Targeted therapy of osteosarcoma with radiolabeled monoclonal antibody to an insulin-like growth factor-2 receptor (IGF2R)

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Abstract

Osteosarcoma (OS) represents 3.4% of all childhood cancers with overall survival of 70% not improving in 30 years. The consistent surface overexpression of insulin-like growth factor-2 receptor (IGF2R) has been reported in commercial and patient-derived xenograft (PDX) OS cell lines. We aimed to assess efficacy and safety of treating PDX and commercial OS tumors in mice with radiolabeled antibody to IGF2R. IGF2R expression on human commercial lines 143B and SaOS2 and PDX lines OS-17, OS-33 and OS-31 was evaluated by FACS. The biodistribution and microSPECT/CT imaging with 111Indium-2G11 mAb was performed in 143B and OS-17 tumor-bearing SCID mice and followed by radioimmunotherapy (RIT) with 177Lutetium-l2G11 and safety evaluation. All OS cell lines expressed IGF2R. Biodistribution and microSPECT/CT revealed selective uptake of 2G11 mAb in 143B and OS17 xenografts. RIT significantly slowed down the growth of OS17 and 143B tumors without local and systemic toxicity. This study demonstrates the feasibility of targeting IGF2R on OS in PDX and sets the stage for further development of RIT of OS.

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LIST OF ABBREVIATIONS

RIT	Radioimmunotherapy
OS	Osteosarcoma
GEP-NETS	Gastroenteropancreatic neuroendocrine tumors
TRT	Targeted Radionuclide Therapy
IGF2R	Insulin-like growth factor- 2 receptor
SNP	Single Nucleotide Polymorphism
EBRT	External beam radiation therapy
CIHR	Canadian Institutes of Health Research
WHO	World Health Organization
HER	Human epidermal growth factor receptor
PDGFR	Platelet-derived growth factor receptor
FGFR	Fibroblast growth factor receptor
VEGFR	Vascular endothelial growth factor receptor
EGFR	Epidermal growth factor receptor
mAb	Monoclonal Antibody
LDH	Lactate Dehydrogenase
LET	Linear energy transfer
MRI	Magnetic resonance imaging
CT	Computed tomography
SPECT	Single Photon Emission Computed Tomography
PET	Positron Emission Tomography
FDG	Fluorodeoxyglucose
ADC	Antibody Drug Conjugate
SCID	Severe combined immunodeficiency
MEM/EBSS	Minimum Essential Medium
CHXA	(R)-2-Amino-3-(4-isothiocyanatophenyl)propyl]-trans-(S,S)-cyclohexane-1,2-diamine-pentaacetic acid
BCA	Bifunctional chelating agent
SG-iTLC	Silica gel instant thin layer chromatography

ATCC	American Type Culture Collection
FACS	Fluorescence-activated cell sorting
PBS	Phosphate buffered saline
FBS	Fetal Bovine Serum
IP	Intraperitoneal
XUHS-M	Extra Ultra High Sensitivity Mouse
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
BUN	Blood Urea Nitrogen
EPR	Enhanced permeability and retention
DOTA	Tetraxetan
^{225}Ac	Actinium-225
^{177}Lu	Lutetium-177
IgG	Immunoglobulin G
PDX	Patient Derived Xenografts
^{188}Re	Rhenium-188
Ab	Antibody
SSTR1	Somatostatin Receptor 1

1.0 Introduction

Standard care for patients

Osteosarcoma is a high-grade primary skeletal malignancy characterized by spindle cells of mesenchymal origin depositing immature osteoid matrix. [1,2] It is the most common type of cancer that arises in bones, and it is usually found at the end of long bones. However, the common site of metastasis occurs in the lungs with the bone being the second most common site of distant disease. It ranges from low grade tumors that only require surgery to high tumors that require an aggressive treatment regimen. The disease is generally treated with a combination of therapies that include either surgery, chemotherapy and radiation therapy. In addition, patients that have high number of high-grade tumors receive neo-adjuvant therapy before surgery. Through surgery, it is possible to remove the tumor safely when located on the bone or the joint and restore the patient to a fully functional life. [3] After surgery, the percentage of tumor necrosis is measured to check if any additional treatment is needed. If such a situation arises, drugs are chosen for additional chemotherapy treatment which could last until a few months. However, a pediatric oncology group raised concerns regarding the possibility that if a large tumor is present it could lead to resistant cells or an increase in micro-metastatic disease during neoadjuvant chemotherapy. [4] Treatments options such as radiation therapy use high energy rays or particles to kill cancer cells. However, osteosarcoma cells are not easily killed by radiation. [5] Therefore, radiation therapy may not be widely used in osteosarcoma treatment. Nevertheless, it has shown to be effective when a tumor is difficult to remove through surgery or when some tumor cells continue to reside after surgery. [3] The treatment option of chemotherapy is often administered after the radiation. This importance of chemotherapeutic response in patients affected with osteosarcoma was emphasized by the effectiveness of radiation therapy. [6] It was shown that it is possible to control osteosarcoma by giving standard doses of radiation in spite of radiation sensitivity. Bone seeking radioactive drugs such as samarium-153 or radium-233 are known to slow down the tumor growth and even help treat symptoms such as pain in patients affected with advanced osteosarcoma. These drugs are helpful especially when the cancer has spread to all the bones however there are major side effects to using these drugs such as the blood cell counts decrease drastically which could increase the risk for bleeding or infections. [5] Despite of all these treatment options and good

prognosis, the benefit of improved quality and function of life must be measured minutely against the potential side effects of the drug treatments, risk of relapse or the development of additional malignancies. [6] Hence depending on the extent, grade and location of the cancer there are several different treatment options for osteosarcoma. For the more debilitating kinds of osteosarcoma such as (metastatic osteosarcoma and recurrent osteosarcoma) that are difficult to treat, novel therapies may be a good option. While chemotherapy drugs are effective for treating osteosarcoma, in some cases the drugs are not as effective as the cancer becomes resistant to the regimen over time. As alternative options, research is in progress towards discovering newer types of drugs that could attack the cancer cells in different ways. Some common examples are immunotherapy and targeted therapy. Recent regulatory approvals of Xofigo for treatment of prostate cancer metastasized to the bone and Lutathera for treatment of somatostatin receptor-positive gastroenteropancreatic neuroendocrine tumors (GEP-NETs) attests the great potential and promise of Targeted Radionuclide Therapy (TRT). Radioimmunotherapy, a subset of TRT has shown great promise as well since drugs such as Zevalin where approved for treatment of non-hodgkin's lymphoma over a decade ago along with the treatment regimen being successfully employed for a number of clinical trials. [7,8]

Osteosarcoma is the most common non-hematologic primary bone malignancy. [9,10] It has been reported that it is the most common primary malignant bone tumor and the fifth most common primary malignancy among adolescents and young adults. [11] About 900 new cases are reported within the Unites States per year (American Cancer Society) representing about 3.4 % of all childhood cancers. [12] Unfortunately, overall survival remains stagnant at approximately 70%, and treatment has not evolved in a meaningful way in over 30 years. [13, 14] Regardless of therapy, patients who exhibit overt metastatic disease continue to suffer dismal outcomes, with metastases to the lungs and to the bone portending an overall survival less than 40% and 20% respectively. Osteosarcoma demonstrates tremendous genetic variability [15] precluding the use of more conventional targeted therapies and illustrating the need for alternate approaches.

Thus, there is a need for alternative novel treatment approaches for osteosarcoma. It has been demonstrated that insulin-like growth factor- 2 receptor (IGF2R) has been over-expressed across a panel of Osteosarcoma cell lines. [16] Moreover, a single nucleotide polymorphism (SNP) within a haplotype block in IGF2R was previously linked to an increased osteosarcoma risk. [17]

Although IGF2R is expressed normally across various tissue types, the constant over-expression in Osteosarcoma suggests that it may serve as a valuable therapeutic target.

Targeted radionuclide therapy (TRT) delivers cytocidal radiation in the form of alpha- or beta-particle emitting radionuclides to the tumor with high precision, thus avoiding many of the side effects associated with external beam radiation therapy (EBRT). Recent regulatory approvals of ^{223}Radium chloride (Xofigo) for treatment of prostate cancer metastasized to the bone, and of ^{177}Lutetium-labeled peptide (Lutathera) for treatment of somatostatin receptor-positive gastroenteropancreatic neuroendocrine tumors (GEP-NETs), attests to the great promise and flexibility of TRT. Radioimmunotherapy (RIT) is a subset of TRT and is a method of delivering cytotoxic radiation in a targeted approach where an antigen-specific antibody is bound to either an alpha or beta- emitting radioisotope. [7,8] RIT was regulatory approved more than a decade ago for refractory and recurrent non-Hodgkin's lymphoma (Zevalin) [18] as well as has been successfully employed in a number of clinical trials [19]. It allows systemic administration, antibody-mediated specificity, and physical cytocidal damage in a manner which is well-tolerated by the patients.

Recently Dadachova's laboratory demonstrated that IGF2R can be a potential target for RIT of osteosarcoma. [20] The purpose of this study is to investigate a novel therapy for Osteosarcoma, using RIT targeted to IGF2R. Lutetium-177, a powerful β-emitter with a physical half-life of 6.6 days has been selected for radiolabeling the IGF2R- specific antibody.

My contribution to the field of radioimmunotherapy (RIT) of osteosarcoma.

When I joined Dadachova's lab to work on the project of RIT of osteosarcoma – the antibody which binds to both human and murine IGF2R – 2G11 – has just become available commercially. Having this antibody in hand allowed me not only to evaluate the RIT of osteosarcoma in mice bearing osteosarcoma xenografts, but also to measure any possible toxic effects of RIT on the healthy tissues which express IGF2R. The results of my experiments on the comprehensive evaluation of RIT of osteosarcoma in mice helped our laboratory to obtain the CIHR grant to take this project further and to evaluate the comparative oncology in companion dogs with osteosarcoma.

2.0 LITERATURE REVIEW

2.1 Cancer

Cancer is a group of diseases that involves abnormal cell growth and have potential to spread to other parts of the body. A cancerous (malignant) tumor is a group of cancer cells that can grow into and destroy nearby tissue. It can also metastasize to other parts of the body. There are several signs and symptoms associated with cancer but they are not specific to cancer. The most common ones being an abnormal growing mass, sudden weight loss, abnormal bleeding etc. [21] According to WHO cancer statistics in the year 2007, cancer was responsible for 7.9 million deaths, resulting in approximately 13% of all deaths worldwide. It is expected that the number will increase to over 11 million deaths by 2030. [22]

Cancers are classified by the type of cell that the tumor cells originated from.

TUMOR TYPE	ORIGIN	CANCER
Carcinoma	Epithelial cells	Breast, lung, prostrate, pancreas and colon
Sarcoma	Connective tissues (bone, cartilage, fat, nerve)	Osteosarcoma, chondrosarcoma and lipo sarcoma
Lymphoma and Leukemia	Hematopoietic (blood forming) cells	Hodgkin lymphoma, Non-Hodgkin lymphomas
Blastoma	Immature “precursor cells or embryonic tissue	Nephroblastoma, medulloblastoma and retinoblastoma
Germ cell tumor	Pluripotent cells (testicle and ovary)	Seminoma and dysgerminoma

TABLE 2.1 Types of Cancer.

2.2 Bone Cancer

Bone cancers start in the cells of the bone or cartilage and are mostly called sarcomas. This kind of cancer that originates in the bone is rare and is known as the primary bone cancer. This cancer is more common in children and young adults than in older people. The secondary bone cancer or bone metastases spread to the bone from other parts of the body and are more common in older adults. This kind of cancer is more common than primary bone cancer. In Canada, the five-year relative survival for primary bone cancer is 68% [11], whereas the average five-year survival rate in the United States after being diagnosed with bone and joint cancer is 67%.[23]

TABLE 2.2 Bone Cancer Survival

TUMOR TYPE	5 YEAR SURVIVAL RATE, %
Chondrosarcoma (most common adult bone cancer)	80
Osteosarcoma, localized (stages 1,2 and 3)	60 to 80
Osteosarcoma, metastatic to lungs only (stage 4)	40
Osteosarcoma, metastatic to other organs (stage 4)	15 to 30

Canadian Cancer Society (2017)

2.3 Types of Bone Cancer

Bone tumors may be classified as “primary tumors” and “secondary tumors” depending on the origin of site. The primary bone tumors originate in the bone or from bone-derived cells and tissues

whereas the secondary bone tumors originate in other sites of the body and spread or metastasize the bone. The secondary bone tumors are most commonly carcinomas of breast, lung and prostate.

2.3.1 Primary bone tumors

Primary bone tumors can be divided into two categories 1) Benign tumors 2) Malignant tumors. In etiology, the benign bone tumors can be neoplastic, developmental, traumatic, infectious or inflammatory. They account for 0.2% of all tumors in humans. [11] The primary tumors, both malignant and benign are most commonly located in the distal femur and around the knee joint. Primary bone tumors are an uncommon malignancy but are primarily found in young people. It is the sixth most common tumor form in children, however it is the third most frequent in adolescents and young adults. [11] Some examples of benign bone tumors are osteoma, osteochondroma, osteoblastoma, fibrous dysplasia etc. Malignant primary bone tumors include osteosarcoma, chondrosarcoma, Ewing's sarcoma, fibro sarcoma, angiosarcoma etc. Multiple myeloma is a hematologic malignant tumor which frequently presents as one or more bone tumors. It is a very common primary bone cancer.

2.3.2 Secondary bone tumors

Metastatic cancer is the most common type of bone malignancy, which metastasizes to the liver, lungs, breast and prostate first and then targets the skeleton. [24,25] The malignant bone lesions are characterized by abnormal bone formation, bone loss or even both. [25,26] These tumors that originate in the soft tissues may secondarily involve bones through direct invasion.

2.3.3 Carcinomas

Carcinomas are a type of cancer that originates from epithelial cells. Carcinomas of prostate, breasts, lungs, thyroid and kidneys are the most common types of cancer that metastasize to the bone.

2.3.4 Germ cell tumors

The most common being teratoma are often present and originate in the midline of the sacrum, coccyx or both.

2.4 Osteosarcoma

Osteosarcoma is the most common non-hematologic primary bone malignancy. [9,10] It is a malignant tumor that originates from primitive bone-forming mesenchymal cells. [27] It has been reported that it is the most common primary malignant bone tumor and the fifth most common primary malignancy among adolescents and young adults. [28] An observation was made that approximately 1,000 new cases are seen per year in North America and a similar number in Europe. [29] Several important pathogenic roles of p53, RB and mdm2 genes have been reported already, however, the role and pathogenesis of osteosarcoma in bone microenvironment has not yet been fully understood. [30] The overall 5-year survival rate has plateaued at approximately 70% with no meaningful improvement in over 30 years. [10, 28, 13, 31] Patients with metastatic disease have poor outcomes, with 35-40% survival rate. Thus, there is a need for alternative novel treatment approaches for osteosarcoma. [32] Understanding the biological mechanisms that govern the osteosarcoma growth at the molecule level is highly important and essential for developing new strategies of treatment.

2.4.1 Site of disease

Osteosarcoma occurs in any bone of the body but usually in areas of the long bones of the extremities such as the distal femur and proximal tibia. [27] The tumor most commonly occurs in the femur (most tumors in the distal femur), in the tibia (most tumors in the proximal tibia) and in the humerus (most tumors in the proximal humerus). The other locations of the site of disease are in the pelvis, skull or jaw and ribs. [27]

2.4.2 Age

There are large differences in the incidence rates of osteosarcoma in patients depending on age. [10] The incidence rate is higher in older children than in younger children. It occurs frequently in adolescents and children between the ages of 10-20 years old. [32]

2.4.3 Gender

The incidence rate of osteosarcoma is higher in males than in females with the rate being 5.4 per year per million males vs. 4.0 per year per million females. [27, 33] However, while the incidence rate is higher in males, the survival rate of osteosarcoma is higher in females.

2.4.4 Diagnosis of Osteosarcoma

The diagnosis of osteosarcoma requires the identification of malignant osteoblasts and neoplastic bone. [13] The diagnosis should be accurate as it is important that the patients are treated with specific protocols which have been associated with improved survival. [34-40] Osteosarcoma is mostly easily diagnosed by light microscopy, but its identification can be difficult due to various reasons such as the wide array of its histologic features might be mimicked by other primary and metastatic bone tumors etc. Another potential tool that can aid in the diagnosis of osteosarcoma could be immunohistochemistry. This is possible since osteoblasts are known to express specific marker proteins. The role of percutaneous image-guided biopsy in the diagnosis of bone-skeletal tumors have been well established [41-42] The placement of biopsy needles for the diagnosis are well aided and guided by the computed tomography and fluoroscopy. However, in recent times, non-invasive imaging techniques such as magnetic resonance imaging (MR) and ultrasonography (US) have been used for the guidance of the biopsies. [43-45]

2.4.5 Potential therapeutic targets for the treatment of osteosarcoma

The microenvironment surrounding tumor cells has a significant impact on the phenotype, growth, invasiveness of malignant cells influencing metastatic properties and responses to drugs. Recent examples of potential therapeutic targets for treatment of the disease are the transmembrane surface receptors that have been implicated in tumorigenesis, [46] and it has been shown recently in other cancers that therapies targeted at transmembrane surface receptors have generated a substantial increase in both the quality of life and long-term survival. [47,48] Receptors such as human epidermal growth factor receptor HER-2, HER-3, HER-4, platelet-derived growth factor receptor (PDGFR)- β, c-Met, fibroblast growth factor receptor (FGFR)-2, FGFR-3, vascular endothelial growth factor receptor (VEGFR)-1, VEGFR-2, VEGFR-3, insulin like growth factor receptor 1 (IGF-1R), IGF-2R, insulin receptor (IR), and epidermal growth factor receptor (EGFR) have been shown to be expressed on several types of tumors as well as osteosarcoma. [49-51] It has been demonstrated that although insulin-like growth factor-2 receptor (IGF2R) is expressed normally across various tissue types, there has been an over-expression across a panel of osteosarcoma cell lines. [16] This constant over-expression of IGF2R may serve as a valuable therapeutic target for the treatment of osteosarcoma. In our earlier studies we have demonstrated the preferential tumor localization of the radiolabeled monoclonal antibody (mAb) to IGF2R in Osteosarcoma (OS) xenografts in mice in comparison with the control mAb. Additionally, we showed that treatment of OS tumors using Radiolabeled IGF2R- specific mAb MEM-238 resulted in tumor growth inhibition and possibly regression using a single administration and non-optimized dosing in comparison with multiple controls [20]. The limitation of that work was the nature of MEM-238 mAb which binds only to human IGF2R thus precluding evaluation of mAb binding to IGF2R expressing healthy tissues as well as the use of one OS cell line. Recently, a novel murine mAb 2G11 that binds to both human and murine IGF2R became commercially available which was used in this study to evaluate the effectiveness of RIT.

2.4.6 Prognosis and survival of patients with osteosarcoma

Osteosarcoma is the most common non-haematopoietic cancer of the bone and has a poor prognosis in patients with relapse or recurrence [52-54]
Some of the prognostic factors would be:

- Patients with osteosarcoma as a secondary neoplasm share the same prognosis as patients with newly diagnosed osteosarcoma if they are treated aggressively with surgical resection and chemotherapy. [55-58]
- Patients belonging to the older adolescent and young adult age group, typically between the ages of 18 to 40 years tend to have a worse prognosis. [59-60]
- Patients suffering from high grade osteosarcoma could be identified with the laboratory tests such as LDH (Lactate Dehydrogenase) levels, alkaline phosphatase levels and histologic subtype. [61,59,6,62,63]
    - Body mass index: Patient with higher body mass index at the initial presentation have worse prognosis [64]

2.5 Treatments for metastatic osteosarcoma

Osteosarcoma is the most common malignant primary tumor of bone [65] and is characterized by the presence of metastases in a majority of patients. The cure of osteosarcoma in a sizable number of patients was reported consistently first in the 1970s and was achieved through the combination of surgical resection of tumors with multi-drug chemotherapy. [66] The results were improved further but since then no clinically significant advances have been made although many patients still access combination chemotherapy within and outside clinical trials. The number, site and size of the metastasis determine the overall management, prognosis and treatment of the disease. [67] Osteosarcoma is often treated with a combination of therapies that include surgery, chemotherapy and radiation therapy. Most patients with high grade tumors receive around three months of chemotherapy which is known as neo-adjuvant therapy. Today there has been an increasing focus on personalized medicine in therapy, exploiting antibodies that target cancer related antigens as one of the approaches. [65] The marketed immunotherapeutic antibodies targeting antigens such as HER2 and EGFR (trastuzumab and cetuximab) have been successful for treatment of other cancers and therefore could be of relevance for the targeted therapy of osteosarcoma. [68-70] Monoclonal antibodies (mAbs) by themselves can show antitumor activity but the effect is often clinically modest. Because of this the antibodies have been also conjugated to drugs, toxins or radionuclides to improve the efficacy. The use of antibody-toxin conjugates has previously been suggested as a modality in osteosarcoma. [71] Nanotechnology can potentially serve as a targeted

delivery vehicle which is capable of delivering large doses of radionuclides or chemotherapeutic agents into malignant cells and reduce the side-effects.

2.5.1 Targeted drug delivery

Radiolabeled nanocarriers can be designed and used for cancer diagnostic and therapeutic purposes when labelled with appropriate radionuclides. Targeted drug delivery systems such as radiolabeled nanocarriers have been developed to improve the biodistribution, pharmacology, toxicity and therapeutic properties of agents used in cancer diagnostics and therapeutics. [72]

2.5.2 Nuclear medicine

Millions of nuclear medicine procedures using radiopharmaceuticals are performed each year in more than 10,000 hospitals worldwide. The field of the nuclear medicine is evolving towards molecular medicine which is focusing more on the cellular and molecular pathways and mechanisms of disease. The most common radionuclide used in diagnosis is Technetium-99m. [73]
Nuclear medicine plays a crucial role in the treatment of cancer by helping to determine the extent or severity of the disease and by selecting the most effective therapy based on the molecular properties of the tumor and the biological characteristics of the patient. [74]

2.5.3 Radionuclide therapy

The selection of potential radionuclides for radionuclide therapy depends on the physical half-life, decay mode and the emission properties of the radionuclides. For targeted radionuclide therapy, the use of high and low energy β emitters as radioisotopes is ideal for the treatment of all sizes of tumor cells. The tissue penetration range (1-10mm) and cross-fire effect of β particles can kill the tumor cells in close proximity. The use of α-emitters holds great promise for the treatment of small cancer lesions and small-metastatic cancer due to their high linear energy transfer and tissue penetration range of 50-100 μm. [72] Radiopharmaceuticals consist of two components: 1) Carrier and 2) Trace amount of radionuclide with a defined radiation type. [72] Biological carriers

in radiopharmaceuticals range from antibodies to various proteins, peptides and nucleic acids. Production of a radiopharmaceutical must include a stable oxidation state, well defined coordination chemistry in an aqueous environment and relatively fast complexation kinetics at physiological pH. [75] The ideal radiopharmaceutical should deliver the radionuclide to the tumor tissue precisely with no radiation reaching the healthy tissues. [72] The main characteristic of a radiopharmaceutical in oncology is a high ratio of activity accumulated per gram of tumor to the activity taken up per gram of tissue. The labelled agent should be retained in the blood for an optimal amount of time for reaching the tumors. The radiopharmaceutical must be excreted from the body rapidly to avoid accumulation in the gastrointestinal tract and kidneys. This is of importance since accumulation of the radionuclide in the gastrointestinal tract and kidneys leads to increased radiation dose to these organs. [76]

2.5.4 Radioimmunotherapy

Radioimmunotherapy (RIT) is a method of delivering cytotoxic radiation therapy in a targeted approach where an antigen-specific antibody is bound to either an alpha or beta- emitting radioisotope. [7,8] It combines the advantages of radiation therapy and specific delivery to the tumors using mAbs. The therapeutic effect on the tumor tissue is achieved by absorption of the radiation energy emitted by the radionuclides that are attached to the mAbs. The choice of radionuclide for radioimmunotherapy depends on a number of characteristics such as: 1) Radiation characteristics of the radionuclide, 2) Radiolabeling chemistry and 3) Type of malignancy or cells targeted. [8] β-emitters are more suitable for targeting solid tumors because of their high energy and long path length whereas α-emitters are more suited for targeting single cells as in micro-metastatic diseases etc. due to their high LET (Linear Energy Transfer) and short distance energy deposition (Fig.1). [74] RIT delivers cytocidal radiation to the targeted cell and is less affected by the multidrug resistance mechanisms in contrast to chemotherapy and does not depend on the immune system of a patient. It allows systemic administration, antibody-mediated specificity, and physical cytocidal damage in a manner which is well-tolerated by the patient. [20]

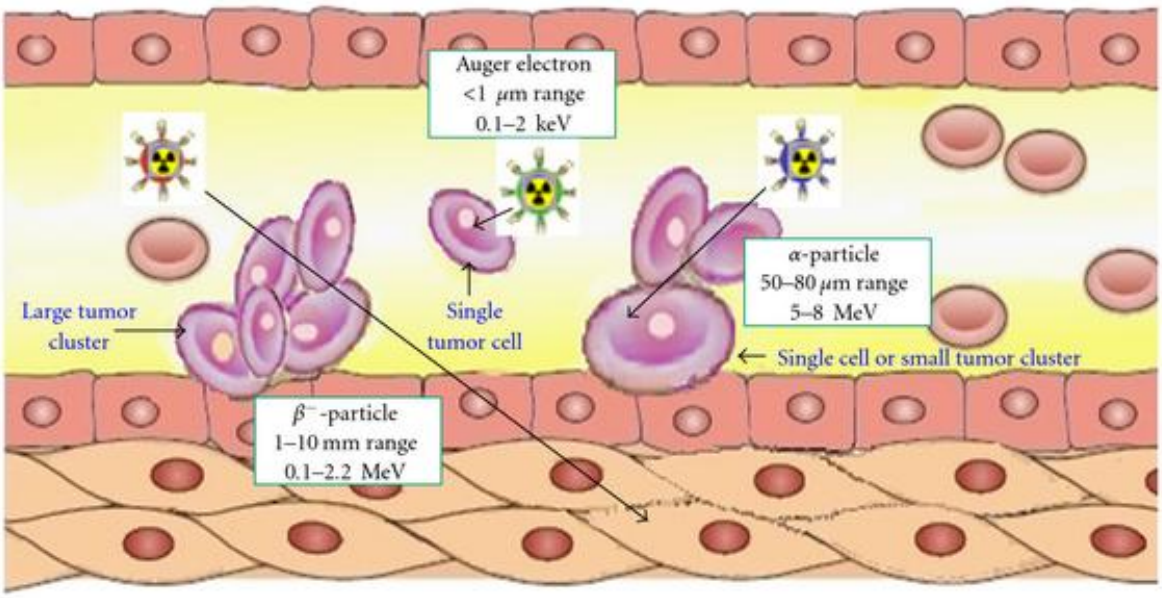


Figure 2.1 Schematic diagram of tumor tissue penetration range of internal radiotherapy. [72]

2.6 Nuclear imaging methods

The diagnostic agents enhance the visibility of specific tissues by increasing the signal to noise ratio relative to surrounding tissues and are optimized to provide a quick, high-quality picture or image of the living system. Some of the imaging modalities are: MRI, CT, US, optical imaging, single photon emission computed tomography (SPECT) and positron emission tomography (PET) (Fig. 2). [77]

2.6.1 Positron emission tomography (PET)

PET provides information about the biochemical and physiological processes in the human body. For PET applications, positron emitting radionuclides are required such as F-18, C-11, N-13 and O-15. One of the most widely used radionuclides for PET imaging is Fluorine-18 (F-18) and because of its convenient half-life of 110 min. The radiopharmaceutical FDG-18 (please give the full name here) is important for PET imaging in oncology. [78]

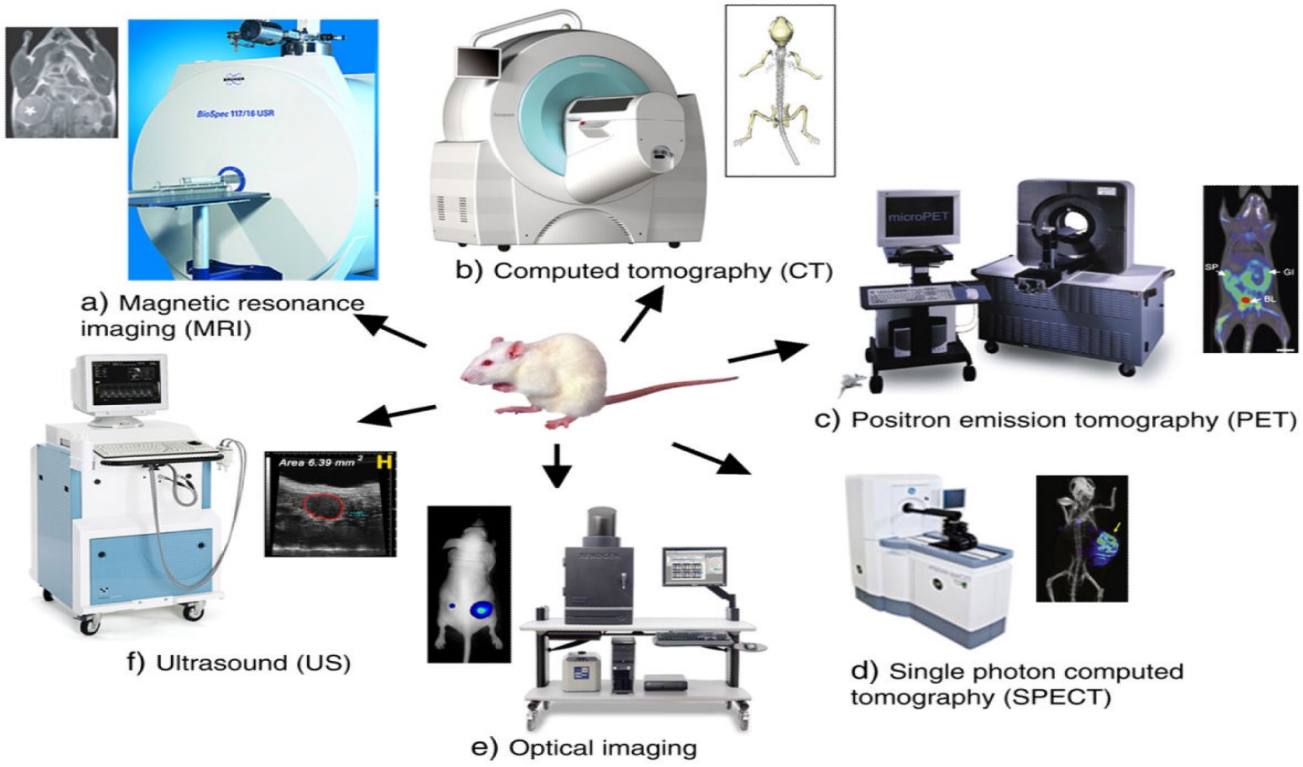


Fig 2.2 Typical molecular imaging instruments and images representative of each modality. [77]

2.6.2 Single photon emission computed tomography (SPECT)

The SPECT is a nuclear medicine tomographic technique using gamma rays wherein a SPECT scan analyzes the function of some of the internal organs. It forms a three-dimensional image of the distribution of gamma-emitting radiotracers injected into the body. An advantage of SPECT is that multiple probes when labelled with different isotopes may be studies in parallel since each is associated with its unique γ spectrum. [74] SPECT-CT combines anatomical and functional imaging and the images from each modality are merged together.

2.6.3 Internalization of antibodies into cancer cells

To develop therapeutically potent anti-cancer antibody drugs such as antibody-drug conjugates (ADCs), it is important to identify antibodies that internalize into cells efficiently and not just bind to the antigens on the cell surface. Antibody internalization should be efficient as the delivery of cytotoxic drugs into the target cells is vital for the development of ADCs [79] The efficacy of ADCs depends not only on the binding affinity and specificity to the antigen, but also on the

internalization of the antibody into the cells.[80,81] **However, for radiolabeled antibodies the internalization is NOT a vital requirement, as the beta or alpha particles travel certain distances, penetrate the cellular membrane and exert the damage on the cells. Nevertheless, internalization can contribute to the retention of the radionuclides within the tumor, which is important for longer lived radionuclides from the safety and efficacy point of view.**

3. Hypothesis

We hypothesize that Lutetium-177 labelled IGF2R–specific monoclonal antibody will help to effectively target osteosarcoma tumor cells and that Lutetium -177 will be able to deliver high tumoricidal doses to the tumours without causing toxicity to healthy tissues.

3.1 Objectives

1) To evaluate the expression of IGF2R on commercial and patient derived osteosarcoma cell lines by flow cytometry and to select the cell lines for in vivo work.

2) To perform biodistribution experiments with Indium-111 labeled IGF2R-specific monoclonal antibody in xenografted osteosarcoma tumors in SCID mice to evaluate the pharmacokinetics.

3) To perform RIT experiments with Lutetium-177 labeled IGF2R-specific monoclonal antibody in xenografted osteosarcoma tumors in SCID mice to evaluate the efficacy and safety of the approach.

4) To perform RIT experiment in murine osteosarcoma bearing immunocompetent C57Bl6 mice to assess possible synergy or antagonism between RIT and functional immune system.

4. Materials and Methods

4.1 Reagents and Antibodies

MEM/EBSS medium that was supplemented with 10% Fetal Bovine Serum, 100 U Penicillin/0.1 mg/ml Streptomycin, 1% Sodium Pyruvate solution and 1% Non-essential amino acids and Phosphatase buffered saline (1X), Trypsin 0.25% (1X) were purchased from HyClone, USA. A murine monoclonal IgG2a antibody 2G11 which binds to murine and human IGF2R and murine monoclonal IgG antibody MOPC-21 to be used as an isotype matching control were purchased from Invitrogen. MOPC-21 antibody was used as a negative control for the flow cytometry experiments, biodistribution experiment and therapy studies. MOPC-21 is produced by mineral oil induced plasmacytoma tumor cell line, according to the manufacturer, The antigen for the antibody is unknown, but MOPC-21 has demonstrated negative binding after screening on a variety of resting, activated, live, and fixed murine and human tissues, and is widely used as non-specific murine IgG control. (R)-2-Amino-3-(4-isothiocyanatophenyl)propyl]-trans-(S,S)-cyclohexane-1,2-diamine-pentaacetic acid (CHXA'') bifunctional chelating agent (BCA) used for the conjugation of the antibody was purchased from Macrocyclics (USA). ^{111}In was obtained from Nordion (Canada); ^{177}Lu was purchased from RadioMedix (USA); and ^{225}Ac/^{213}Bi generator – from Oak Ridge National Laboratory (USA). Silica gel instant thin layer chromatography (SG-iTLC) strips were obtained from Agilent (Canada). The internalization of the IGF2R specific 2G11 monoclonal antibody into the 143B cell line was determined using the IncuCyte live-cell analysis system. The IncuCyte FabFluor Red Antibody Labeling Reagent for labeling the 2G11 antibody was purchased from Sartorius.

4.2 Cell Lines

143B and SaOS2 are human osteosarcoma cell lines obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). OS-17, OS-33 and OS-31 are well characterized patient-derived osteosarcoma xenograft models maintained in our laboratories. [77] IGF2R is known to express normally across a wide array of tissue types but has shown constant over-expression in osteosarcoma cell lines. [16] These cell lines were cultured into a MEM medium that was

supplemented with 10% Fetal Bovine Serum, 100 U Penicillin/0.1 mg/ml Streptomycin, 1% Sodium Pyruvate solution and 1% Non-essential amino acids. The cells were grown under humidified conditions of 95% air and 5% CO_2 at 37°C. Once confluent, cells were washed with PBS twice, trypsinized and re-suspended in media. The cell growth was monitored using a light microscope. An automated cell counter from Bio-Rad with cell counting slide was used to count the cells at different steps of the experiments.

4.3 Measurement of IGF2R expression by Flow cytometry

Basics of flow cytometry: Prior to testing and even depending on the cells being analyzed, the sample may be treated with special dyes to further define cell sub-types. The dyes (fluorochromes) that are used are attached to monoclonal antibodies that bind to particular cells or key components of cells. In the instrument, the fluid in which the cells are suspended pass through very narrow channels so that the cells are organized in a single line as they pass the detectors. This is accomplished at a high rate (hundreds to thousands of cells per second.) The flow cytometer contains one or more lasers and a series of photodetectors that are able to identify certain characteristics unique to various cell types. The single cell suspension creates unique light shattering events that occur when each cell passes through the laser light. These initial events are characteristic of the size and shape of the cell, as well as the intensity of the signal that is generated by the specific dyes, thus creating patterns that reflect cell type. The signals from the detectors are then amplified and sent to a computer wherein they are converted to digital read-outs displayed on a computer screen or in the form of a printout. [78] The Cytoflex flow cytometer instrument used has a computer software associated with it. The computer program controls the flow cytometer instrument during data acquisition where the parameters for measurement are selected. The acquisition conditions for the parameter measurement are selected based on the stopping rules such as the volume of the sample liquid aspirated, flow rate of the sample and number of events to be displayed for the cell populations which are entered into the software. Additionally, area, width or height on different parameters are selected as well. The data is generally presented in the form of graphs and histogram plots. Flow cytometry data analysis is based upon the principle of gating wherein the gates and regions are placed around populations of cells with common characteristics such as forward scatter, side scatter and receptor or marker expression to quantify and investigate the populations (cells) of interest.

Flow cytometry of osteosarcoma cell lines: This experiment was performed to assess the binding efficiency of the IGF2R specific monoclonal antibody 2G11 to the osteosarcoma cells lines with the purpose of selecting the cell lines with the lowest and highest IGF2R expression for the follow-up biodistribution studies. The osteosarcoma cell lines such as 143B, SaOS2, OS-17, OS-33 AND OS-31 were harvested, and cell numbers were determined by utilizing the cell counter followed by suspension in FACS buffer (100 µl). The cell suspension was distributed in a U-bottom 96 well plate with $5 * 10^5$ cells per well. Primary antibodies such as the IGF2R specific 2G11 and isotype matching control (negative control) MOPC-21 were added in a concentration of 1 µg/µl and cells were incubated on ice for 20 mins before 2 washes with 1 ml of FACS buffer. Secondary antibody anti-mouse IgG2a (APC) was added in a concentration of 1 µg/µl with 100 µl of FACS buffer to the cells and incubated for 20 mins and washed as in the previous step. The wash steps were performed by centrifugation at 500 g for 5 mins at 4°C. The cell pellets were washed, taken up in FACS buffer and analyzed in a Cytoflex flow cytometer instrument. This flow cytometry analysis was replicated three times for all the cell lines. The data was analyzed by the Flowjo software.

Statistical Analysis: The statistics can be either be descriptive or inferential. The descriptive statistics allow us to describe our flow cytometry data and to test the hypothesis to check whether there is binding between the receptor IGF2R expressed by the cells and the IGF2R specific monoclonal antibody 2G11 and to see if there is an increase or decrease in the particular population of cells. 1.) Percentage of positive cells: If the cells that express the particular receptor or marker of interest are positive cells. Thus, we can say that those percentage of cells have positive and effective binding of the IGF2R specific monoclonal antibody 2G11 to the osteosarcoma cells. 2.) Measurement of median or mean of the distribution: These parameters are useful when comparing distribution of cells that overlap since we need to compare the entire population of cells and not just a part of it.

4.4 Animal model

The animal work was approved by the University of Saskatchewan's Animal Research Ethics Board (AUP 20170006) and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

Cell lines were grown in Eagle's Minimum Essential medium and supplemented with 10% FBS, Sodium pyruvate, Non-essential Amino acids and Pen/strep. The cells were grown in a humidified condition of 95% air and 5% CO_2 at 37°C. Once confluent, cells were washed PBS twice, trypsinized and resuspended in media. Approximately 3 million cells in 100 µL of Eagle's Minimum Essential medium were implanted per animal.

1)Tumor bearing mouse model: Six to eight weeks old female SCID (CB17/Icr-*Prkdc*^{scid}/IcrIcoCrl) mice obtained from Charles River Laboratories (USA) were anesthetized with isoflurane and injected subcutaneously with three million of either 143B or OS-17 cells into the right flanks.

2)Tumor monitoring: After 143B and OS-17 cells were implanted into the mice, tumors were allowed to grow until palpable and measuring approximately 5 mm in diameter. Tumor volume was calculated every 3 days until the desired volume of the tumor (50-100 mm^3) was achieved. Electronic calipers were utilized to measure the length and width of the tumor. Volume was calculated utilizing the formula (V= L*W^2/2). It was noticed that for the mice injected with the 143B cell line around 80% of the mice developed palpable tumors by day 12 and for the OS-17 cell line by day 5.

4.5 Conjugation of Bifunctional Chelating agent CHXA'' to mAB's

For radiolabeling with ^{111}In, the antibodies were first conjugated to a bifunctional chelator, CHXA". The bifunctional chelator CHXA" was added to the antibody at 5 molar equivalents excess of 2 mg/ml solution. Five mL of 10X conjugation buffer (0.05 M Carbonate/Bicarbonate, 0.15 M NaCl, 5 mM EDTA, pH 8.6 - 8.7) was combined with 0.5 M EDTA, pH = 8.0 (0.5 mL) and was diluted to 50 mL in a 50 mL Falcon tube with deionized water to give the 1X buffer. An Amicon Ultra 0.5 mL centrifugal filter (30K MW cut off, Fisher) was loaded with 2 mg of either 2G11 or MOPC-21 antibody. The antibody was exchanged into the above conjugation buffer by

performing 6 x 1.5 mL washes using an Amicon concentrator in a refrigerated centrifuge at 4°C. A solution of bifunctional CHXA" ligand with 2 mg/mL concentration was prepared by dissolving CHXA" in conjugation buffer. The antibody was recovered from the Amicon and 23.6 µL of 2 mg/mL CHXA" solution in conjugation buffer is added to provide 5-fold molar equivalents excess of CHXA" over the antibody. The reaction mixture was incubated at 37°C for 1.5 hrs. The reaction mixtures were then purified into 0.15 M ammonium acetate buffer, pH=6.5-7.0, with 6 x 1.5 mL washes on Amicon concentrators in a refrigerated centrifuge at 4°C. The sample were stored at 4°C. A Bradford assay was performed to determine protein recovery and concentration.

4.6 Radiolabeling of antibodies

The IGF2R-specific mAb 2G11 and the isotype matching control mAb MOPC21 were then labelled with the radioisotope ^{111}In to yield ^{111}In-2G11 and ^{111}In-MOPC-21, and with ^{177}Lu to yield ^{177}Lu-2G11 and ^{177}Lu-MOPC-21, or with ^{213}Bi to yield ^{213}Bi-2G11. The radiolabeling of an antibody-CHXA" conjugate with ^{111}In was performed to achieve the specific activity of approximately 5 µCi/µg of the antibody. For example, 600 µCi of ^{111}In chloride was added to 10 µL 0.15 M ammonium acetate buffer and added to a microcentrifuge tube containing 120 µg 2G11-CHXA" conjugate in 0.15 M ammonium acetate buffer. The reaction mixture was incubated for 60 min at 37°C, and then the reaction was quenched by the addition of 3 µL 0.05 M EDTA solution. The percentage of radiolabeling was measured by SG-iTLC using 0.15 M ammonium acetate buffer as the eluent. SG-iTLCs were cut in half and read on a Perkin Elmer 2470 Automatic Gamma Counter (top containing unlabeled ^{111}In, bottom containing antibody conjugated ^{111}In). ^{177}Lu labeling was performed with an identical protocol using ^{177}Lu as the radioisotope and labeling with ^{213}Bi was carried out as in (79). Both ^{111}In and ^{177}Lu incorporation was >95%. The antibody was labelled with ^{111}In radionuclide for the biodistribution study since it has a physical half-life of 2.8 days and is widely used as a radiotracer for various diagnostic applications.

4.7 Biodistribution Study

The biodistribution was performed to assess whether IGF2R- specific mAb 2G11 will localize in the tumor preferentially when compared to non-specific isotypes matching control MOPC-21. As murine and human IGF2R are not similar, the mAb 2G11 was used since it can bind to both human as well as murine IGF2R. The IGF2R-specific mAb 2G11 and isotype matching control mAb MOPC-21 were radiolabeled with ^{111}In as above. Mice were separated into two different groups according to the cell lines i.e. 143B and OS-17. Tumors were allowed to grow until they reached an average size of 70-100 mm^3. The volume was calculated assuming elliptical geometry and using the following formula: $V = (LxW^2)/2$. The mice in each group were then randomized and administered with 20 μCi each via an intraperitoneal injection, with 18 mice (8 mice implanted with OS-17 cell line and 10 mice implanted with 143B cell line) receiving ^{111}In- 2G11 and 16 mice (8 mice implanted with OS-17 cell line and 8 mice implanted with 143B cell line) receiving ^{111}In-MOPC-21. At 24 and 48 hr. post injection mice were sacrificed from each group: 143B tumor bearing mice injected with ^{111}In- 2G11 or MOPC21, and OS17 tumor bearing mice injected with ^{111}In- 2G11 or MOPC21. Once sacrificed, the tumor, blood, heart, lungs, pancreas, spleen, kidney, liver, brain, a section of the small and large intestines, thigh muscle, and femur were collected, weighed, and counted in a gamma counter (Perkin Elmer). The percent of injected dose per gram (%ID/g) for each sample was calculated to determine the biodistribution.

4.8 MicroSPECT/CT IMAGING (micro single photon emission computed tomography/computer tomography)

The images were collected on a MILabs VECTor^4 (Netherlands) microSPECT/CT scanner and processed using the comprehensive image analysis software package PMOD (version 3.9, PMOD Technologies, Inc, Switzerland). Imaging studies were conducted using 200 μCi ^{111}In at a 5:1 mCi/mg specific activity with a CHXA'' conjugated 2G11. Tumor-bearing mice were administered ^{111}In- 2G11 via IP injection and imaged in the prone position at 2, 24, and 48 hours post injection. SPECT data was collected for 20 minutes using an Extra Ultra High Sensitivity Mouse (XUHS-M) collimator for 20-350 keV range using spiral trajectories. All SPECT images were reconstructed using both 245 keV and 171 keV ^{111}In gamma emissions on a 0.4 mm voxel grid with MILabs reconstruction software.

4.9 Radioimmunotherapy Study

OS17 and 143B Tumor bearing mice were monitored until the tumor size reached 70-100 mm^3. The mice were randomized into groups of 5 mice per group. Group 1 received 80 μCi ^{177}Lu-2G11, group 2 - 80 μCi ^{177}Lu-MOPC-21, group 3 - unlabeled (cold) 2G11, and group 4 was left untreated. In addition, a group of mice with 143B tumors was given 80 μCi ^{213}Bi-2G11. Tumors were monitored as previously described and average tumor volume was calculated for the mice in each group over a 12-day period. At the completion of the observation period the mice were humanely sacrificed, their spleens removed and analyzed histologically for signs of radiation damage. Their blood was analyzed for AST, ALT, BUN and creatinine as biomarkers of possible liver and kidney toxicity.

4.9.1 Internalization Assay

The internalization of the IGF2R specific 2G11 monoclonal antibody into the 143B cell line was determined using the IncuCyte Antibody Internalization Assay provided by Essen Biosciences (Sartorius). The cells of the 143B cell line were typically seeded at 50 μL/well, with 5,000-30,000 cells/well into a 96-well plate and were left to adhere for between 2-24 hours. The 2G11 antibody was then mixed with the IncuCyte FabFluor Red Antibody Labeling Reagent purchased from Sartorius for labeling the antibody with the regent. They were mixed at a molar ratio of 1:3 in DMEM media with a 2x final assay concentration. The mixture was incubated for 15 mins to allow conjugation. This IncuCyte FabFluor-labeled Antibody was then added to the cell plate at 50 μL/well and the images were captured every 15-30 mins in the IncuCyte live-cell analysis system. These images were analyzed using the same integrated software. Subsequently the graphs representing internalization of the IGF2R specific 2G11 monoclonal antibody into the 143B cells was obtained with the GraphPad prism statistical software. To enable the quantitative measurement of labeled antibodies, assays based on the internalized fluorescence pH sensitive fluorescent dyes have been developed. Those dyes are used for labeling the antibodies specific for the targeted receptor. This pH sensitive dye can be helpful in monitoring the internalization quantitatively over time. When internalization is measured by a single step labeling protocol, the Fc-targeted Fab coupled pH-sensitive dye (IncuCyte FabFluor) is used for tagging the antibody of interest. These pH sensitive dyes are non-fluorescent at neutral pH and exhibit increasing

fluorescence as the pH become more acidic in the endocytic compartment. However, the relatively small increase in fluorescence over the cellular pH range in question limits their applicability. [82,83] The assay developed by Sartorius which we utilized in this work, measures internalization with high specificity, sensitivity and morphological information. However, there are certain limitations of this approach, such as high background fluorescence, quenching of the fluorescent dyes over time, etc. which often limit the use of this technique to the qualitative only evaluation of internalization.

4.9.2 Statistical Analysis

The differences between the biodistribution groups were analyzed using Kruskal-Wallis and/or Mann-Whitney tests. Differences between the treatment groups were similarly analyzed using the Kruskal-Wallis and/or Mann Whitney tests.

5.0 Results

5.1 IGF2R Expression in human Osteosarcoma cell lines

2G11 mAb specifically binds to IGF2R-expressing cell lines and preserves its immunoreactivity post-conjugation of the bifunctional chelating agent. We first sought to establish if 2G11 mAb to human and murine IGF2R would specifically bind to OS across multiple cell lines. We utilized flow cytometry technique to analyze the expression of IGF2R across two standard cell lines from ATCC such as 143B and SaOS2 and three patient-derived cell lines such as OS17, OS31 and OS33. The 2G11 mAb bound specifically to all 5 cell lines when compared with the isotype matching control mAb MOPC21 (Fig 3 a-e.). The expression of IGF2R by ATCC cells lines SaOS2 and 143B was 1.5-2 times higher than that of the patient-derived osteosarcoma cell lines. Of the patient-derived cell lines, OS17 had the lowest expression of IGF2R (Fig. 3e) and OS33 exhibited the highest (Fig. 3c).

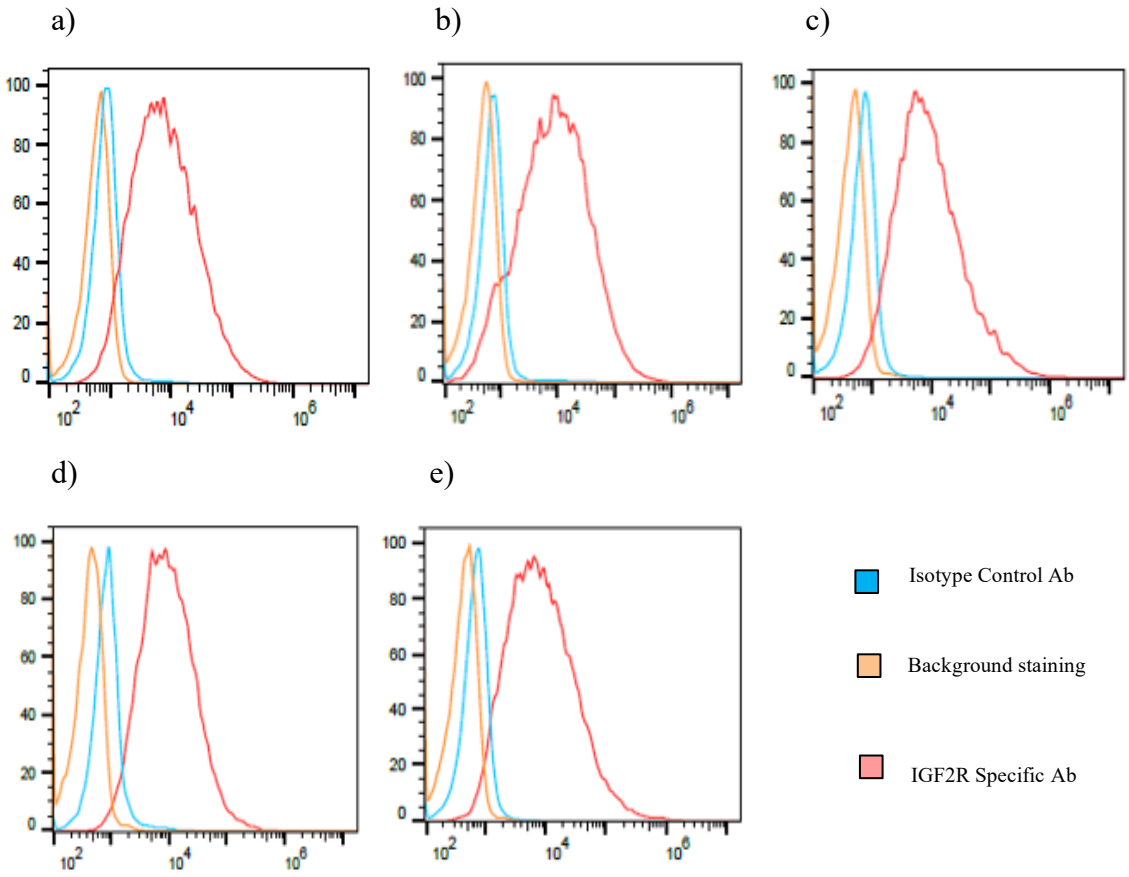


Fig 5.1 Flow cytometry detection of IGF2R expression on 5.1.1) 143B, 5.1.2) SaOS2, 5.1.3) OS-33, 5.1.4) OS-31 and 5.1.5) OS-17 cell lines.

On comparing the binding results between the cell lines, we selected 143B and OS-17 as the cell lines with highest and lowest IGF2R expression respectively. We further used these cell lines for the biodistribution and RIT experiments.

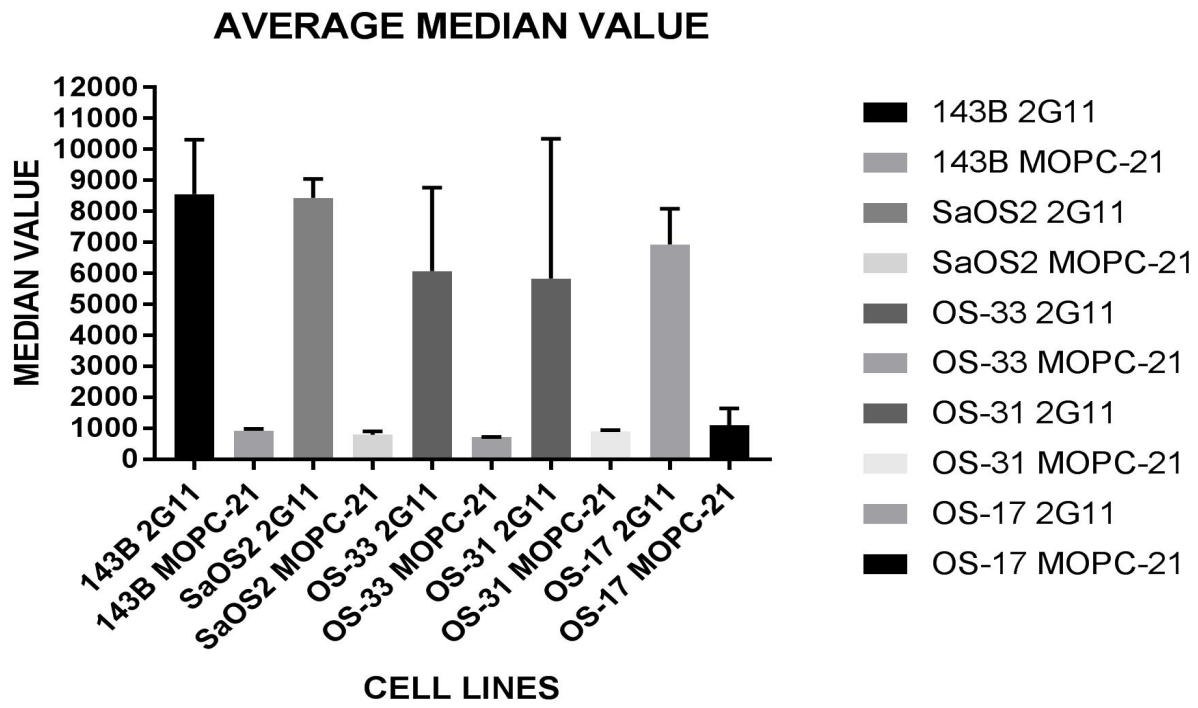
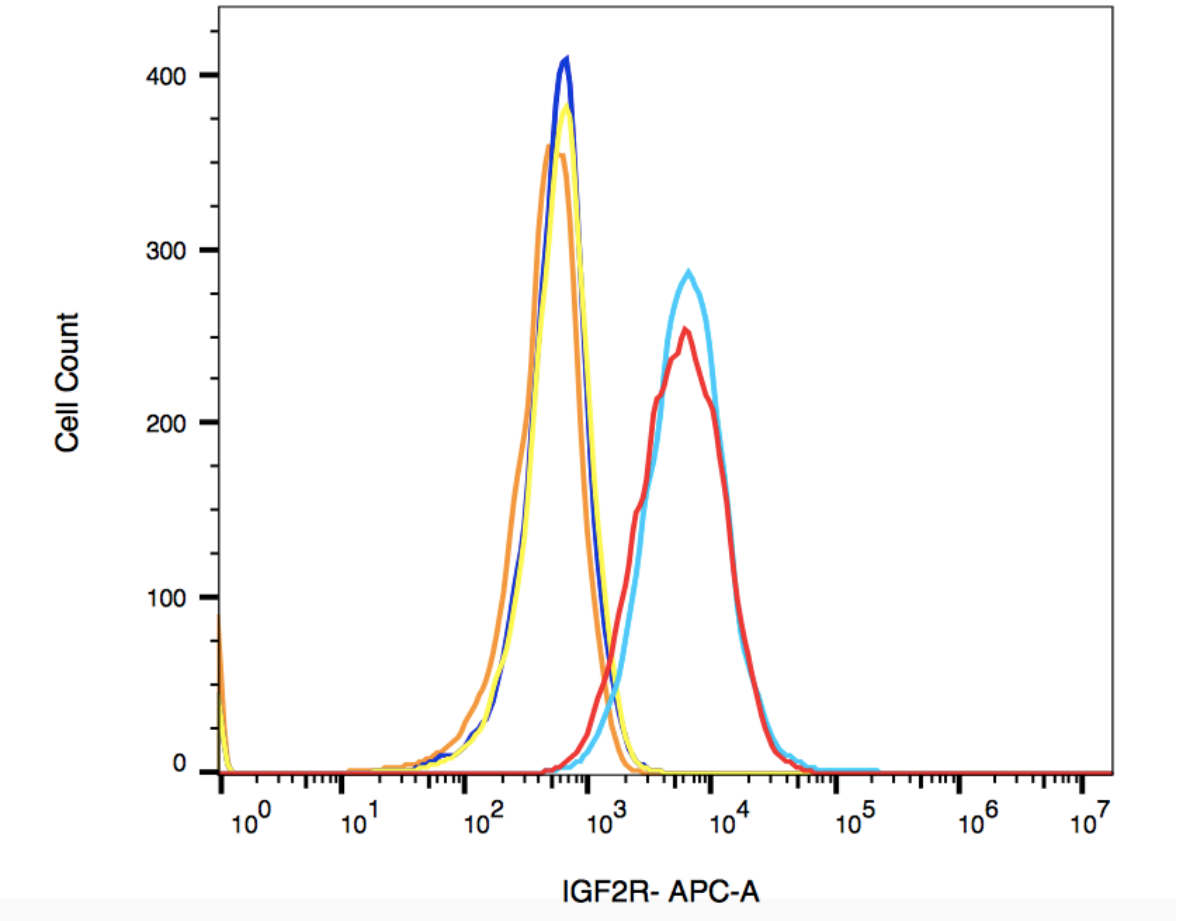


Fig 5.2 Average median value calculated for each cell line on the basis of the values calculated from the flow cytometry experiment.

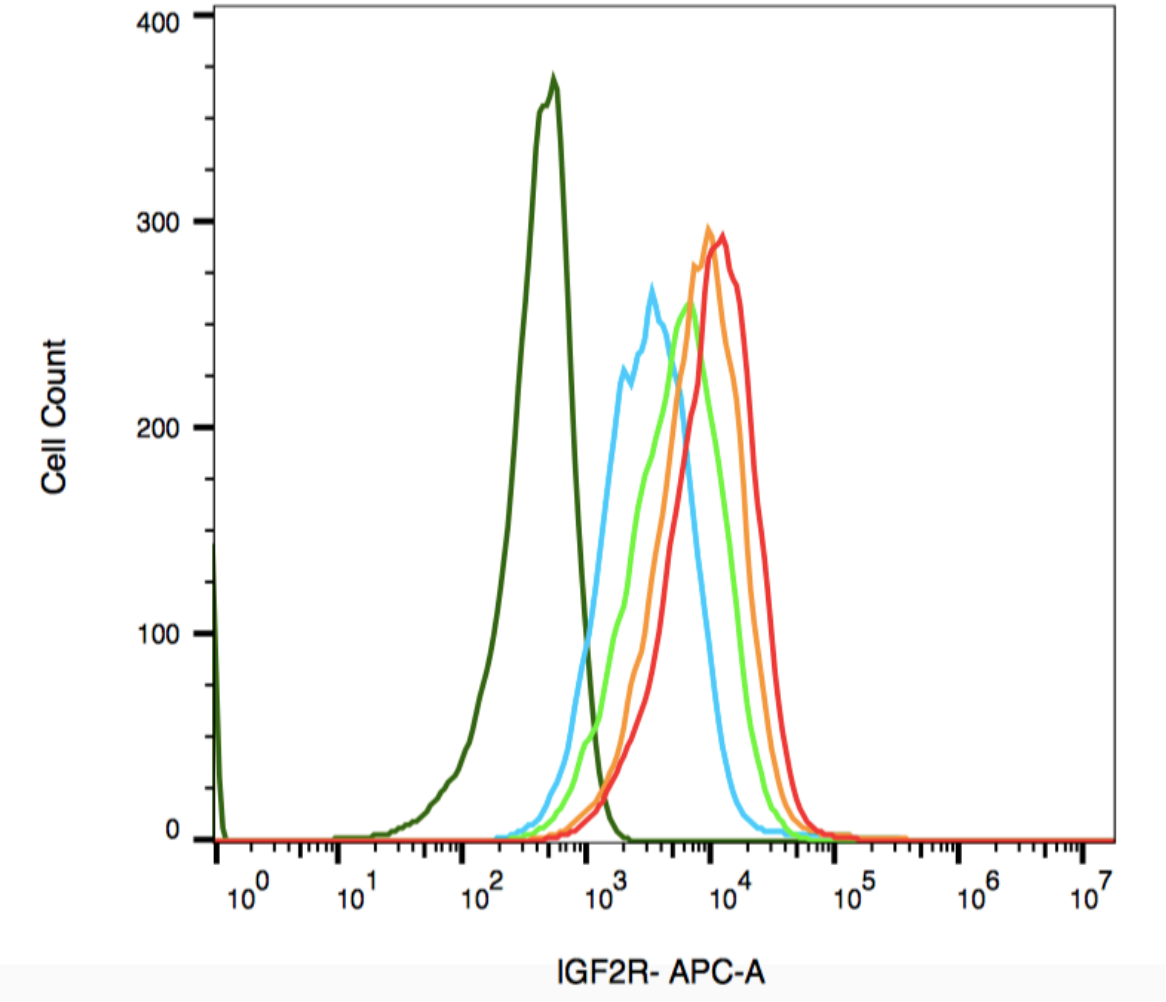
5.2 Binding of conjugated and unconjugated 2G11 antibody to OS-17 and 143B cells

To enable the radiolabeling of 2G11 monoclonal antibody with ^{111}In (^{111}Indium) for biodistribution and imaging study, 2G11 was conjugated to the bifunctional chelating agent CHXA". The flow cytometry experiment was carried out to demonstrate that after conjugating 2G11 with CHXA" it was still able to bind to 143B and OS-17 cells to the same extent as the naked 2G11 monoclonal antibody.



	Sample ID	Count
	OS-17 2G11 CHXA	8024
	OS-17 2G11	8077
	OS-17 MOPC-21 CHXA	8067
	OS-17 MOPC-21	8223
	OS-17	8214

Fig 5.3 Binding of conjugated and unconjugated antibody 2G11 mAb to OS-17 cells.



	Sample ID	Count
	143B 2G11 CHXA	8581
	143B 2G11	8544
	143B MOPC-21 CHXA	8473
	143B MOPC-21	8497
	143B	8439

Fig 5.4 Binding of conjugated and unconjugated antibody 2G11 mAb to 143B cells.

5.3 Biodistribution of ^{111}In – labeled monoclonal antibodies in 143B and OS-17 osteosarcoma xenografts

To find out if 2G11 mAb is capable of selectively targeting IGF2R-expressing osteosarcoma tumors, this mAb and the isotype matching murine mAb MOPC21 were radiolabeled with the imaging radionuclide 111In and administered by intraperitoneal injection to 143B and OS17 tumor-bearing mice. The mice were sacrificed at 24 and 48 hours after the administration of the radiolabeled mAbs and the biodistribution was performed. The results are shown in Fig 7a and 7b. The uptake of 2G11 mAb in the tumors at 24 and 48 hours was significantly higher than that of MOPC-21 for both the types of tumors. Some uptake of MOPC-21 control mAb in the tumors can be explained by the enhanced permeability and retention (EPR) effect by which molecules of certain sizes (typically liposomes, nanoparticles, and macromolecular drugs) tend to accumulate in tumor tissue much more than they do in normal tissues. The uptake of IGF2R specific 2G11 mAb into the tumors was relatively similar for both cell lines with the uptake in the tumor being slightly but not significantly for the OS-17 cell line tumors. There was a very high uptake of the IGF2R specific 2G11 mAb in the spleens of mice with both tumor types in comparison to the isotype control MOPC-21.

48 hr

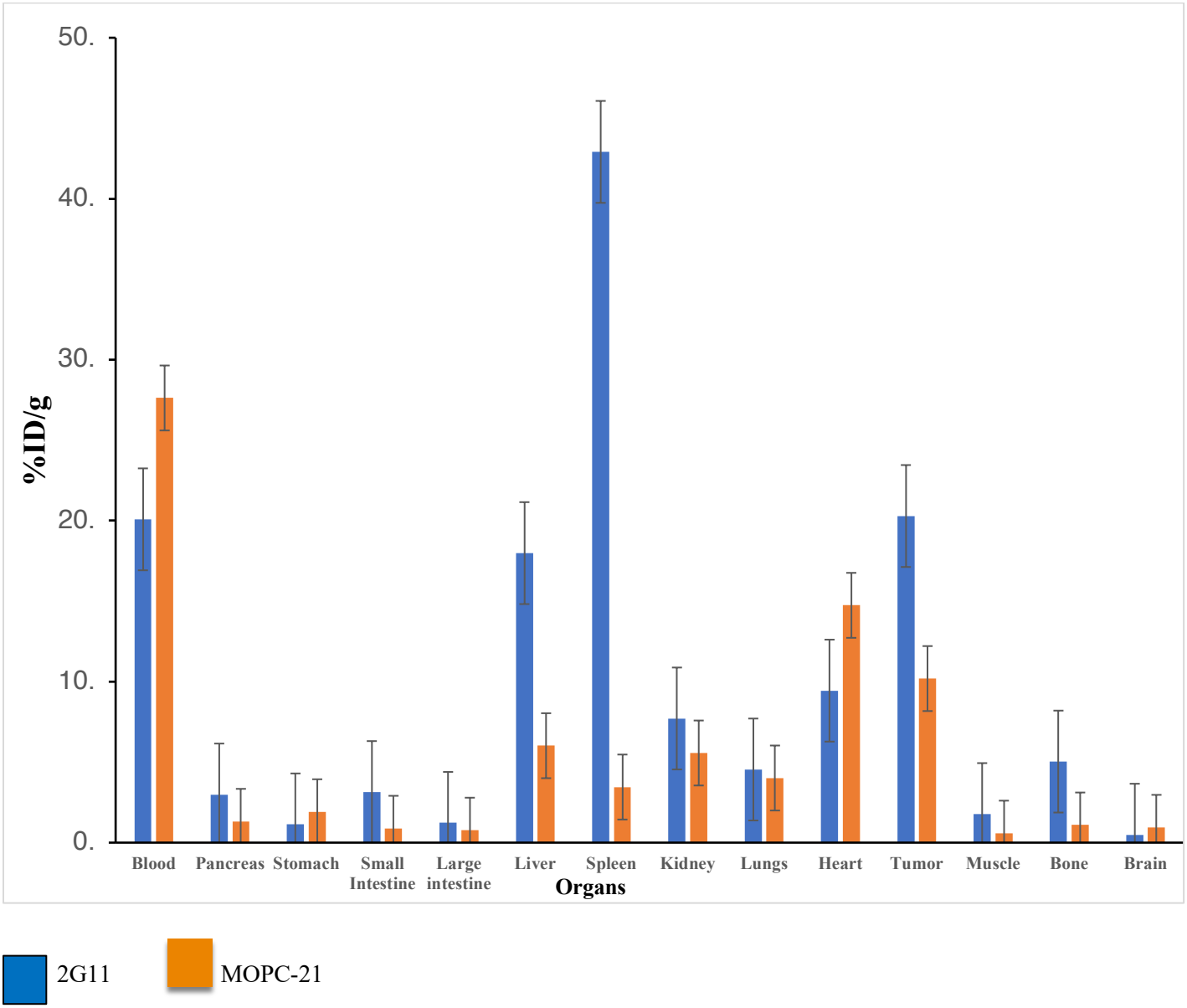


Fig 5.5 Biodistribution of ^{111}In – labeled monoclonal antibodies in OS-17 xenografts at 48 hours post antibody administration.

24 hr

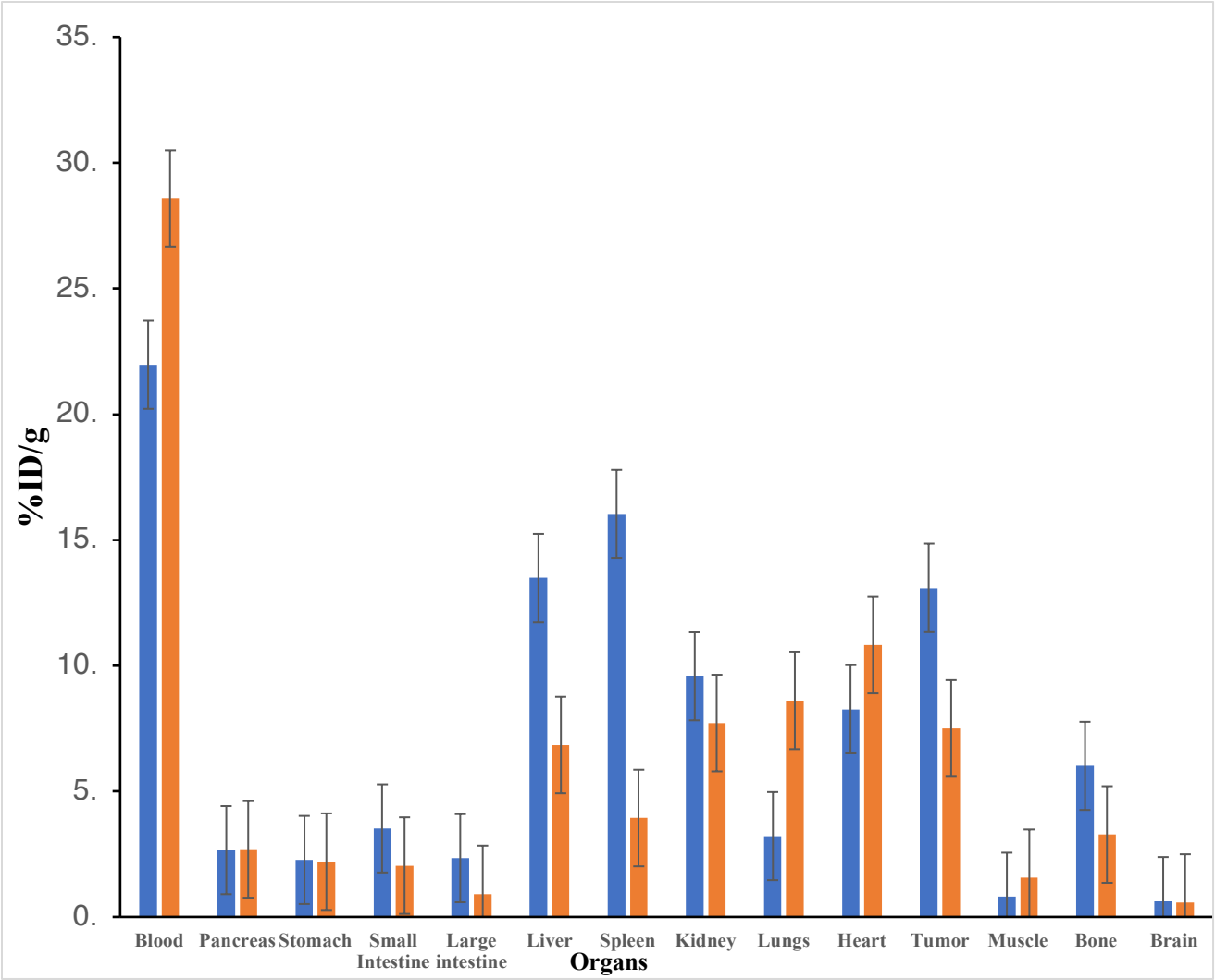


Fig 5.6 Biodistribution of ^{111}In – labeled monoclonal antibodies in OS-17 xenografts at 24 hours post antibody administration.

48 hr

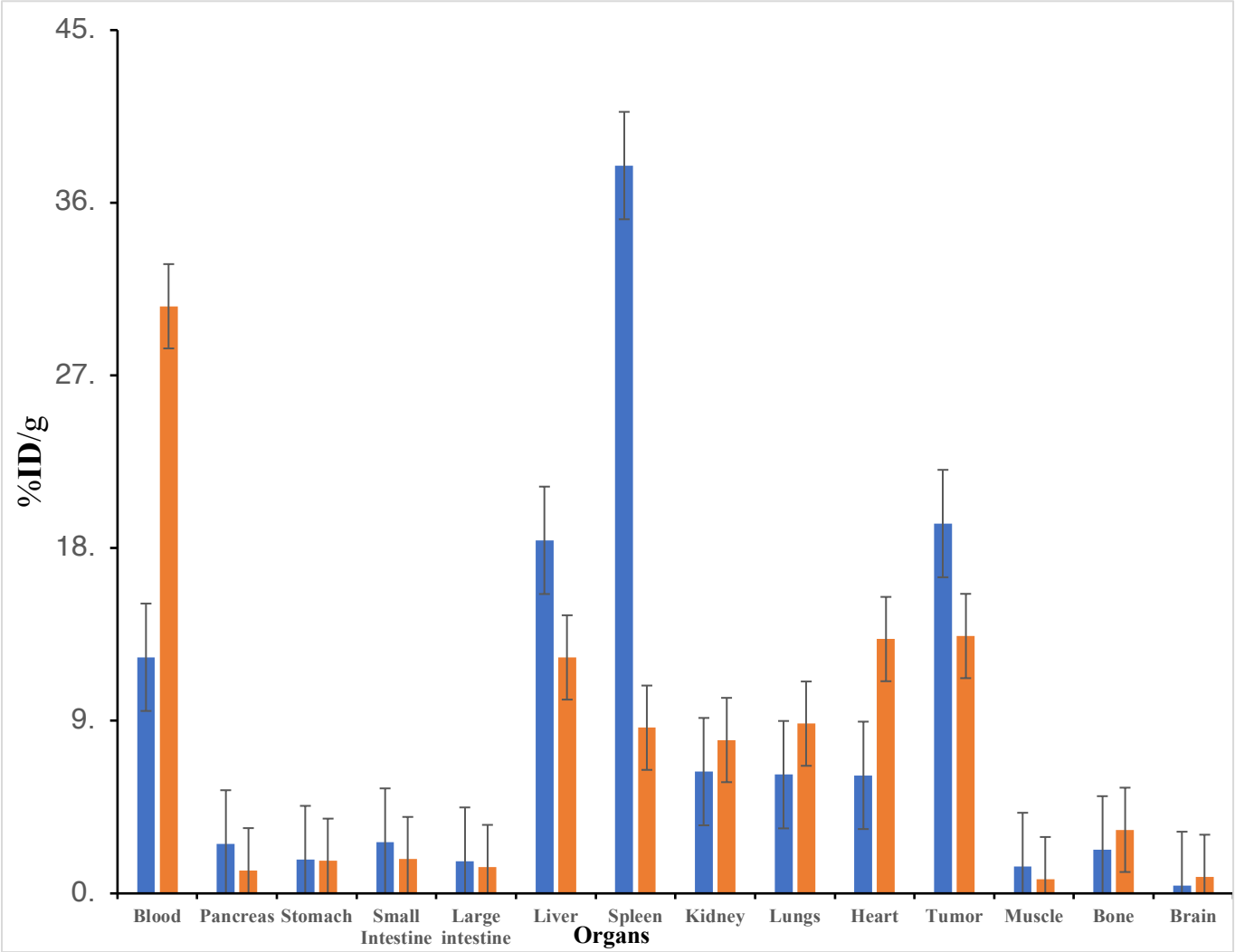


Fig 5.7 Biodistribution of ^{111}In – labeled monoclonal antibodies in 143B xenografts at 48 hours post antibody administration.

24 hr

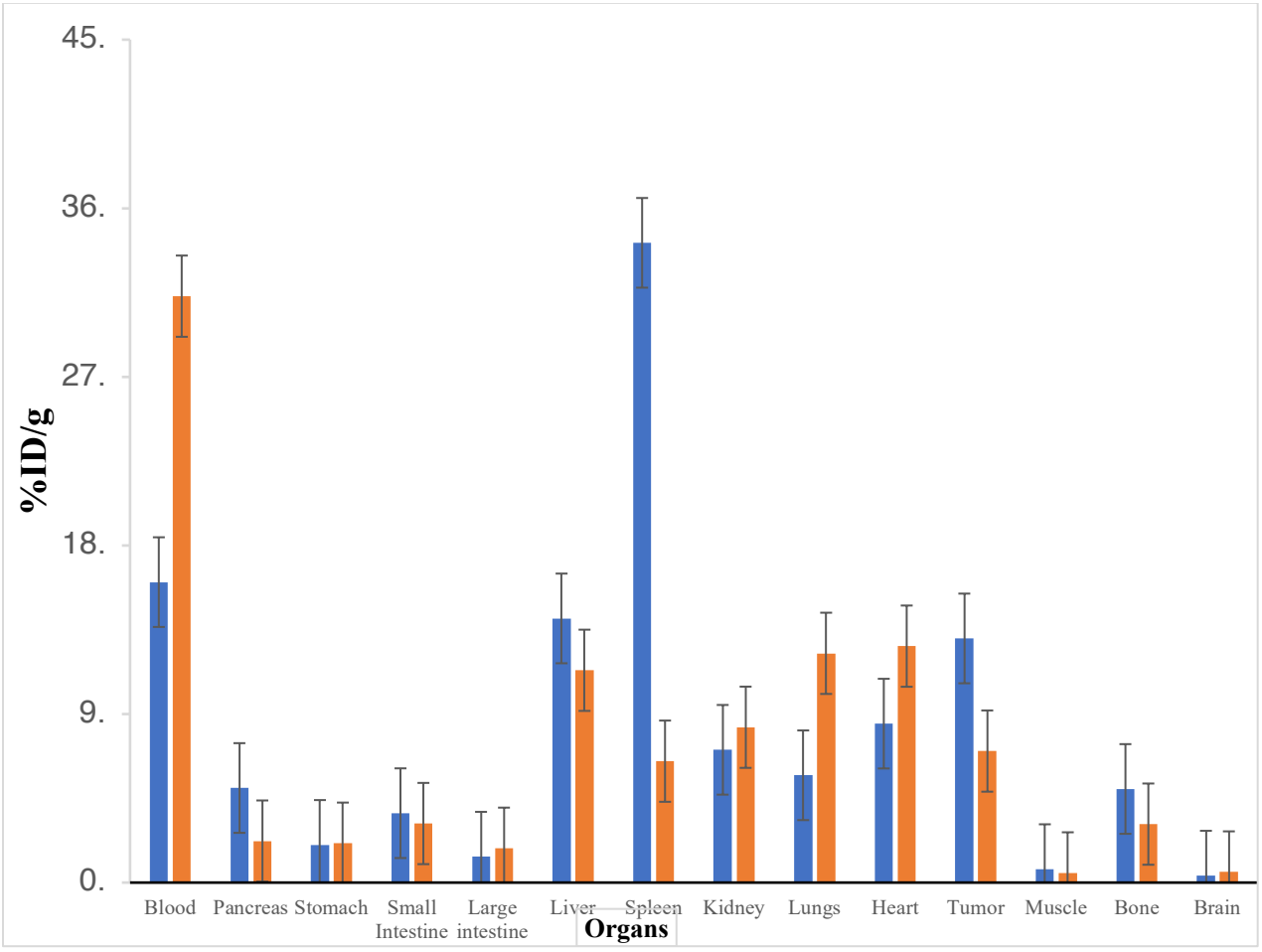
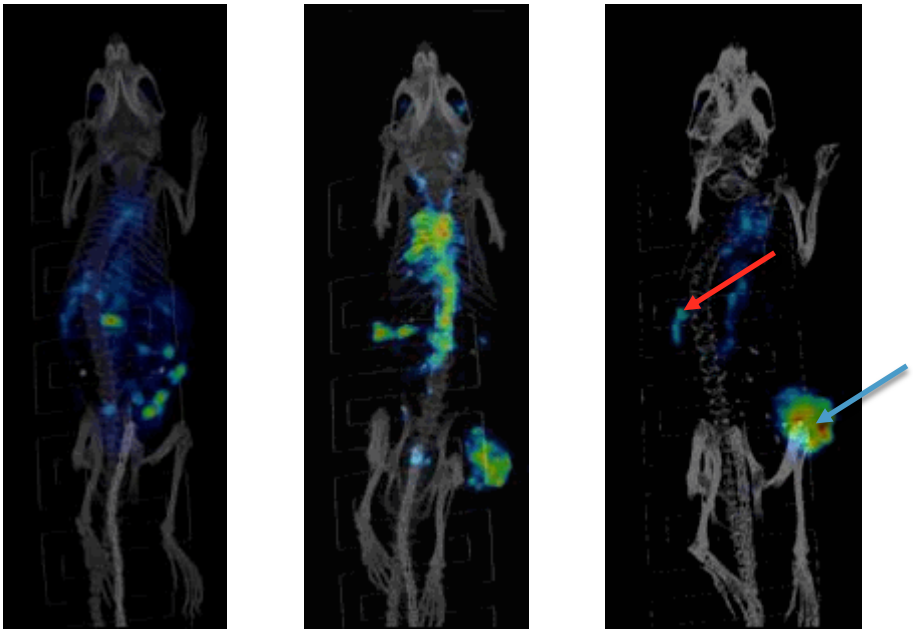


Fig5.8 Biodistribution of ^{111}In – labeled monoclonal antibodies in 143B xenografts at 24 hours post antibody administration.

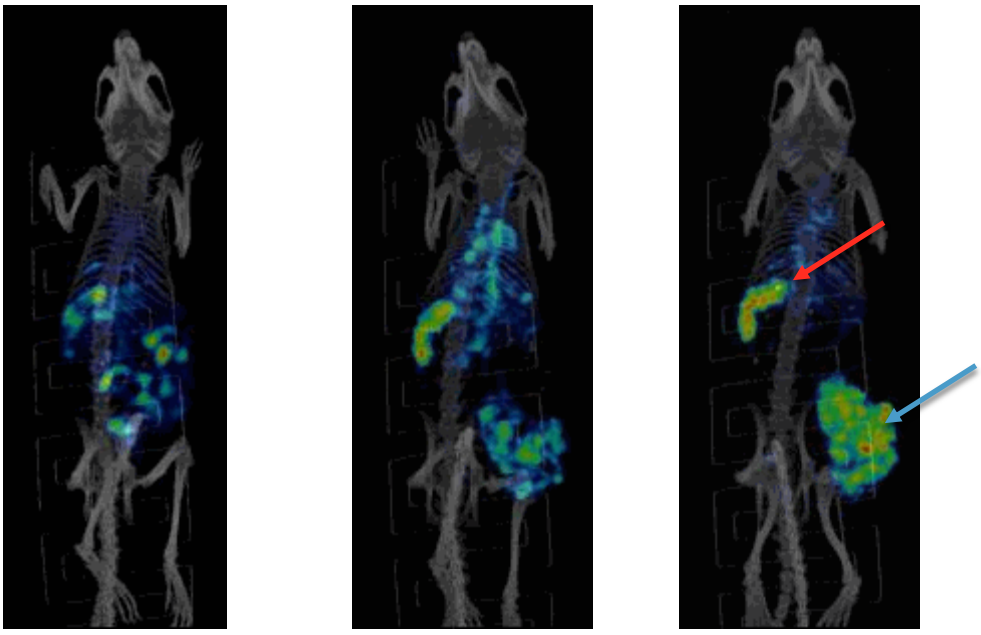
5.4 microSPECT/CT imaging of ^{111}In–labeled monoclonal antibodies in 143B and OS-17 osteosarcoma xenografts

The microSPECT imaging of OS17 tumor-bearing mice is shown in Fig. 8a and 143B tumor-bearing mice is shown in Fig. 8b. The imaging was done at 2, 24 and 48 hours post administration of the radiolabeled 2G11 mAb. At 2 hours post administration, the mAb was still circulating in the blood and through the organs with the tumors practically not visible, at 24 hours the tumors were clearly visible, and the spleens were visible as well. By 48 hours, the mAb cleared almost completely from the circulation with only the tumors and the spleens retaining high amounts of the 2G11 mAb. To find out if this high uptake of 2G11 mAb in the spleen was due to the possible metastatic spread of tumor cells into the spleen, we performed microSPECT/CT imaging of the healthy SCID mice with radiolabeled 2G11 mAb at 2 and 24 hours and 4 days post mAb administration (Fig. 9). High spleen uptake was seen in the images starting at 24 hours, confirming that the 2G11 mAb was binding to the IGF2R expressed by the spleen cells of SCID mice.



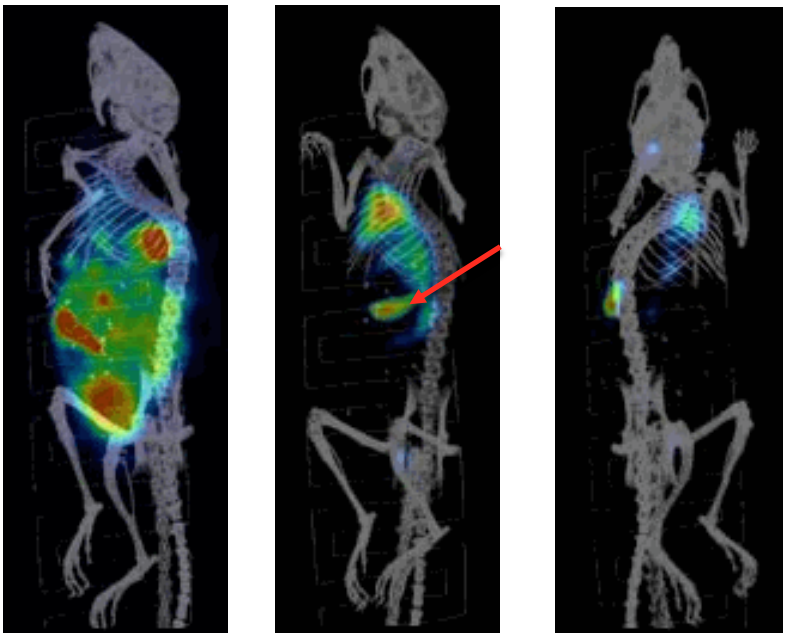
2 HOURS 24 HOURS 48 HOURS

Fig 5.9 microSPECT imaging of OS17 tumor-bearing SCID mice labelled with ^{111}Indium-CHXA" 2G11. The red arrow indicates the spleen and the blue arrow indicates the tumor in the image of the mouse post 48 hours.



2 HOURS 24 HOURS 48 HOURS

Fig 5.10 microSPECT imaging of 143B tumor-bearing SCID mice labelled with ^{111}Indium-CHXA" 2G11. The red arrow indicates the spleen and the blue arrow indicates the tumor in the image of the mouse post 48 hours.

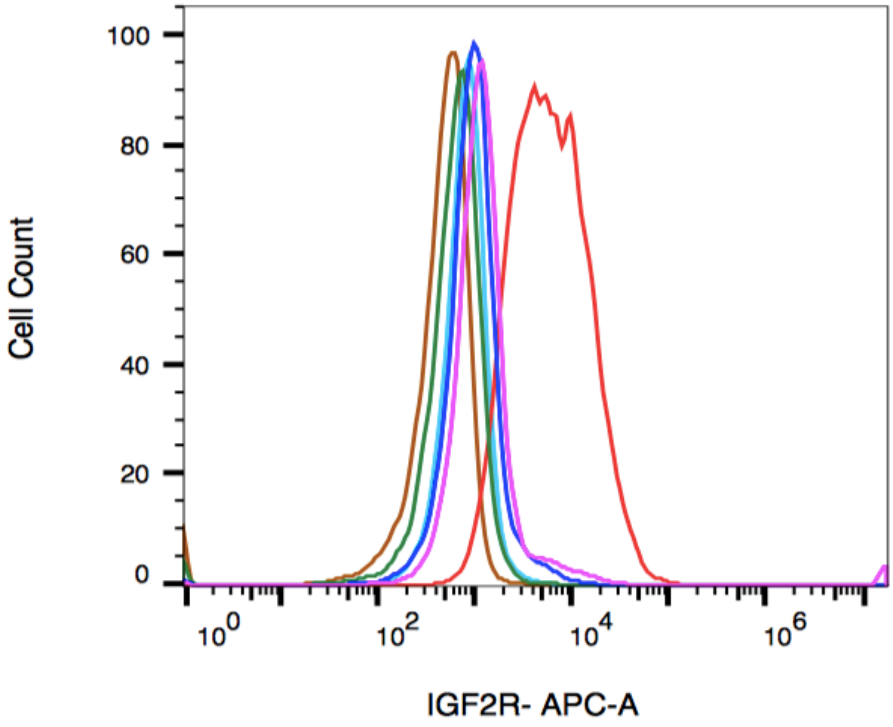


2 HOURS 24 HOURS 4 DAYS

Fig 5.11 microSPECT imaging of healthy SCID mice with radiolabeled 2G11 mAb. The arrow pointing to the organ in the image post 24 hours is the spleen of the healthy SCID mouse.

5.5 IGF2R expression in murine osteosarcoma cell line

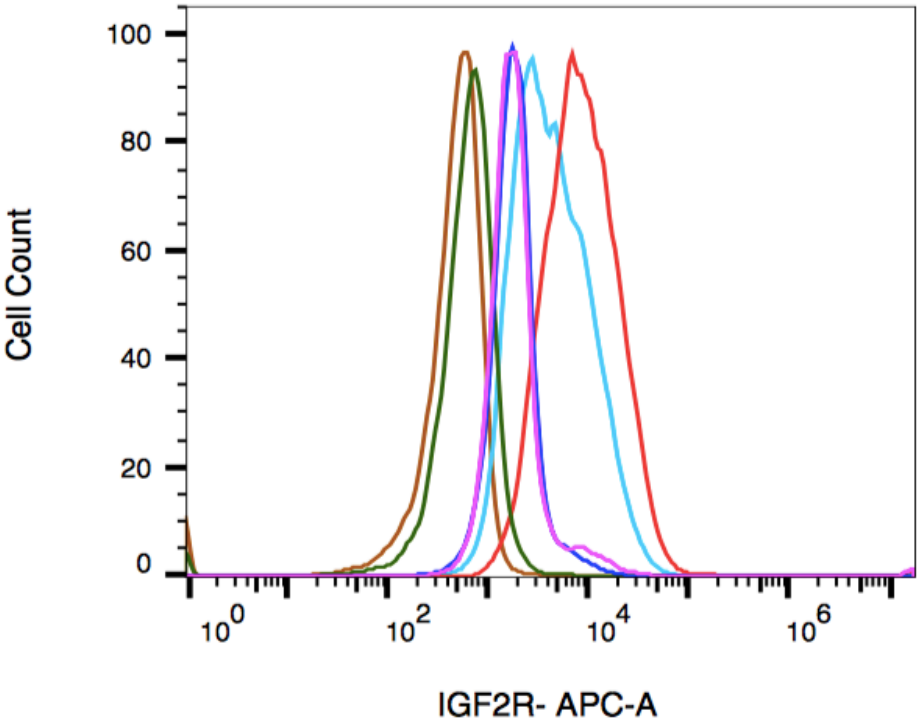
We set out to establish if 2G11 mAb specific for human and murine IGF2R would specifically bind to the murine osteosarcoma cell line K7M2. For this purpose, we utilized flow cytometry technique to analyze the expression of IGF2R on K7M2 cells when compared to 143B cell line as a positive control. On analyzing the results, the K7M2 cell line showed no IGF2R expression.



	Sample ID	Count
	K7M2 2G11 1μg/μl	7340
	K7M2 MOPC-21 1μg/μl	7175
	K7M2	7150
	143B 2G11 1μg/μl	8553

	143B MOPC-21 1µg/µl	8517
	143B	8134

Fig 5.12 IGF2R expression by K7M2 cell line at 1µg/µl concentration



	Sample ID	Count
	K7M2 2G11 5µg/µl	7478
	K7M2 MOPC-21 5µg/µl	7147
	K7M2	7150
	143B 2G115µg/µl	8594
	143B MOPC-21 5µg/µl	8523
	143B	8134

Fig 5.13 IGF2R expression by K7M2 cell line at 5µg/µl concentration

5.6 Radioimmunotherapy (RIT) with radiolabeled 2G11 mAb significantly slowed down the growth of OS-17 and 143B OS tumors

To investigate if radiolabeling 2G11 with therapeutic radionuclides will confer therapeutic properties, we radiolabeled it with an intermediate energy beta-emitter ^{177}Lu. Pilot experiments demonstrated that the maximum tolerated dose for ^{177}Lu-2G11 is below 250 µCi, and in the follow up experiments the mice were given IP either 80 µCi ^{177}Lu-2G11, 80 µCi ^{177}Lu-MOPC-21, unlabeled (cold) 2G11, or were left untreated. Administration of ^{177}Lu-2G11 significantly ($P<0.05$) slowed down the growth of both 143B (Fig. 12a) and OS17 tumors (Fig. 12b) in comparison with the untreated tumors and cold 2G11. The treatment effect was IGF2R-specific as the control mAb MOPC-21 radiolabeled with the same activity of ^{177}Lu, had significantly less effect on the tumor growth rate. In addition, a group of mice with 143B tumors received 80 µCi of the alpha-emitter ^{213}Bi-2G11 mAb, and its retardation effect on the tumor growth became more profound, though not significantly, than that of ^{177}Lu-2G11 by day 12 post treatment. RIT treatment of tumors resulted in tumors developing necrosis by day 12 and necessitated euthanizing the animals in compliance with the Animal Research Ethics regulation.

a)

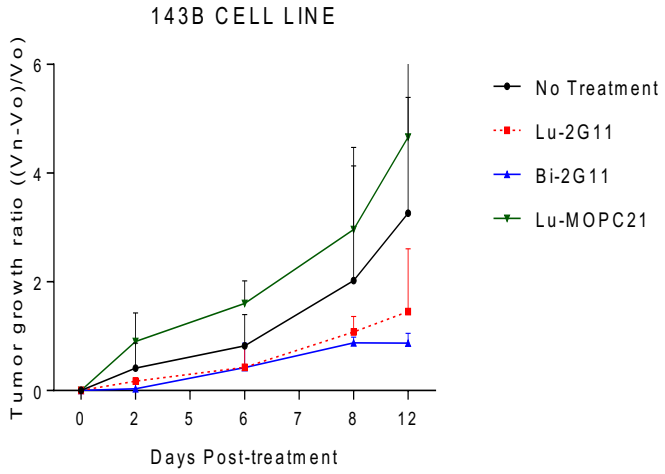


Fig 5.14 Radioimmunotherapy of OS tumor-bearing SCID mice: a) 143B tumors. The mice were given IP either 80 µCi ^{177}Lu-2G11, or 80 µCi ^{177}Lu-MOPC-21 or left untreated. A group of mice were also given 80 µCi ^{213}Bi-2G11.

b)

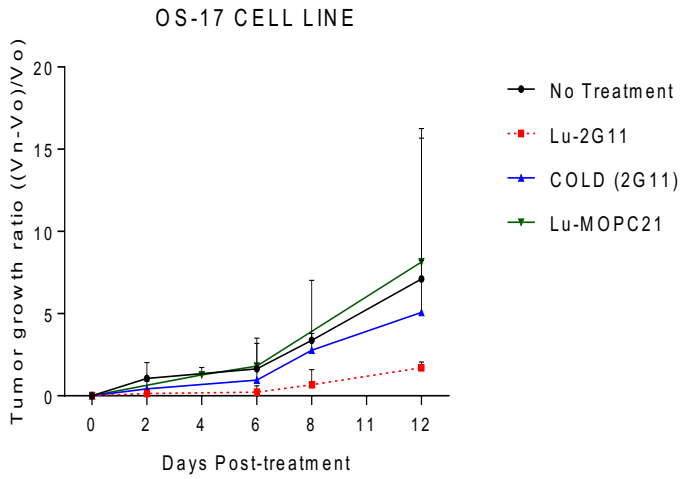
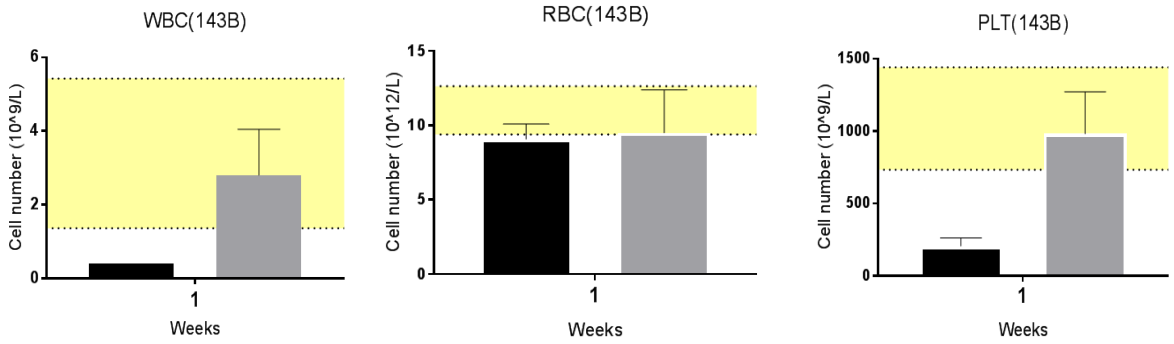


Fig 5.14 Radioimmunotherapy of OS tumor-bearing SCID mice: b) OS17 tumors. The mice were given IP either 80 µCi ^{177}Lu-2G11, or 80 µCi ^{177}Lu-MOPC-21, or unlabeled (cold) 2G11, or was left untreated.

Findings demonstrate that neither ^{177}Lu-2G11 nor ^{213}Bi-2G11 produced histologically overt splenic damage (Fig. 12a-12b). The levels of liver enzymes (ALT and AST) as well as of creatinine and BUN were within the normal range for SCID mice (Fig. 15a-g) attesting to the high tolerability of RIT.

a) b) c)



Lu-2G11
No Treatment(SHAM)

WBC :1.36 - 5.42(10^9/L)
RBC : 9.39 – 12.64 (10^{12}/L)
PLATELET : 733 – 1441 (10^9/L)

Fig 5.15 Hematologic toxicity was evaluated in 143B tumor bearing mice treated with ^{177}Lu-2G11 and Untreated after Radioimmunotherapy. a) White blood cells (WBC), b) Red blood cells (RBC) and c) Platelets (PLT) were analyzed after 1 week using the automated blood analyzer. There was a four-fold decrease in the counts which were well within the limits attesting to the high tolerability of RIT.
The ranges given above were provided by the Charles River Laboratories.

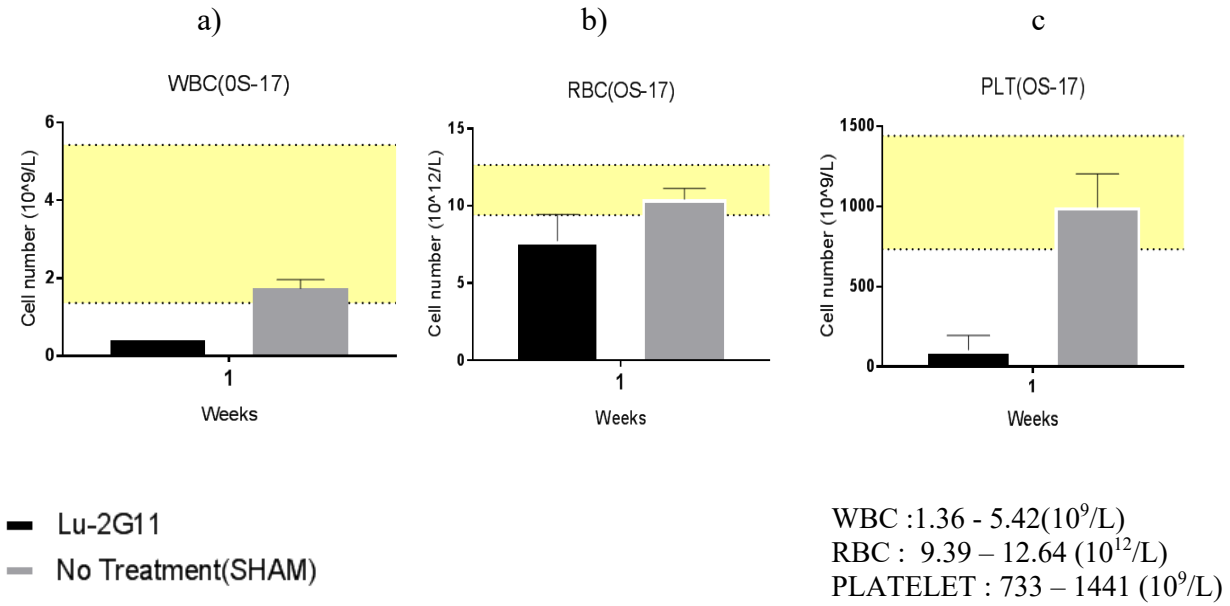
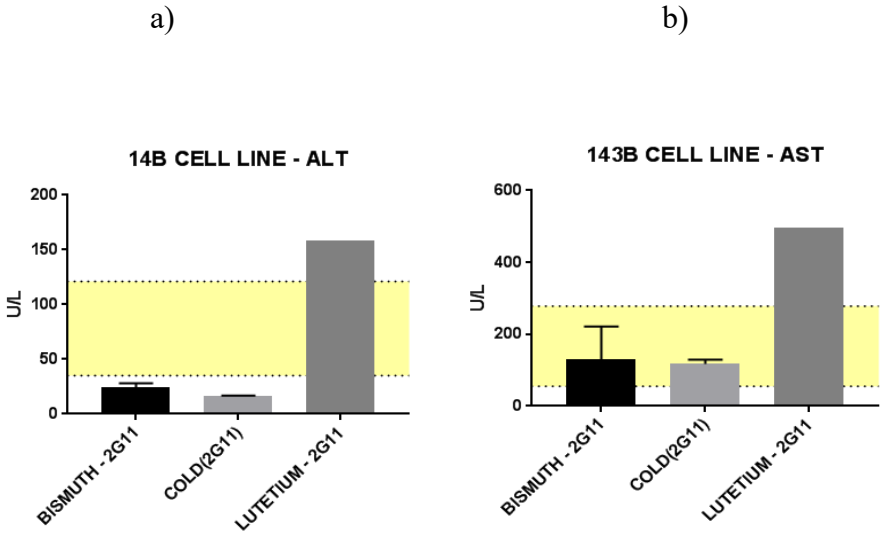
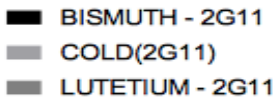
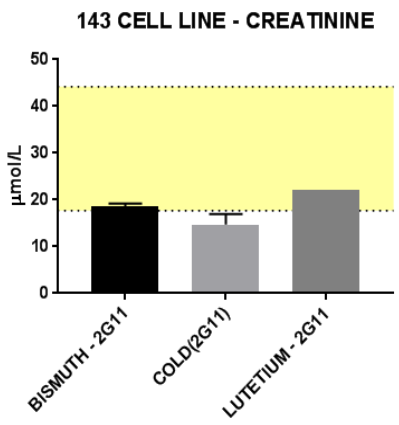


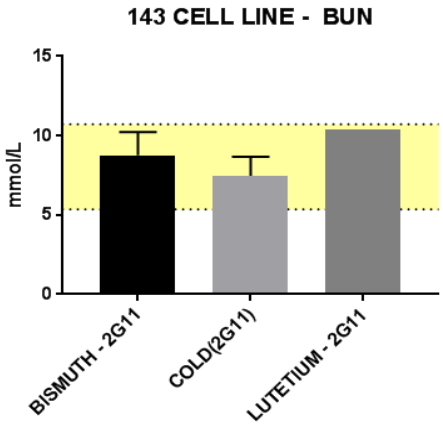
Fig 5.16. Hematologic toxicity was evaluated in OS-17 tumor bearing mice treated with ^{177}Lu-2G11 and Untreated after Radioimmunotherapy. a) White blood cells (WBC), b) Red blood cells (RBC) and c) Platelets (PLT) were analyzed after 1 week using the automated blood analyzer. There was a four-fold decrease in the counts which were well within the limits attesting to the high tolerability of RIT.

The ranges given above were provided by the Charles River Laboratories.





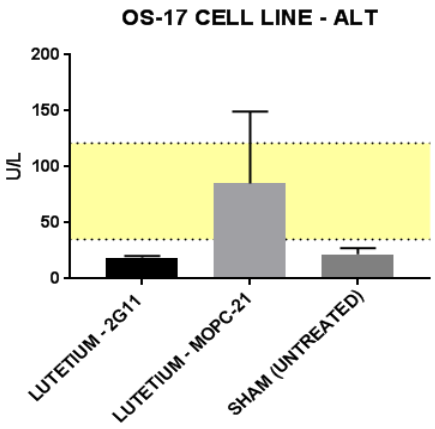


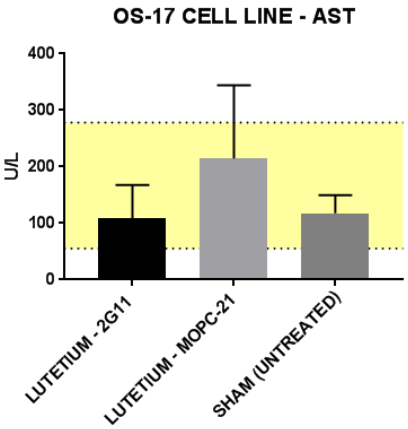


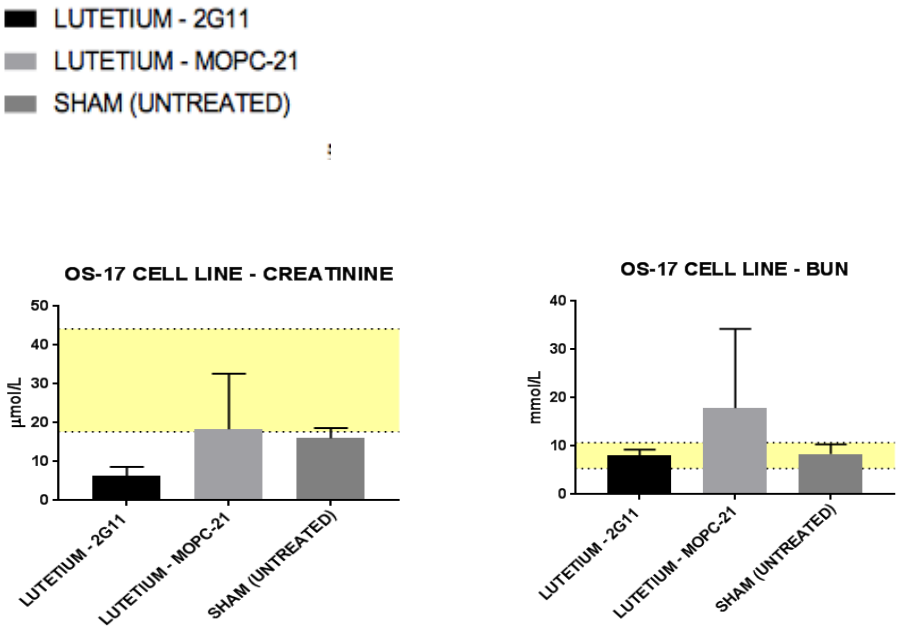
c) d)

ALT : 35 – 121 (U/L)
AST : 55 – 278 (U/L)
CREATININE : 17.6803 – 44.2008 (µmol/L)
BLOOD UREA NITROGEN (BUN) : 5.3571 – 10.7143 (mmol/L)

e) f)







g) h)

ALT : 35 – 121 (U/L)
AST : 55 – 278 (U/L)
CREATININE : 17.6803 – 44.2008 (µmol/L)
BLOOD UREA NITROGEN (BUN) : 5.3571 – 10.7143 (mmol/L)

Fig 5.17 Evaluation of the liver and kidney toxicity for the 143B tumor bearing mice: a) Alanine aminotransferase (ALT); b) Aspartate aminotransferase (AST) ; c) Creatinine ; d) Blood Urea Nitrogen (BUN) and OS-17 tumor bearing mice: e) Alanine aminotransferase (ALT) f) Aspartate aminotransferase (AST) g) Creatinine h) Blood Urea Nitrogen (BUN). There was a four-fold decrease in the counts which were well within the limits attesting to the high tolerability of RIT.

The ranges given above were provided by the Charles River Laboratories.

5.7 Internalization Assay

We utilized the IncuCyte Antibody Internalization Assay to determine the internalization of the IGF2R specific 2G11 monoclonal antibody into the 143B cell line. The method allows the antibody to be screened for their internalization properties. This method uses pH sensor dyes which are low in fluorescence at neutral pH but become highly fluorescent at acidic pH. For receptor mediated

internalization, the antibody against the particular receptor is conjugated with the pH sensor dye and incubated with the cells expressing the receptor. When this antibody labeled with the pH sensor fluorescent dye binds to its antigen (receptor) on the cancer cells, they do not become fluorescent but when internalization into the endosomal and lysosome vehicles takes place, the pH drops and the dye becomes fluorescent. It follows the process from early endosomes (pH 6.3) to lysosomes (pH 4.7). The IncuCyte FabFluor Red Antibody was tested at a final concentration of 4 µg/ml and was incubated for 15 mins at 37°C in dark for conjugation. This labelled antibody was added to the 143B cells and was analyzed using the IncuCyte live-cell analysis system. On analyzing the results, the 143B cells showed positive internalization of the IGF2R specific 2G11 monoclonal antibody. (Fig 16a) A positive result is indicated when the cells present in the well emit a red fluorescent light showing that the receptor mediated antibody is being internalized into the cells. Fig 16b shows the image for the non-specific control MOPC-21 that was used for comparison to the IGF2R specific 2G11 monoclonal antibody. Fig 16c indicates a graphical representation of the internalization of the IGF2R specific 2G11 monoclonal antibody into the 143B cells. The images were captured at the end of 48 hours to check for the internalization and the graphs were plotted using the values obtained at the end of 48 hours.

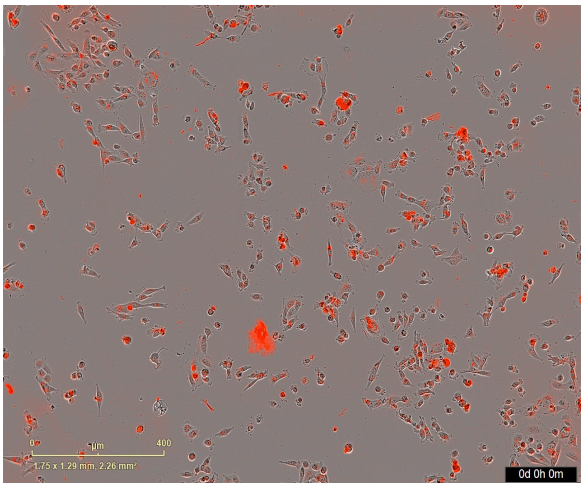


Fig 5.18 143B Cells stained with 2G11

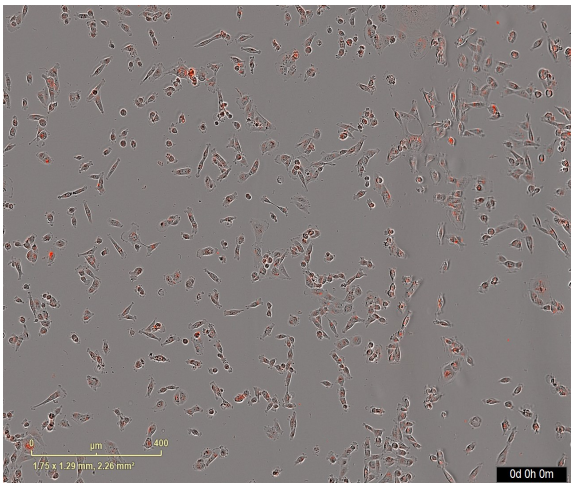


Fig 5.19 143B Cells stained with MOPC-21

IGF2R is mostly intracellular and gets cycled between the surface and internally as a transport mechanism. The reason it remains on the surface of the cancer cell is still unclear and additional

experiments such as CRISPR-CAS9 experiments are now being performed to test if absence of IGF2R will affect cellular proliferation (Dr. David Geller, personal communications).

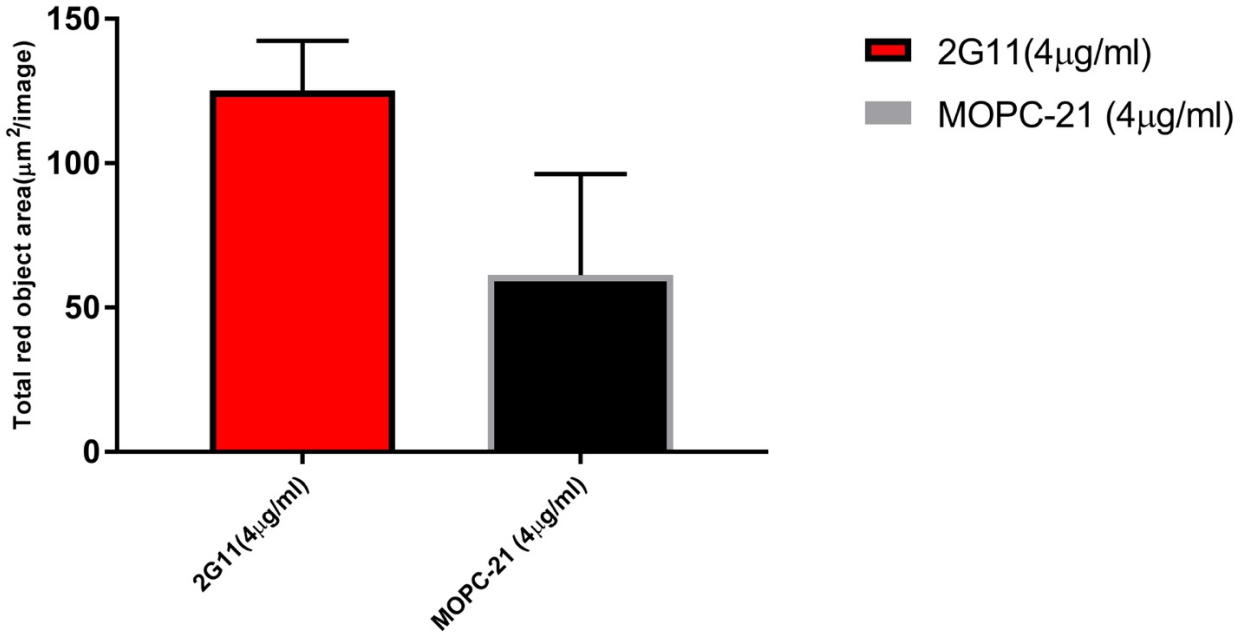


Fig 5.20 Graphical representation of the internalization of the IGF2R specific 2G11 monoclonal antibody into the 143B cells.

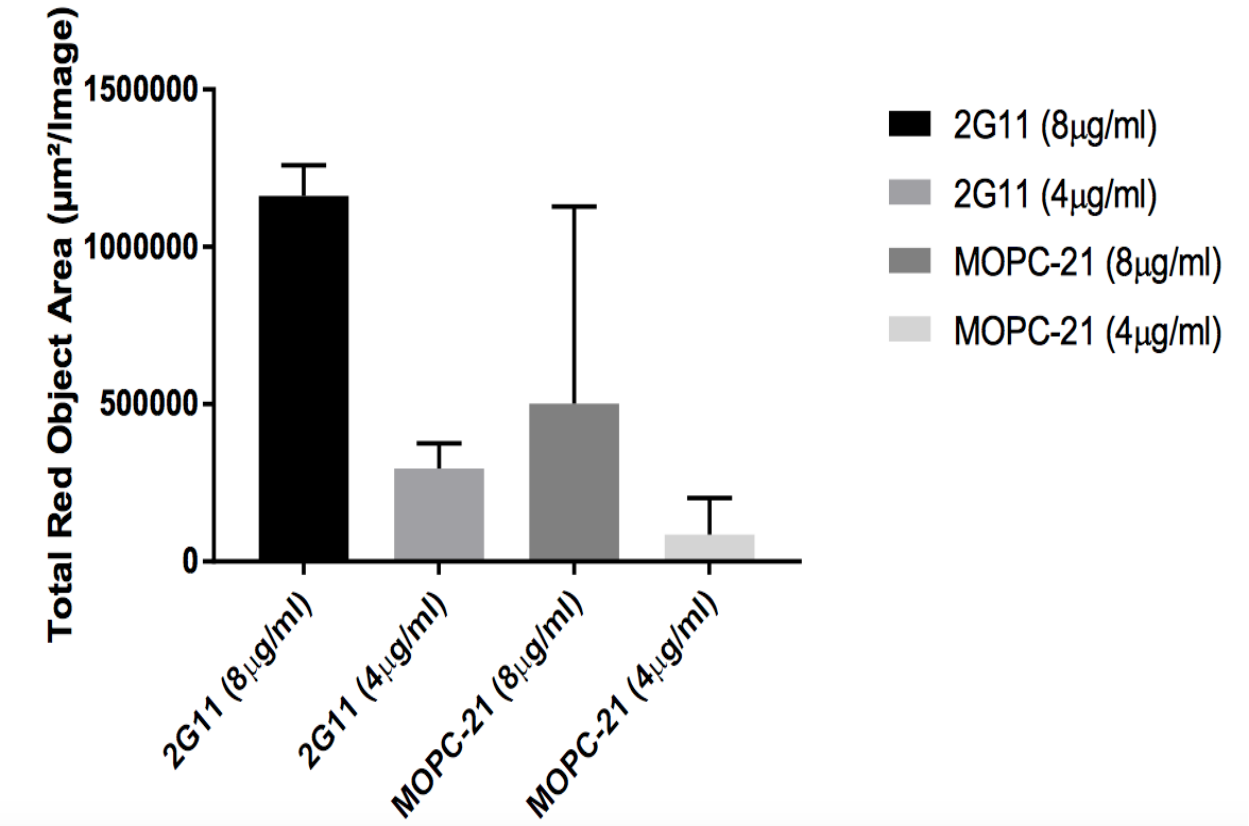


Fig 5.21 Concentration dependent internalization of the IGF2R specific 2G11 monoclonal antibody into the 143B cells.

5.8 Biodistribution of different excess molar equivalents concentrations of a DOTA conjugated monoclonal antibody in tumor free mice

To find out whether Human IgG A' (Propriety Antibody) conjugated at different molar equivalents excess concentrations of bifunctional chelating agent DOTA will display the same biodistribution in healthy mice. The antibodies were conjugated at 10 molar equivalents and 109 molar equivalents excess of DOTA and the biodistribution experiment was performed at four different time points for each of the excess concentrations of DOTA. The CB17 black mice were divided into two groups with one group receiving the 10 molar equivalents excess DOTA conjugated antibody and the other group receiving 109 molar equivalents excess DOTA conjugated antibody. The mice were sacrificed at 4, 24, 48 and 96 hours after the administration of the ^{225}Ac -radiolabeled mAbs and the biodistribution was performed. The results are shown in

Fig 16e. The uptake of the 10 molar equivalents excess conjugated Hum195 mAb was significant in the organs at 4 and 24 hours however by 48 hours and 96 hours the Human IgG 'A' started to excrete out from the body. The uptake of the 109 molar equivalents excess conjugated Human IgG 'A' was relatively similar with a slight difference in the excretion out of the organs but not significantly. The main difference was really fast clearance of 109 molar equivalents excess conjugate from the blood, which might have implications for the tumor uptake in future studies in mouse tumor models.

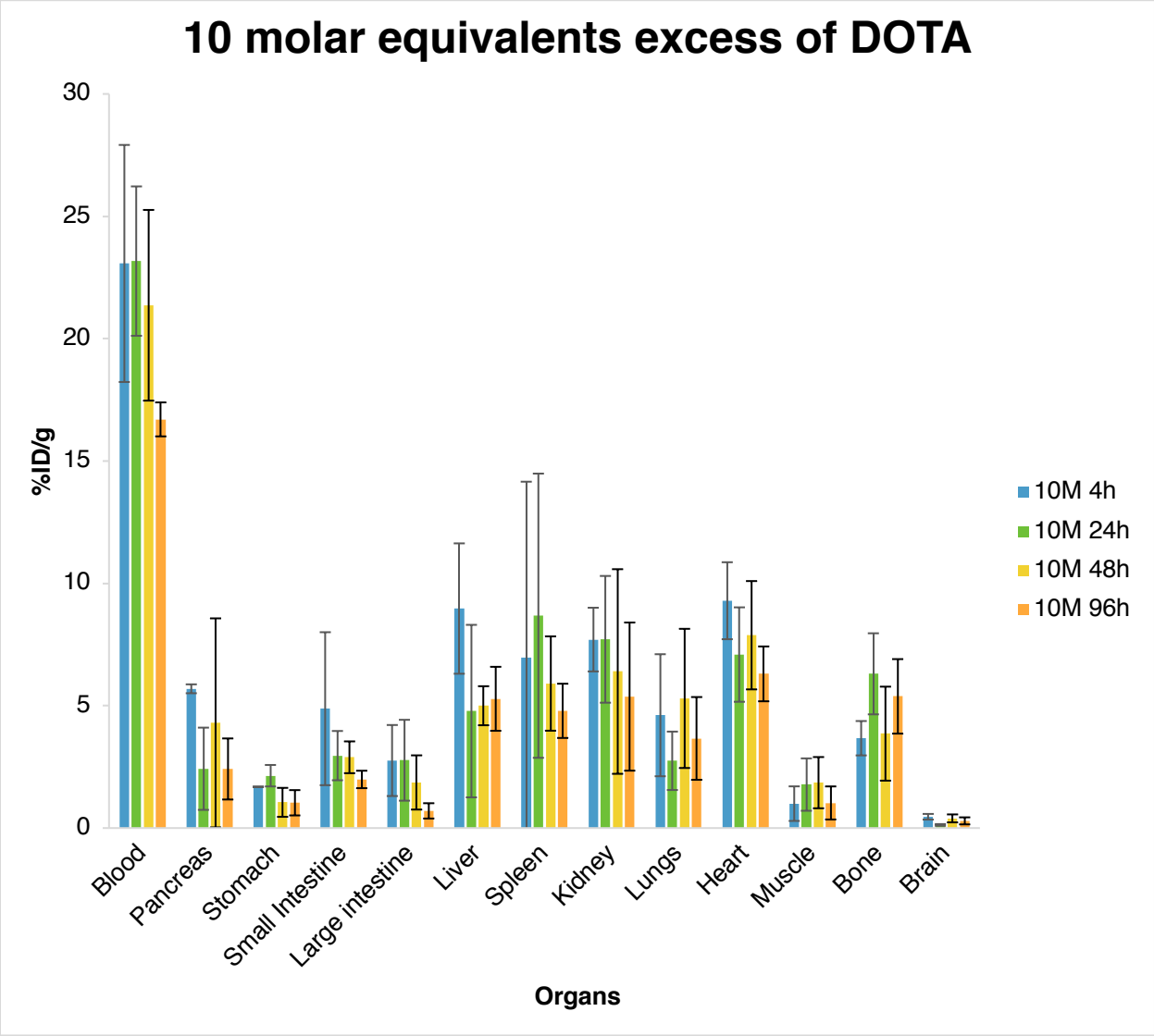


Fig 5.22 Biodistribution of ^{225} Ac – labeled Human IgG 'A' at 10 molar equivalents excess concentration of DOTA

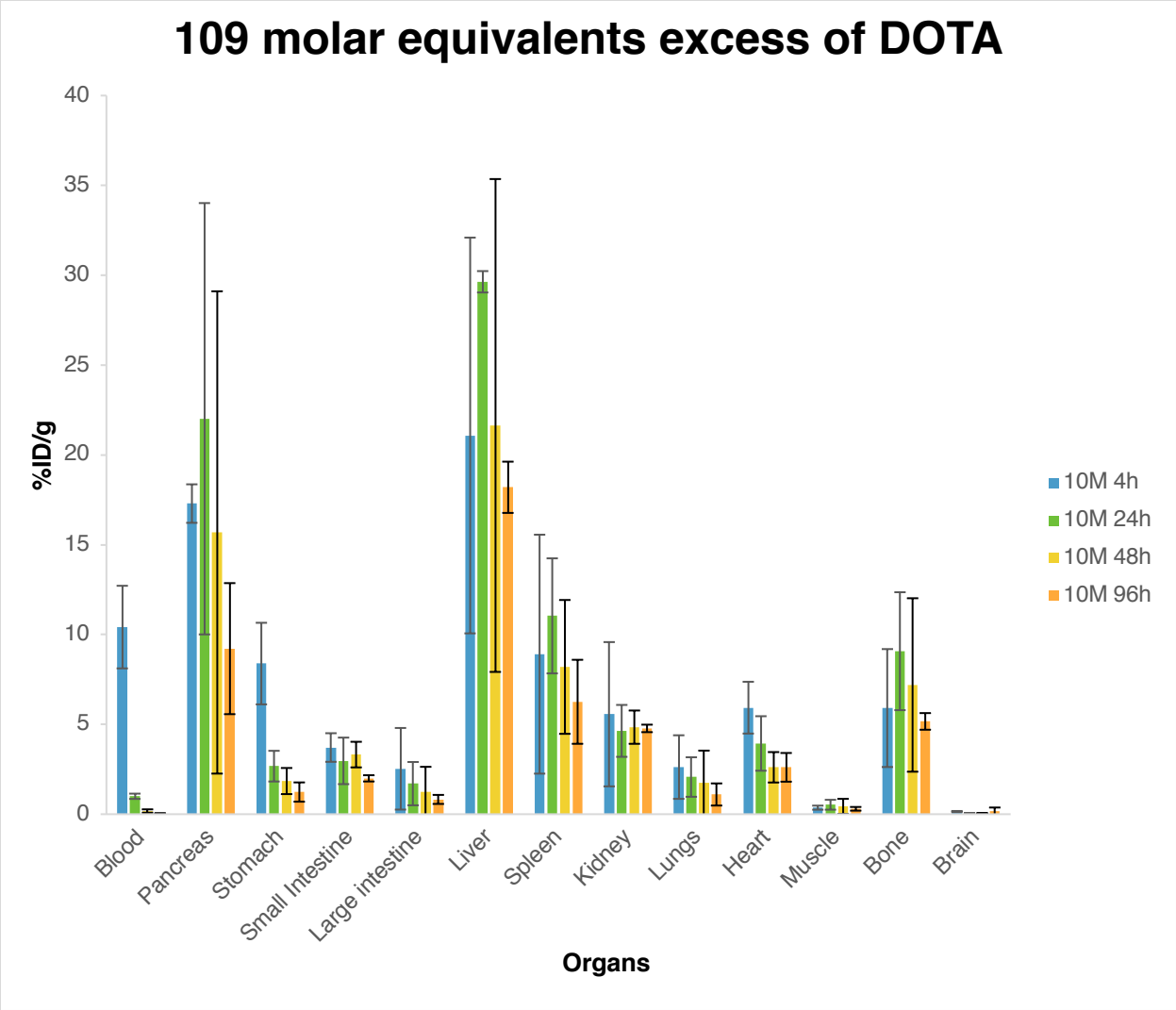


Fig 5.23 Biodistribution of ^{225} Ac – labeled Human IgG 'A' at 109 molar equivalents excess concentration of DOTA

6.0 Conclusion

In conclusion, given the lack of new effective therapies RIT might prove beneficial. Since IGF2R is expressed normally across various tissue types, the constant over-expression in Osteosarcoma recommends that it may serve as a valuable therapeutic target in the treatment of Osteosarcoma. Through the study, it was shown that Radioimmunotherapy with the use of both the ^{177}Lu-2G11 mAb conjugate and the alpha emitting ^{213}Bi-2G11 mAb conjugate showed substantial reduction in the tumor growth rate in vivo using both the patient derived cell line as

well as the standard derived cell line. The selective targeting of the radionuclides such as Lutetium-177 or Bismuth-213 to the cancer cell antigen IGF2R using 2G11 monoclonal antibodies exhibited a great potential in treating the tumors.

Future Work

This radiolabeled monoclonal antibody hence can be used in the development of a therapeutic agent for the treatment of Osteosarcoma. In addition, as there are overwhelming similarities between human and canine osteosarcomas [96] - presence of IGF2R in canine tumors helps to further develop IGF2R-targeting RIT of OS using the comparative oncology approach.

6.1 Discussion

This study demonstrates the feasibility of targeting OS using a commercially available mAb to IGF2R within a xenografted murine model. Biodistribution and microSPECT/CT imaging reveal ed selective uptake of the radiolabeled IGF2R-specific mAb 2G11 within the tumor as well as the spleen, with the latter resulting in no detectable untoward effect within the experimental time course. Moreover, the treatment using both the beta emitting ^{177}Lu-2G11 mAb conjugate and the alpha emitting ^{213}Bi-2G11 mAb conjugate showed substantially slowed tumor growth *in vivo* using both a standard cell line and a PDX tumor line. No local or systemic toxicity was noted.

Use of the beta-emitting radionuclide, ^{177}Lu (maximum beta energy 0.13 MeV) offers multiple benefits over ^{188}Re, which was previously utilized. Firstly, ^{177}Lu has a longer physical half-life of 6.7 days, extending the therapeutic effect for a longer period. Secondly, the process of labeling ^{177}Lu is simpler and more reliable [84]. Finally, ^{177}Lu is readily available from several vendors, likely is due to the recent approval of the ^{177}Lu-labeled peptide (Lutathera) in Europe and the United States for the treatment of somatostatin receptor positive neuroendocrine tumors. Importantly, our laboratory has recently demonstrated the equivalency of ^{188}Re- and ^{177}Lu-labeled mAbs in the treatment of experimental cervical cancer [85]. Taken together, the use of ^{177}Lu appears to support the treatments' technical feasibility and clinical translation. Additionally, while previous work has demonstrated tight Ab binding to IGF2R, Scatchard plot analysis has shown only a moderate number of available IGF2R binding sites. Antibodies labeled with alpha emitters

are, at least theoretically, less dependent on the antigen density as one or two hits of an alpha-particle can kill a cell. [7]

The biodistribution and microSPECT/CT imaging experiments in the two OS xenograft models showed high IGF2R-specific uptake of ^{111}In-2G11 in the tumors that was not dependent on the *in vitro* levels of IGF2R expression by the respective cell line. This observation suggests that even tumors with lower IGF2R expression will be effectively targeted with the IGF2R-specific mAb. The uptake of ^{111}In-2G11 into the tumors at both 24 and 48 hrs was significantly higher than the previously observed uptake of ^{188}Re-MEM-238 [20]. This might be due to the residualizing nature of trivalent radiometals such as ^{111}In or ^{177}Lu that tend to remain in the tumor even after separating from the mAb, as opposed to ^{188}Re, which leaves the tumors relatively fast after oxidizing into the inert perrhenate anion. The high uptake of radiolabeled 2G11 mAb into tumors translated into an impressive therapeutic response for both OS17 and 143B xenograft models. Interestingly, a very short-lived alpha-emitter ^{213}Bi (46 min physical half-life versus 6.7 days for ^{177}Lu) had a similar effect to ^{177}Lu treated mice for the 143B tumor xenografts, highlighting the enormous therapeutic potential of alpha-emitters in RIT.

While systemic chemotherapy combined with wide surgical resection remains the mainstay of OS treatment [13], there remains no meaningful alternative therapy for those patients who relapse following standard first-line therapy. The addition of conventional cytotoxic chemotherapy to the standard three-drug backbone consisting of high-dose methotrexate, doxorubicin and cisplatin, has failed to increase overall survival. This stark reality underscores the need for innovative disruptive approaches to OS therapy, particularly for patients who present with overt metastases or who relapse following standard therapy. While OS has been historically considered a radiation-resistant histology, the entire radiation oncology field has enjoyed numerous advances and improvements. Currently, the application of radiation therapy has been well-established in specific instances. For example, patients unable to undergo an R0 resection, particularly in the spine and in the pelvis, benefit from the addition of external beam radiation, realizing improved outcomes when compared with chemotherapy alone [86, 87] External beam radiation also offers a well-tolerated palliative option in cases where cure is no longer feasible and where the main treatment objective shifts to improving pain control and quality of life.

Radionuclide therapy has been utilized for the management of metastatic disease to the bone in both the setting of carcinoma and OS. Both Samarium-153 and Radium-223 localize to areas of increased bone turnover, thereby delivering relatively high doses of selective treatment and yielding a reduction in pain and a decrease in analgesia dependence in a number of prospective double-blinded placebo-controlled randomized studies. [88-91] However, these radiopharmaceuticals are only used for the palliation of painful bone metastases and are not viewed as standard of care. Combination of radionuclide and monoclonal antibodies is a recognized strategy used for a number of hematopoietic and solid tumors, combining the targeted specificity of the monoclonal antibody with cytotoxic impact exerted by the radionuclide. [92] This approach may offer further benefit via the abscopal effect, a well-recognized phenomenon, described as a radiation-mediated tumor response in disease outside of or distant from the field of radiation.

Limitation of the current study includes the relatively short experimental time course, which resulted from the need to euthanize the animals owing to local tumor ulceration despite tumor response. It is unclear if this simply represents the natural course of a growing tumor or a treatment-related event and further characterization of tumor histology may offer insight. The use of immunocompromised animals is an inherent limitation in that it discounts or artificially nulls any immune-mediated response. For this reason and despite being a recognized model, it simply does not recapitulate the human condition. Planned utilization of murine OS within an immunocompetent murine model was not feasible, given the lack of IGF2R overexpression within murine OS. Taken together, these limitations illustrate the need for improved preclinical models which allow for the evaluation of human tumor within immune-intact animals. The observation of high spleen uptake of ^{111}In-2G11 during biodistribution and microSPECT/CT imaging of OS tumor-bearing and healthy SCID mice raised the concern about potential undesirable effects of RIT on this organ and may represent a translational limitation despite the histological splenic analysis which failed to demonstrate any evidence of organ injury.

As with all therapeutic measures, safety remains a paramount and critical consideration. In addition to the normal splenic histology, no abnormalities in the liver enzymes and kidney biomarkers such as creatinine and BUN were detected. It has been shown that 2G11 mAb was able to mimic the effect of mannose-6-phosphate on the receptor up-regulation. [93] As 2G11 stays in circulation for several days, it upregulates the IGF2R expression in the tumor and the spleen, which results in its increased uptake in those tissues. Interestingly, according to [93] mAb MEM-238

which was also used in our initial RIT of OS experiments [20] does not upregulates IGF2R. This observation can explain why the tumor uptake of radiolabeled MEM-238 at 48 hours was only 3.5% ID/g, while the uptake of 2G11 at 48 hours in the current study was 5 times higher at 18% ID/g. These findings corroborate the Human Atlas Database contention that IGF2R is only minimally or moderately expressed in all normal organs, suggesting that using it as a target for RIT should be relatively safe: https://www.proteinatlas.org/ENSG00000197081-IGF2R/tissue. For comparison, SSTR1, which is targeted by the recently approved Lutathera, is highly expressed in several crucial organs and is still safe to administer to patients: https://www.proteinatlas.org/ENSG00000139874-SSTR1/tissue.

Safety can be further demonstrated within the context companion animals, which serve as a great resource, as shown by the remarkable growth of comparative oncology over the last 30 years. In this regard, canine and human OS share certain antigens and can be targeted with the same mAbs [94], and the mAbs to human cation independent mannose-6-phosphate receptor also bind to canine one. [95] In fact, a sequence alignment of human, mouse and canine IGF2R genes encompassing the IGFII binding region (containing domains 11-FNII), shows that this region is highly conserved across these species with 82% sequence identity. Randomly selected cases of canine OS from the companion dogs treated in our institution, demonstrated significant expression of IGF2R (unpublished data). As there are overwhelming similarities between human and canine osteosarcomas [96] - presence of IGF2R in canine tumors sets the stage of further development of IGF2R-targeting RIT of OS using the comparative oncology approach.

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