
g) the acquired data evaluation in MS Office Excel and GraphPad Prism programs
h) the comparison of obtained data with previously published scientific works in discussion chapter

5 Experimental part

5.1 Employed devices and software

Table 1 Devices and Software Used in Laboratory

Device/Software	Model/Brand
Shaker	Vortex V1 plus (Biosan)
Analytical Balances	Sartorius Analytic
Automatic Pipettes	Eppendorf Research Plus (0.5-10 µL, 2-20 µL, 20-200 µL, 100-1000 µL)
Bürker's Cell Counting Chamber	Brand
Cell Culture Incubator	Sartorius Stedim Biotech Certomat® CS-18
Centrifuge	U-32 R (Boeco)
Chemical Extractor Fan	Polon
Dry-Bath	MS Science
Gamma Counter	Automatic Gamma Counter, Waltec Wizard2 3", 2480 Perkin Elmer
HPLC System	Agilent 1100 Series (Agilent Technologies Inc.)
Laminar Flow Box	Bio Air Instruments
Magnetic Stirrer	IKA RTC basic
Microcentrifuge	Sprout (Heathrow Scientific)
Microscope	XDS-IR (OPTICA)
Multipette E3	Eppendorf Research Plus (up to 50 mL)
Multichannel Pipette	Comfort
Non-Sterile Cell Culture Incubator	Sanyo Incubator MIR-153

pH Meter	Eutech Instruments pH510
Preliminary Weights	Kern PLB 200-3
Software	GraphPad Prism (version 10.1.2)
Software	Microsoft Excel (Office 365)
Raytest	Elysia
Spectrophotometer	TECAN INFINITE M200 PRO
Surface Radiation Contamination Detector	Polon
ThermoShaker	Biosan
Water Bath	TW12 (Julabo)

5.2 Used materials

Table 2 Comprehensive list of laboratory items used within this thesis.

Item	**Brand**
24-well cell culture plate	TPP
96-well cell culture plate	TPP
Cell culture flask (75 cm2)	TPP
Gas burner	Schuett Phoenix
Glass Pasteur's pipette	Brand
HPLC vials	Agilent Technologies
iTLC SG chromatography paper impregnated with silica gel	Varian
LC column	Bio-Sil SEC 250, 300 mm x 7.8 mm
Pipette tips (of volume 10 µL, 200µL, 1000 µL)	Eppendorf

PD10 column	Sigma-Aldrich
Sterile serological pipettes (of volume 5, 10, or 25 mL)	TPP
Ultrafiltration spin columns Vivaspin (membrane cut-off 50 kDa)	Sartorius
Vial inserts (of volume 250 µL)	Agilent Technologies
Volumetric flask (volume 250 mL and 1 L)	Simax

5.3 Used chemicals

Table 3 list of used chemicals within this thesis.

Chemical/Reagent	**Brand/Source**
70% ethanol	Penta
37% HCl	Sigma-Aldrich
Argon (90%)	Linde Gas
Borate acid	Penta
CaCl2	Penta
Cell culture medium (DMEM, high glucose)	Sigma Aldrich
Copper atomic absorption solution	Merck
Dimethylsulfoxid (DMSO)	Merck
Distilled water	Millipore
Ethylenediaminetetraacetic acid (EDTA)	Merck
Fetal bovine serum	Merck

Glucose	Penta
Glycine	Merck
HCl	Penta
HEPES	Thermo Fisher Scientific
KCl	Penta
L-glutamine cell culture sterile solution	Sigma-Aldrich
$MgCl_2$	Penta
NaCl	Penta
$Na_2HPO_4.12H_2O$	Penta
$NaH_2PO_4.2H_2O$	Penta
NaOH	Penta
Na_3PO4	Merck
Non-essential amino acids solution (sterile, cell culture)	Sigma Aldrich
Penicillin Streptomycin solution for cell culture	Sigma-Aldrich
Pierce BCA Protein Assay Reagent A	Thermo Scientific
Pierce BCA Protein Assay Reagent B	Thermo Scientific
Pyruvate solution (sterile, for cell culture)	Sigma Aldrich
p-SCN-Bn-DOTA	Macrocyclics
Ramucirumab (Cyramza)	Eli Lilly
Sodium citrate	Sigma Aldrich

Terbium-161 ([161Tb]TbCl in HCl 0.1 M)	Czech Technical University in Prague and ÚJV Řež
Triton X	Sigma-Aldrich
Trypsin EDTA solution (sterile, cell culture)	Sigma-Aldrich

5.4 The employed cell culture

In the study used human glioblastoma cell line (U-87 MG, under the code HTB-14) (Figure 7) was acquired from the American Type Culture Collection (ATCC).

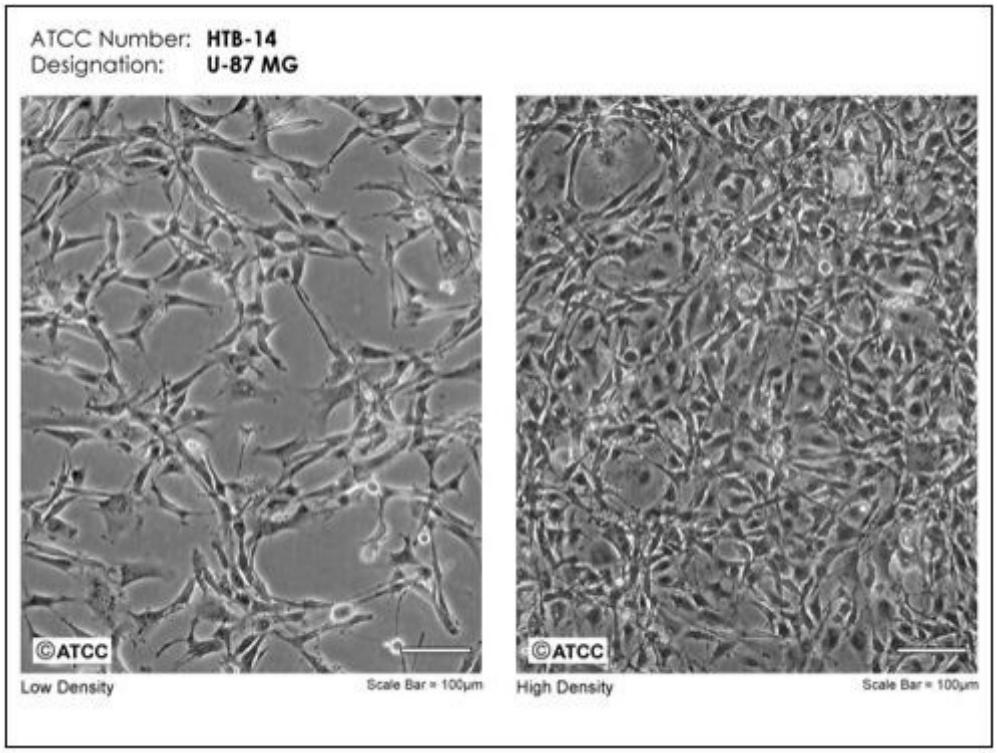

Figure 7 The morphology of low and high-density growth of human glioblastoma cell line U-87 MG (HGT-14) (Reference: https://www.atcc.org/products/htb-14).

5.5 Protocols

5.5.1 Monoclonal antibody ramucirumab

Monoclonal antibody ramucirumab (RAM) was purchased in infusion solution. Before its use in further experiments, RAM (10 mg/1 mL) was purified into borate buffer (0.1 M, pH 8.63) with the use of PD10 column to remove additional substances. The Bradford Assay analysis followed to assess the quantity of purified antibody, when 5 µL of purified solution was pipetted into 96-well plate (made in triplate of wells), Bradford Assay solution was added (250 µL) and incubated for 15 min. The analysis on a spectrophotometer followed at the wavelength 595 nm.

5.5.2 Ramucirumab conjugation with DOTA

The purified monoclonal antibody ramucirumab (in borate buffer) was conjugated with chelating agent DOTA in the molar ratio 1:5 (RAM:DOTA). For example, 1 mg of RAM was conjugated in the presence of 24 µg of DOTA. First, DOTA was weighed and dissolved in dimethylsulfide and then the adequate quantity of dissolved DOTA was added to the solution with RAM. The pH value was adjusted to 9-10 with the addition of Na_3PO_4 solution (1 M). The mixture of RAM with DOTA was incubated for 24 h in dark at 4 °C while stirred. When the incubation was over, the mixture was filtrated with the use of ultrafiltration spin columns VivaSpin to remove non-conjugated DOTA molecules. This filtration was accompanied with the transfer of conjugated RAM (DOTA-RAM) into ammonium acetate buffer (0.1 M, pH 7.48). The number of conjugated DOTA molecules per one molecule of monoclonal antibody RAM was quantified with the use of Arsenazo III spectrophotometric assay. Besides, the quantity of RAM in the immunoconjugate was analysed with the Bradford Assay as described in the previous Chapter.

5.5.3 Arsenazo III Spectrophotometric Assay

First, the working solution of Arsenazo III (A(III)) was prepared (40 µM), when A(III) adequate quantity was weighed and dissolved in ammonium acetate (0.15 M, pH 7.0). To this prepared solution of A(III) the solution of copper ions (2 µL per 1 mL of A(III) solution) was added. A(III) solution (190 µL) with copper ions was transferred into each well with sample in 96-well plate and incubated for 30 min at 37 °C. Besides the triplicate of DOTA-RAM sample, the DOTA standards were also analysed in the chosen concentrations (0, 10, 25, 50, 100, 250,

500, 750 and 1000 μg/mL) to prepare the calibration curve for conjugated DOTA to RAM molecule for the quantification.

When the incubation was over, each well was analysed in a spectrophotometer at the wavelength 630 nm. The calibration curve of DOTA standards was created in MS Office Excel. Based on the linear plot calibration curve, the number of DOTA molecules conjugated to RAM was quantified.

5.5.4 Radiolabelling of DOTA-ramucirumab and the radiochemical purity control

The prepared immunoconjugate DOTA-ramucirumab (DOTA-RAM) in ammonium acetate (0.1 M, pH 7.48) was radiolabelled with terbium-161. After the previous optimization of radiolabelling conditions (data not shown), DOTA-RAM (26.4 nmol) was radiolabelled with terbium-161 ([^{161}Tb]TbCl in HCl (0.1 M)) of the activity A = 48 MBq for 25 min at the temperature 37 °C while constantly stirred. When the incubation was over, EDTA solution (10 μL, 10 mM) was added and the reaction solution was incubated for next 5 min. The radiochemical purity of the prepared radioimmunoconjugate was assessed with the use of HPLC with radio detection. Gel permeable column was used for the separation of [^{161}Tb]Tb-DOTA-RAM from the possible present radiochemical impurity [^{161}Tb]Tb-EDTA with the use of the mobile phase PBS (pH 7.4) at isocratic elution and flow rate 1 mL/min.

5.5.5 Stability control of [^{161}Tb]Tb-DOTA-RAM in saline and mouse serum

The stability of prepared [^{161}Tb]Tb-DOTA-RAM was tested in saline and serum with the use of instant thin layer chromatography (iTLC). Radioimmunoconjugate solution was transferred into saline and serum in ratio 1:4 and incubated for 144 h at 37 °C. At the specific time points (0, 24, 48, 72, 96, 120 and 144 h) the sample (5 μL) of [^{161}Tb]Tb-DOTA-RAM in either saline or serum was applied on iTLC chromatographic paper and eluted in sodium citrate buffer (0.1 M).

5.5.6 Cell Culturing

In this study used U-87 MG cells were maintained and cultured in DMEM (high glucose) supplemented with penicillin (100 U/mL), streptomycin (100 mg/mL), non-essential amino acids (1 %), pyruvate (1 %) and FBS (10 %) at 37°C in a humidified atmosphere with CO_2 (5 %). U-87 MG cells are isolated from human glioblastoma and abundantly express the target VEGFR-2 on their cell surface. The cells were cultured in cultivation bottles with an area of 75 cm^2. The work with cells proceeded under sterile conditions in the laminar box. All tools needed for cell culturing work have been sterilized with 70% ethanol before being placed in

the laminar box to maintain a sterile environment. Cells were cultured until became confluent and they underwent trypsinization and seeding in a new culture flask. When needed for *in vitro* experiments, cells were seeded in the 24-well plate 48 hours before an experiment, when $1x10^5$ cells were transferred in each well. The exact number of cells after their release by trypsin was determined using Bürker's counting chamber. Cells deployed in this way were cultured under conditions identical to conventional cell line cultivation.

5.5.7 The preparation of Phosphate buffer

Phosphate buffer (PBS) of pH about 7.4 was used for a cell wash and as a mobile phase for HPLC analysis. The protocol for the preparation of 1 litre buffer solution included the weighing of NaCl (8.01 g), KCl (0.21 g), Na_2HPO_4 (3.58 g), and NaH_2PO_4 (1.56 g). The compounds were dissolved in distilled water and once dissolved transferred into a volumetric flask (1 L), which was then topped up with distilled. Finally, the pH value of the buffer solution was adjusted to 7.4 using either NaOH (1 M) or HCl (1 M). PBS solution was kept in a refrigerator to avoid bacterial degradation until the use.

5.5.8 The preparation of Glycine buffer

Glycine buffer was used for *in vitro* internalization study of radioimmunoconjugate. Glycine buffer triggers the release of a non-internalized receptor-radioligand complex from a cell surface. For the preparation of 200 mL of glycine buffer of the concentration 0.2 M with pH 2.8, 3 g of glycine were dissolved in 0.2 L of distilled water, and HCl (1 M) was used to adjust the pH value.

5.5.9 The preparation of Krebs-Ringer´s solution

Krebs-Ringer´s solution was used in *in vitro* binding studies. It served as a cell medium with a tested radioimmunoconjugate. One litre of Krebs-Ringer´s solution was prepared with the following compounds: NaCl (7.2 g), KCl (0.40 g), $CaCl_2$ (0.13 g), $MgCl_2$ (0.16 g), Na_2HPO_4 (0.29 g), NaH_2PO_4 (0.03 g), glucose (0.99 g) and HEPES (2.38 g). The weighed compounds were dissolved in distilled water in the final volume of 1 L. Finally, the pH value of Krebs-Ringer´s solution was adjusted to 7.4 using either NaOH (1 M) or HCl (1 M).

5.5.10 The preparation of disintegration solution

Cells after *in vitro* experiments were lysed with the disintegration solution, when the produced cell lysate was used to assess cell protein concentration. To prepare the disintegration

solution of the volume 200 mL, NaOH (0.8 g) was weighed, dissolved in distilled water, and Triton X (1 mL) was finally added.

5.5.11 BCA Protein Assay

BCA potein assay is the commercial method and was used for the assessment of cellular protein content. The assay is based on bicinchoninic acid (BCA) colourful complex formation with reduced copper ion (Cu^{1+}), which is reduced from oxidation state 2^+ by peptide bonds present in a sample. Duplicates of cell lysate samples (20 µL) were taken from each well of the 24-well plate used in an *in vitro* experiment and placed in the 96-well plate. The amount of cell protein was determined from the linear dependence equation of the calibration series, which was prepared using bovine serum as a standard with a concentration in the range of 0-2000 µg/mL. BCA Protein Assay Mixed Reagent Solution (200 µL) was added to each lysate sample. The incubation followed at 37°C for 30 min. When incubation proceeded, the intensities of colour complex were measured in a spectrophotometer at a wavelength of 590 nm. The amount of cellular protein was calculated according to the calibration curve in MS Office 365 Excel. When data of measured activity (CPM) were obtained, they were calculated per cell protein concentration.

5.5.12 The in vitro internalization study

The internalization of VEGFR-2 bound with [^{161}Tb]Tb-DOTA-RAM was analysed in U-87 MG cells at 37°C. First, 5×10^5 of cells were seeded in a 24-well plate. After 48-h incubation, cells were washed twice with PBS (0.7 mL) and then incubated with Krebs-Ringer´s solution (0.5 mL) containing [^{161}Tb]Tb-DOTA-RAM (90 nM) for the following time points: 30, 60, 90, 120, 150 and 180 min. Each time point was made in triplicates. Non-specific binding was analysed simultaneously in the same experimental setting with the difference of the presence of non-labelled RAM (1 µM) in each well. When the incubation was over, cells were washed twice with ice-cold PBS (0.7 mL) and incubated with ice-cold glycine buffer (0.5 mL) for 1 min. Glycine wash was collected into centrifugal microtubes and cells were further lysed in disintegration buffer (0.5 mL). Cell lysate was collected into centrifugal microtubes, from which a sample (2 x 20 µL) was pipetted into a 96-well plate for cell protein analysis. The gamma counter was used to measure the radioactivity in both glycine buffer wash (representing receptor-bound activity) and cell lysate (representing internalized activity). The combined radioactivity, including cell surface-bound and cell-internalized, counts (CPM) corrected on CPM measured in non-specific binding

samples, was plotted against the time points in the GraphPad Prism program. Each time point was made in triplicate of wells and each experiment was made in three independent repetitions.

5.5.13 The in vitro saturation study

The binding affinity of [^{161}Tb]Tb-DOTA-RAM to targeted receptor was assessed with the use of the saturation binding assay. First, 5×10^5 of U-87 MG cells were seeded in a 24-well plate. After 48 h incubation, cells were twice washed with PBS (0.7 mL). The addition of Krebs Ringer´s solution (0.5 mL) containing [^{161}Tb]Tb-DOTA-RAM with increasing concentrations (0, 3, 15, 30, 45, 90, and 180 nM) followed. Non-specific binding was made in the same experimental setting with extra addition of non-labelled RAM (1 uM). The incubation proceeded at 37°C for 180 min. When the incubation was over, cells were twice washed with ice-cold PBS (0.7 mL) and lysed in a disintegration solution (0.5 mL). Then, cell lysate was pipetted into centrifugal microtubes, when a sample (2 x 20 µL) was transferred into a 96-well plate for cell protein analysis. Cell lysate in centrifugal microtubes was measured in a gamma counter, and the binding data were analysed using MS Office Excel and GraphPad Software Prism, in which the final activity values (CPM) corrected on non-specific binding were plotted against concentration values (nM). The result of the affinity analysis was the determination of equilibrium dissociation constant (K_D), which reflects radioligand binding activity. Each concentration value was made in triplicate of wells and each experiment was made in three independent repetitions.

5.5.14 The in vitro competition study

To determine the concentration of ligand inhibiting 50 % of maximum specific binding (IC_{50}) of a competitor, competition experiments were conducted using [^{161}Tb]Tb-DOTA-RAM and its non-radiolabelled counterpart RAM. First, $5x10^5$ U-87 MG cells were seeded in a 24-well plate. After 48-h incubation, cells were washed twice with PBS (0.7 mL) and the addition of Krebs-Ringer´s solution (0.5 mL) with [^{161}Tb]Tb-DOTA-RAM (90 nM) followed together with non-radiolabelled RAM at varying concentrations (0.1, 1, 10, 100, 1000 and 10 000 nM). The incubation proceeded at 37°C for 90 min. After the incubation, each well with cells was twice washed with ice-cold PBS (0.7 mL) and the addition of disintegration solution (0.5 mL) followed. Cell lysate was pipetted into centrifugal microtubes when a sample (2 x 20 µL) for cell protein assessment was taken into a 96-well plate. Radioactivity (in CPM) in each centrifugal microtube was measured in gamma counter and obtained data were processed in MS Office Excel and GraphPad Prism. As a result, the inhibitory curve was

obtained to characterize the inhibitory concentration of radioligand. Each concentration value of the competitor was made in triplicate of wells and each experiment was made in three independent repetitions.

5.5.15 The preparation of citrate buffer

Sodium citrate buffer (pH 5) was prepared as a mobile phase for iTLC analysis. To create 100 mL of 0.1 mM citrate buffer solution, a precise amount of citric acid monohydrate (0.74 g) and trisodium citrate dihydrate (1.92 g) were weighed on an analytical balance. This weighed quantities were then transferred to a clean beaker and dissolved. Subsequently, approximately 60 mL of distilled water was added to the container. The mixture was gently stirred or swirled until the sodium citrate dihydrate completely dissolved, resulting in a clear, colourless solution. The dissolved compounds were transferred into a volumetric flask and the total volume (100 mL) was completed with the addition if distilled water.

6 Results

The experimental part of this thesis was focused on the preparation and testing of the radiopharmaceutical based on monoclonal antibody ramucirumab, which was labelled with terbium-161. The monoclonal antibody ramucirumab was conjugated with the chelating agent DOTA and consequently radiolabelled with terbium isotope. The prepared radiopharmaceutical was tested on its stability in saline and serum and further tested in human glioblastoma cell line U-87 MG that overexpresses the receptor VEGFR-2. The obtained results are presented in the following chapters.

6.1 The conjugation of mAb ramucirumab with DOTA

The conjugation of ramucirumab with DOTA was performed in the molar ratio 1:5 (RAM:DOTA) and the number of conjugated DOTA molecules per one molecule of ramucirumab was assessed with the use of Arsenazo III spectrophotometric assay. The assay was based on the competition of DOTA for copper ions with Arsenazo III.

As the result, the successful conjugation of RAM with DOTA was confirmed by Arsenazo III spectrophotometric assay. The average number of DOTA molecules conjugated to RAM molecule was about 15.

6.2 The radiochemical purity analysis of [^{161}Tb]Tb-DOTA-RAM

The radiochemical purity of the synthesized [^{161}Tb]Tb-DOTA-ramucirumab was always analysed using HPLC with radiometric detection before any further application of radioimmunoconjugate. The chromatogram of the [^{161}Tb]Tb-DOTA-RAM complex (Figure 8) shows a single prominent peak at a retention time of approximately 7.5 min, indicative of the successful radiolabelling of ramucirumab with terbium-161.

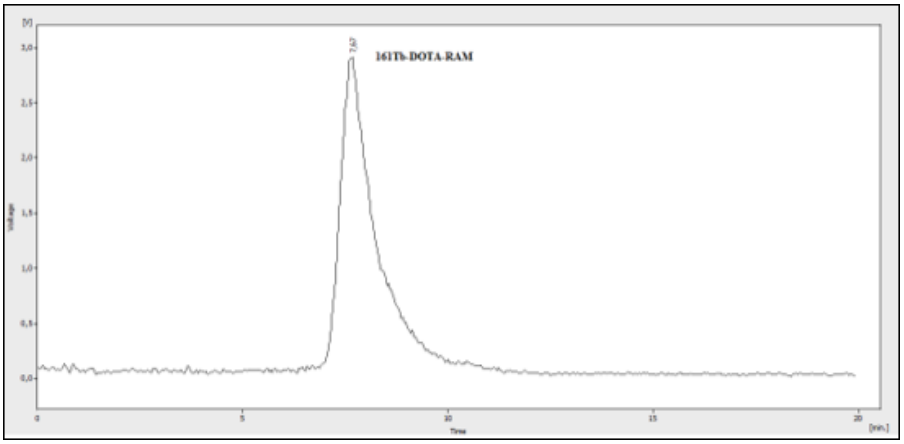

Figure 8 The example radiochromatogram of [^{161}Tb]Tb-DOTA-RAM which eluted about 7.5 min.

The elution time of the possible radiochemical impurities [^{161}Tb]Tb-chloride and [^{161}Tb]Tb-EDTA was also analysed. The illustrative radiochromatograms of [^{161}Tb]Tb-chloride (Figure 9) and [^{161}Tb]Tb-EDTA (Figure 10) are depicted bellow.

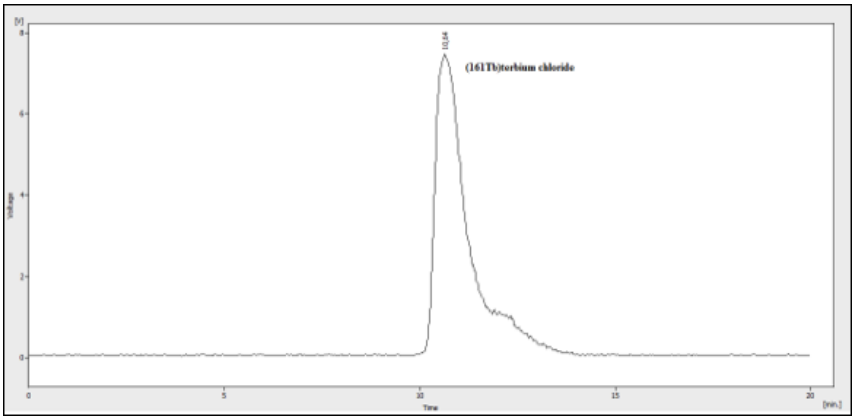

Figure 9 The example radiochromatogram of [^{161}Tb]$TbCl_3$ which eluted at about 10.5 min.

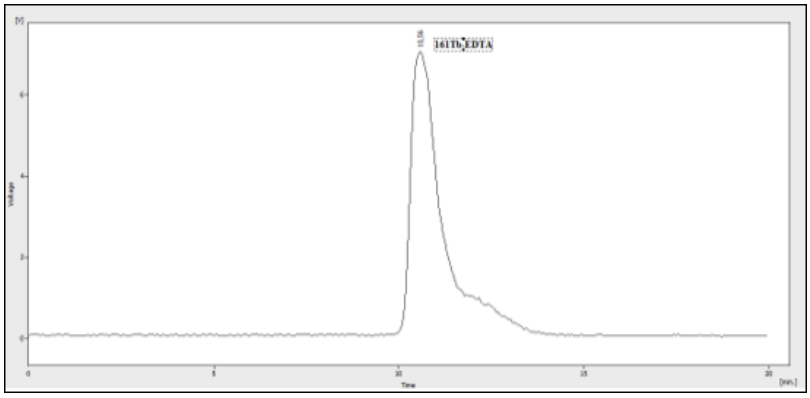

Figure 10 The example radiochromatogram of [¹⁶¹Tb]Tb-EDTA which eluted at about 11.5 min.

6.3 The stability testing of [^{161}Tb]Tb-DOTA-RAM

The stability of [^{161}Tb]Tb-DOTA-RAM was evaluated in both saline and mouse serum for 144 hours at 37°C using iTLC (Table 4).

In saline: The radioimmunoconjugate exhibited high stability in saline, maintaining a radiochemical purity above 94% for the first 120 hours. A slight decrease to 84.4% purity was observed at 144 hours.

In mouse serum: [^{161}Tb]Tb-DOTA-RAM demonstrated excellent stability in mouse serum, with radiochemical purity consistently exceeding 96% throughout the 144-hour study period.

Overall, these results indicate that [^{161}Tb]Tb-DOTA-RAM possesses good stability in both physiological and biological environments, which is crucial for its potential use as a radiopharmaceutical.

Table 4 The summary of [^{161}Tb]Tb-DOTA-RAM stability testing in saline and serum

Time(hours)	Stability in saline [%]	Stability in mouse serum [%]
0	97.3	97.3
24	98.3	99.2
48	94.6	99.8
72	97.5	98.7
96	97.1	96.9
120	96.0	98.7
144	84.4	99.0

6.4 The *in vitro* testing of [^{161}Tb]Tb-DOTA-RAM

The *in vitro* experiments were performed in human glioblastoma cell line U-87 MG which is known to overexpress the VEGFR-2 receptor targeted by ramucirumab. *In vitro* characterization of radioligands is crucial for the their binding ability assessment before administered in animals.

6.4.1 The internalization study of [^{161}Tb]Tb-DOTA-RAM

The internalization of [^{161}Tb]Tb-DOTA-RAM in U-87 MG cells was evaluated in 180-min time course. The graph in Figure 11 demonstrates a time-dependent increase in both receptor-bound and internalized radioimmunoconjugate reaching the plateau at 180 min. The plateau characterizes the reached equilibrium between receptor-bound and unbound form of [^{161}Tb]Tb-DOTA-RAM. The majority of [^{161}Tb]Tb-DOTA-RAM was internalized into cells, which is crucial for terbium-161 delivery into cancer cells.

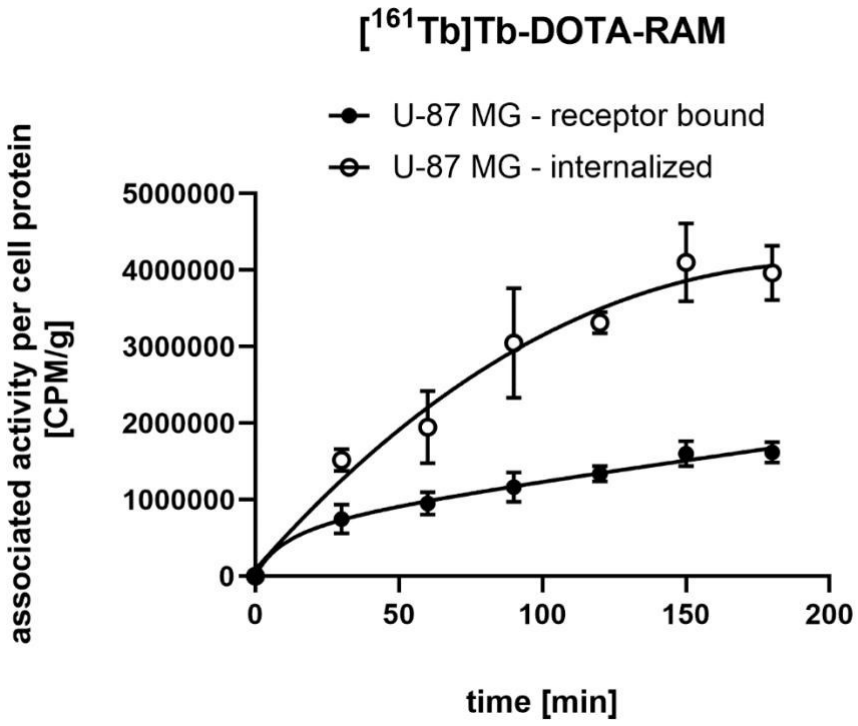

Figure 11 The time-depending internalization of [^{161}Tb]Tb-DOTA-RAM into glioblastoma cells (n = 3).

6.4.2 The saturation study of [^{161}Tb]Tb-DOTA-RAM

The binding affinity of [^{161}Tb]Tb-DOTA-RAM to its target was evaluated using a saturation binding assay. The graph in Figure 12 demonstrates a concentration-dependent increase of radioimmunoconjugate, almost reaching a plateau at the highest concentration (180 nM). The equilibrium dissociation constant (K_D) was determined by the program to be 142.0 ± 3.5 nM, indicating a moderate binding affinity of [^{161}Tb]Tb-DOTA-RAM to its target receptor.

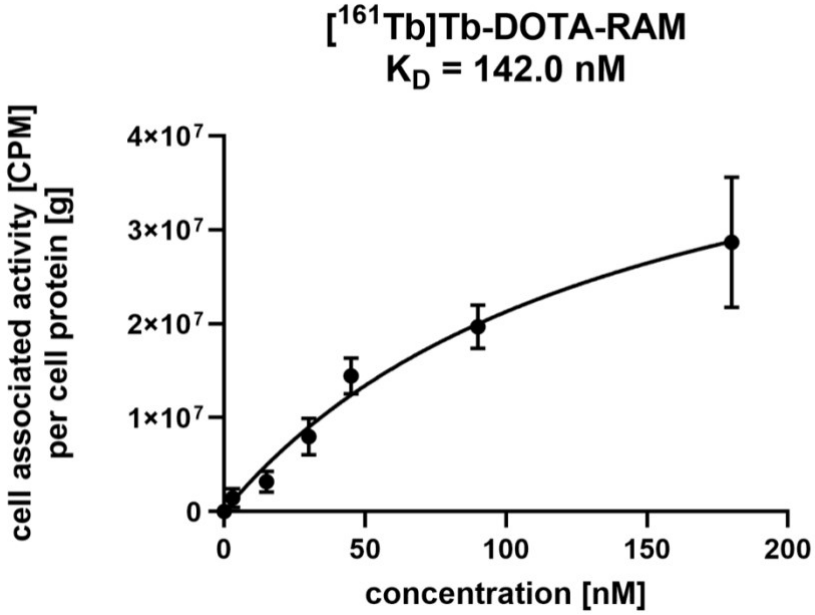

Figure 12 The saturation binding study graph obtained for [^{161}Tb]Tb-DOTA-RAM interaction with VEGFR-2. The found value of the equilibrium dissociation constant was K_D = 142.0 ± 3.5 nM. (n = 3).

6.4.3 The competition study of [^{161}Tb]Tb-DOTA-RAM

The inhibitory concentration value for [^{161}Tb]Tb-DOTA-ramucirumab competing with natural ramucirumab to the common target VEGFR-2 was determined to be IC_{50} = 2.8 ± 0.4 μM, as shown in the competition binding curve in Figure 13. This indicates the concentration of radiolabelled RAM required to reduce the specific binding of the natural RAM as a competitor by 50 %.

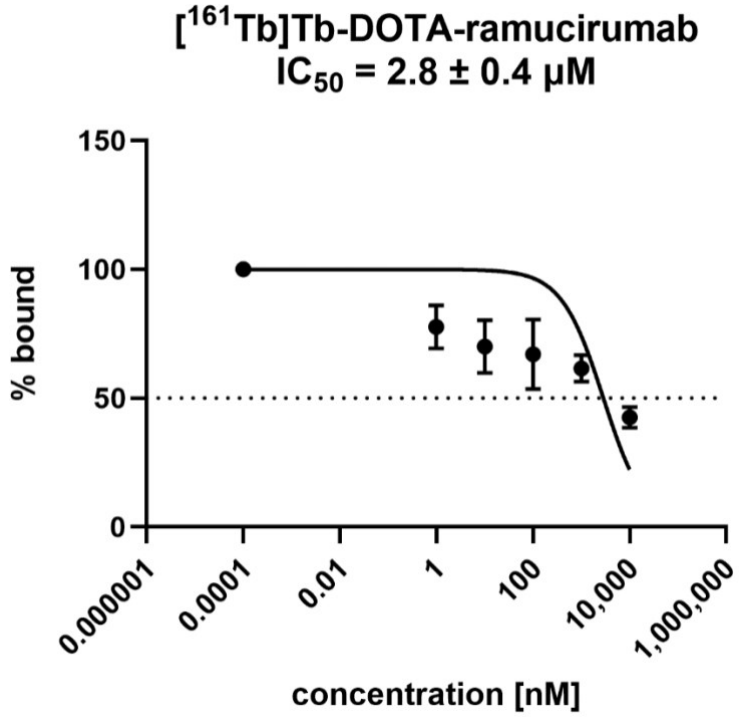

Figure 13 The inhibitory concentration assessment of [^{161}Tb]Tb-DOTA-RAM competing with natural ramucirumab. The found value was IC_{50} = 2.8 ± 0.4 µM, (n = 3).

7 Discussion

The presented thesis aimed to investigate the preparation and *in vitro* behavior of a potential radiotherapeutic agent based on monoclonal antibody ramucirumab labelled with terbium-161. Monoclonal antibodies represent promising specific targeting vectors for targeted radionuclide therapy thanks to their high specificity and affinity to a receptor of interest [76]. Ramucirumab is a monoclonal antibody targeting VEGFR-2, which is overexpressed in many solid tumors including glioblastoma [77]. The overexpression of VEGFR-2 is associated with the poor prognosis of an illness in patients [78]. This study focused on the preparation of ramucirumab radiolabelled form, which could increase the cytotoxic effect to glioblastoma cancer cells without the harm to health tissue.

The first part of thesis research was focused on the ramucirumab conjugation with bifunctional chelator DOTA in the a ratio of 1:5 (RAM:DOTA). The found value of the conjugation level was 15 DOTA molecules per antibody molecule. The ideal quantity is about 5 chelator molecules per antibody molecule . Our quantity was three times higher, which could negatively affect the binding ability of antibody due to the higher risk of chelator presence at the binding sites of protein molecule.

However, the conjugation of ramucirumab with DOTA was successful, which was demonstrated with the efficient radiolabelling controlled by HPLC. The level of conjugation ensured sufficient chelation sites for stable radiolabelling with terbium-161 while preserving the antibody's integrity and binding affinity. The optimized radiolabelling process resulted in [^{161}Tb]Tb-DOTA-RAM with high radiochemical purity, comparable to other studies utilizing terbium-161 for radiolabelling of DOTA-conjugated antibodies [70]. High radiochemical purity is critical for maximizing the therapeutic efficacy of the radioimmunoconjugate and minimizing potential side effects associated with unbound radionuclides. The excellent stability of [^{161}Tb]Tb-DOTA-RAM in both saline and mouse serum, demonstrated by iTLC analysis, is consistent with the reported stability of other ^{161}Tb-labelled DOTA-conjugated antibodies [79]. The radioimmunoconjugate maintained high radiochemical purity over 144 h, indicating its strength and potential for *in vivo* applications. This stability is crucial for achieving effective tumour targeting and minimizing off-target radiation exposure.

The in vitro experiments conducted in U-87 MG cells, which over-express the VEGFR-2 receptor targeted by ramucirumab, provided valuable insights into the behaviour of [^{161}Tb]Tb-DOTA-RAM at the cellular level. In the internalization study: the time-dependent increase in both receptor-bound and internalized radioimmunoconjugate is consistent with the internalization kinetics observed for other radiolabelled antibodies targeting VEGFR-2 [80]. This efficient internalization process is crucial for delivering the therapeutic payload of terbium-161 directly to the tumour cells, maximizing its cytotoxic effect. Saturation binding study discovered that the moderate binding affinity (KD = 142.0 ± 3.5 nM) of [^{161}Tb]Tb-DOTA- RAM to its target receptor and it is comparable to other radiolabelled antibodies targeting VEGFR-2. While a higher affinity would be desirable, this moderate affinity, coupled with efficient internalization, may still be sufficient for effective tumour targeting, especially when considering the potential for enhanced permeability and retention (EPR) effect in tumour tissues. The competition binding assay showed an IC50 value of 2.8 ± 0.4 μM, indicating the radiopharmaceutical's strong competitive binding capability. While these KD and IC50 values are slightly higher than those reported for ^{64}Cu-labelled ramucirumab (KD ≈ 1.6 nM, IC50 ≈ 1.3 nM), the differences could be attributed to variations in radiolabelling conditions, higher number of conjugated DOTA molecules per protein molecule and the distinct properties of [1]terbium-161 and copper-64, which might influence the antibody's binding dynamics and internalization. Overall, the results presented in this thesis support the potential of [^{161}Tb]Tb-DOTA-RAM as a targeted therapeutic agent [81].

8 Conclusion

The investigation of [^{161}Tb]Tb-DOTA-ramucirumab, as a tumour targeting agent, presents a promising strategy for addressing glioblastoma, a particularly aggressive and challenging type of brain cancer. This study effectively highlighted the potential of this compound to improve treatment effectiveness through targeted radionuclide therapy. By using the DOTA chelator to label ramucirumab with terbium-161 the resulting compound shown purity, stability and favorable *in vitro* characteristics such as efficient internalization and strong binding affinity competition. Experiments on internalization demonstrated that U-87 MG glioblastoma cells could efficiently transport [^{161}Tb]Tb-DOTA-ramucirumab maximizing the effects of the radionuclide directly at the tumour site. Despite binding affinity findings its robust competitive binding ability and effective internalization indicate that [^{161}Tb]Tb-DOTA-ramucirumab might serve as an agent in disrupting VEGFR-2 mediated tumor angiogenesis. While this study lays a groundwork for advancing [^{161}Tb]Tb-DOTA-ramucirumab it also underscores the necessity for other *in vivo* investigations and clinical trials to comprehensively evaluate its therapeutic efficacy and safety profile. The insights gained contribute information to the realm of targeted radionuclide therapy and bring hope for efficient treatments, for glioblastoma in the days ahead.

9 References

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