ALBUMIN AS A PLATFORM FOR RADIOTHERAPY AND ANTIBODY-RECRUITING THERAPY

ALBUMIN AS A PLATFORM FOR RADIOTHERAPY AND ANTIBODY-RECRUITING THERAPY

By NATALIE MERCANTI, B.Sc

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AUTHOR: Natalie Mercanti, B.Sc. (McMaster University)

SUPERVISOR: Professors John Valliant, Saman Sadeghi, and Anthony Rullo

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Abstract

The aim of this thesis was to develop and evaluate albumin-based conjugates for their use in radiotherapy and antibody-recruiting therapy which may then be combined to enhance therapeutic efficacy of each monotherapy. The approach taken in order to achieve high tumour uptake of the conjugates and minimize doses to healthy tissues involved the intratumoural administration of therapeutic compounds; a technique which has gained popularity in recent years for the treatment of solid tumours. Despite the promise this method of administration holds, it is often limited by the fast clearance of injected compounds from the tumour. Using albumin-based conjugates allows for the exploitation of the enhanced permeation and retention (EPR) effect which aids in the retention of the compound at the site of interest for longer periods of time, thus allowing the opportunity for enhanced therapeutic efficacy.

Bovine serum albumin conjugated with DOTA chelators was first synthesized and found to possess 3.9 ± 0.4 chelates per BSA molecule. Radiolabelling of the compound with lutetium-177 produced the desired product in radiochemical yields of 74 ±2 % with a radiochemical purity >99%. The stability of the compound was evaluated by monitoring the radiochemical purity over 7 days which was found to be >95% pure over the entirety of the testing period, indicating a stable product. The intratumoural administration of [^{177}Lu]Lu-DOTA-BSA in a triple negative breast cancer (TNBC) tumour model revealed significant tumour retention of 52 ± 12 %ID/g and 35 ± 6 %ID/g at 24 h and 72 h post-injection, respectively, while autoradiography displayed a heterogenous dispersion of the compound throughout the tumour. A multidosing therapy study in which animals received two doses of either 4.44, 5.92, or 7.40 MBq of [^{177}Lu]Lu-DOTA-BSA showed promise, with a strong trend observed between the administration of higher doses and a prolonged lifespan. Histological analysis of tumours excised 7 days post-treatment revealed signs

of necrosis and apoptosis in tumours treated with 7.40 MBq [^{177}Lu]Lu-DOTA-BSA. These preliminary results prove to be a promising approach for use in combination therapy and may be further optimized to enhance its efficacy as a monotherapy.

Next, the *in vivo* evaluation of DNP-BSA was carried out to assess using an intratumourally administered, albumin-based platform for antibody-recruiting in a triple negative breast cancer model. A preliminary antibody-recruiting study administering 35 nmol DNP-BSA three times per week unfortunately did not induce slowed tumour growth nor did it have an impact on lifespan. Treated mice were also unable to tolerate repeated doses of the antigen which indicated too high of a concentration and/or dosing frequency was used. A tolerability study was then carried out in order to determine a treatment schedule which did not lead to adverse effects. Mice treated once per week with low (9 nmol) to moderate (17 nmol) doses of DNP-BSA did not display toxic effects but unfortunately did not exhibit a therapeutic effect nor any indication that an adaptive immune response was achieved. These results suggest that further optimization is required prior to use in combination therapy and moderate doses (17 nmol) DNP should be used to investigate a treatment schedule which is able to induce antibody recruitment.

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Dedication

I would like to dedicate this thesis to all of my friends and family that have sat and listened to me while I cried about science over the past 2.5 years. I would not have made it through without all of your support in those moments (and there have been many) and I am forever grateful for all of you.

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List of Abbreviations

%ID/g	Percent Injected Dose per Gram
ATP	Adenosine Triphosphate
AP	Alkaline Phosphatase
ACK	Ammonium-Chloride-Potassium
ABT	Antibody Binding Terminus
ADCC	Antibody Dependent Cellular Cytotoxicity
ADCP	Antibody Dependent Cellular Phagocytosis
ARM	Antibody Recruiting Molecule
ARP	Antibody Recruiting Polymer
ARGP	Antibody Recruiting Glycopolymer
RGD	Arginylglycylaspartic Acid
BSA	Bovine Serum Albumin
BT	Brachytherapy
BV	Brilliant Violet
CDC	Complement Dependent Cytotoxicity
CTLA-4	Cytotoxic T-Lymphocyte Associated Protein 4
DAMPs	Danger Associated Molecular Patterns
DNA	Deoxyribonucleic Acid
DOTA	1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraaceticacid
DNP	Dinitrophenyl
DaRT	Diffusing Alpha-Emitters Radiation Therapy

EPR	Enhanced Permeation and Retention
ELISA	Enzyme-Linked Immunosorbent Assay
EBRT	External Beam Radiation Therapy
FACS	Fluorescence-Activated Cell Sorting
FDA	Food and Drug Administration
Fc	Fragment Crystallizable
FCA	Freund's Complete Adjuvant
FIA	Freund's Incomplete Adjuvant
α-gal	Galactosyl-(1,3)-galactose
H&E	Hematoxylin & Eosin
HCC	Hepatocellular Carcinoma
HPLC	High Performance Liquid Chromatography
HDR	High-Dose-Rate
HMGB1	High-Mobility Group Box 1
ICI	Immune Checkpoint Inhibitor
irAEs	Immune Related Adverse Events
IgG	Immunoglobulin G
IgG1	Immunoglobulin G1
IHC	Immunohistochemistry
IDO	Indolamine 2,3-dioxygenase
iTLC	Instant Thin Layer Chromatography
IL-2	Interleukin 2
I.P	Intraperitoneal

I.T	Intratumoural
I.V	Intravenous
KLH	Keyhole Limpet Hemocyanin
LET	Linear Energy Transfer
LDR	Low-Dose-Rate
MS	Mass Spectrometry
MALDI	Matrix-Assisted Laser Desorption Ionization
mCRPC	Metastatic Castration-Resistant Prostate Cancer
MDSC	Myeloid Derived Suppressor Cell
NHS	N-Hydroxysuccinimide
NK cell	Natural Killer Cell
NK1R	Neurokinin Type 1 Receptor
OCT	Optimal Cutting Temperature
PNPP	para-Nitrophenylphosphate
PRRT	Peptide Receptor Radionuclide Therapy
PBS	Phosphate Buffered Saline
PD-L1	Programmed Death Ligand 1
PSMA	Prostate Specific Membrane Antigen
PSA	Prostate Specific Antigen
RCF	Relative Centrifugal Force
RT	Radiotherapy
R/M HNSCC	Recurrent/Metastatic Squamous Cell Carcinoma
SPARC	Secreted Protein Acidic and Rich In Cysteine

SEC	Size Exclusion Chromatography
SCC	Squamous Cell Carcinoma
TBT	Target Binding Terminus
TRT	Targeted Radionuclide Therapy
TOF	Time Of Flight
TNBC	Triple Negative Breast Cancer
TAA	Tumour-Associated Antigen
TAM	Tumour-Associated Macrophage
uPAR	Urokinase-Type Plasminogen Activator Receptor
VLA-4	Very Late-Antigen 4
WHO	World Health Organization

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Chapter 1 - Introduction

1.1 Radiotherapy

Since the discovery of radioactivity and the realization of its medical applications in medicine, ionizing radiation has played a key role in inducing DNA damage and subsequent cell death in various cancers.[1] The efficacy of radiotherapy is exemplified by statistics that have shown that despite advancements in many other treatments, up to 60% of cancer patients in the United States continue to receive curative radiation therapy.[1,2] There are three primary methods through which radiotherapy may be delivered: external beam radiotherapy (EBRT), brachytherapy (BT), and targeted radionuclide therapy (TRT).[1,3] Although these methods share similar radiobiological principles, the nature of the radiation used and the route through which it is delivered differs.

External beam radiotherapy is currently the most widely used form of radiation therapy for primary, radiosensitive tumours and delivers ionizing radiation through a beam of linear accelerator generated X-rays or particles to the tumour from outside of the body.[1] External beam therapy is traditionally administered at a high dose rate (~1-2 Gy/min) in the form of low linear energy transfer (LET) photons and electrons (0.2 keV/μm) for a short amount of time. This treatment is often fractionated, meaning several smaller treatments are given over a period of time, and tends to have a uniform dose distribution throughout the tumour.[4]

Brachytherapy, or internal radiotherapy, is the implantation of a sealed radioactive source directly into a tumour in order to induce cell death while minimizing exposure to healthy tissues. BT is often separated into two classes: low-dose-rate (LDR) and high-dose-rate (HDR).[5,6] LDR-BT delivers a dose ≤2 Gy/h and involves the permanent implantation of a radioactive source or "seed" whereas HDR-BT delivers ≥12 Gy/h and involves a temporary insertion of the source.[6]

Brachytherapy, both LDR and HDR, may be used either as a monotherapy or as a means to deliver a "boost" of radiation following EBRT or surgery.[6,7] However, its use is generally limited to a specific subset of patients as preconditions to qualify for BT include a relative ease of tumour access in addition to a small tumour volume (~50-100 cm^3).[5] BT is most often used for cases of gynecologic and prostate cancers, however, it can also be used for other malignancies such as cancers of the skin and bladder.[5]

While EBRT and BT are excellent treatments for primary tumours, these methods tend to lose their utility if the tumour has metastasized as it is not practical to access all lesions without affecting healthy tissues.[8] This obstacle prompted a need for alternative methods of radiotherapy such as targeted radionuclide therapy (TRT). TRT involves the use of a radiolabelled targeting vector to deliver cytotoxic radiation directly to the site of primary tumours and metastatic lesions.[8] Radionuclides used in TRT can be separated into three categories: Auger electron, alpha particle, and beta particle emitters. Auger electrons are emitted as the result of inner-shell electron transitions caused by an electron from a higher energy level falling into a vacancy in an inner shell.[9] The range of these emissions are primarily in the nanometer scale (2-500 nm) and multiple high energy ionizations (4-26 keV/μm) occur in the immediate vicinity of the decay site. As a result, to generate efficacy from Auger electrons, the radiolabelled molecule must be close to the DNA of the tumour cell. Alpha and beta particle emitters are better suited for radiopharmaceuticals that localize in the cell cytoplasm or cell surface as their emission ranges are longer than that of Auger emitters and can induce DNA damage from outside of the cell nucleus.[9] Having a longer range of several cell diameters allows for irradiated cells to impart a dose on cells to which the radiopharmaceutical is not bound, also known as the cross-fire effect, which can further enhance cell death. Alpha emitters have a shorter emission range (40-100 μm)

but higher linear energy transfer (50-200 keV/μm) than beta emitters (0.15-12 mm; 0.2 keV/μm), creating greater radiobiologic damage.[10] However, alpha emitters are generally less commonly used due to their complex decay schemes, unstable daughter nuclides, and short half-lives.[11] As a result, beta emitters have been the primary focus of TRT research.

1.2 Beta Emitting Radiopharmaceuticals

Radiopharmaceuticals consisting of beta emitting isotopes have been used with significant clinical success for years.[9,12–16] Two isotopes that have had early success are iodine-131 and yttrium-90 for their use in the treatment of thyroid cancer and Non-Hodgkin's Lymphoma (NHL), respectively. In 2002, Zevalin®, a ^{90}Y-labelled antibody, gained FDA approval for the treatment of NHL followed shortly thereafter by Bexxar®, an ^{131}I-labelled monoclonal antibody used for the treatment of CD20 positive, follicular NHL resistant to first-line treatments. While Bexxar® was approved, its clinical use was found to be limited and its use has since been discontinued.[17,18]

Additionally, yttrium-90 and lutetium-177 are now commonly used in peptide receptor radionuclide therapy (PRRT) to radiolabel somatostatin analogues for the treatment of neuroendocrine tumours. In early clinical studies [^{90}Y]Y-DOTATOC, was shown to result in partial or complete remissions (10-34%) and prolonged survival in patients.[17,19,20] Clinical trials evaluating Lutathera®, a lutetium-177 based peptide which gained FDA approval in 2018, have shown an overall response of 30-38% as well as a significantly high median overall survival (48 months).[15–17,21] More recently, lutetium-177 was used in a phase 3 clinical trial for the treatment of prostate cancer.[22] In the VISION trial, patients received either standard care which involved treatment with hormones, targeted antibodies, steroids, radiation therapy or bisphosphonates, or [^{177}Lu]Lu-PSMA-617 in addition to standard care. It was found that those receiving [^{177}Lu]Lu-

PSMA-617 had prolonged survival in comparison to the control group, with median overall survival times of 15.3 months and 11.3 months, respectively.

While more a form of brachytherapy, the implantation of radiolabelled microspheres containing yttrium-90 is worth noting given its increased use. TheraSphere® and SIR-Spheres® have gained FDA approval for their use in the treatment of nonresectable hepatocellular carcinoma (HCC) and metastatic colorectal cancer, respectively. These treatments are administered to liver tumours via the hepatic artery and have proven to have positive patient outcomes with minimal instances of observed toxicity.[12,23–25]

1.3 The Biological Effects of Radiotherapy

The effect of radiation on biological tissues is due to the absorption of X-rays, gamma rays, or high energy particles and at high doses, can induce molecular modifications in cells which ultimately lead to cell death.[9,26] In cancer radiotherapy, the primary target upon which to induce biological effects is the DNA within tumour cells and occurs through the formation of molecular lesions such as single-strand and double-strand DNA breaks.[9] This can occur through the direct ionization of DNA or it may occur indirectly, leading to bystander effects which result in adjacent, non-irradiated cells that exhibit the same characteristics as irradiated cells. One such example is the interaction of radiation with other intracellular molecules (most often water) which is known to mediate a bystander effect through the production of free radicals that cause cell death.[4,26]

Tumour cell death primarily occurs through apoptosis and can often lead to necrosis due to the DNA damage that occurs during treatment.[27] In some cases, radiation has been known to induce autophagy and senescence in tumour cells which contribute to its cell-killing properties. While these effects are extremely positive in regard to tumours, these same effects may apply to

radiosensitive immune cells which result in a suppressed immune system. Radiation has been observed to induce apoptosis in natural killer (NK) cells, T cells, and B cells.[27]

Radiation also induces alterations of the tumour microenvironment which can both stimulate and/or suppress the immune system, leading to positive and negative outcomes in terms of anti-tumour immunity.[4] Irradiated cells undergo immunogenic cell death which is characterized by the translocation of calreticulin to the cell surface as well as the release of danger associated molecular patterns (DAMPs) such as ATP and HMGB1. The former mechanism leads to the phagocytosis of tumour cells by dendritic cells while the latter induces the production of tumour-associated antigens (TAAs) as well as the cross-priming of $CD8^+$ T-cell lymphocytes.[4,28] The release of DAMPs can induce the regression of distance, non-irradiated metastases; an occurrence referred to as the abscopal effect.[29] However, observed abscopal effects are uncommon with only 46 reported cases from 1969-2014 which may be attributed to the immunomodulatory effects of radiation that lead to immunosuppression and thus prevent this phenomena from occurring.[30] Radiation has been found to lead to the upregulation of indolamine 2,3-dioxygenase (IDO) as well as immune checkpoints such as PD-L1 and CTLA-4, all of which enable T-cell suppression and other immunosuppressive effects.[31,32] IDO alters the metabolic landscape of the tumour microenvironment by inducing the breakdown of tryptophan in cells which leads to T-cell suppression and the stimulation of immunosuppressive myeloid-derived suppressor cells (MDSCs) and tumour-associated macrophages (TAMs) while PD-L1 and CTLA-4 inhibit T-cell activation and proliferation.[31,33]

1.4 Immunotherapy

The concept of immunotherapy for cancer treatment was first thought of by William B. Coley in the late 19th century when he discovered that cancer patients with post-operative wound

infections also exhibited regression of their unresected tumours.[34] He began to administer a mixture of bacteria, later known as "Coley's toxin", in an attempt to induce sepsis and consequently an increased immune response followed by anti-tumour effects. Using this method, long lasting complete remissions were seen in various different types of cancer, however, the associated risk led oncologists to implement alternative forms of therapy such as chemotherapy and radiotherapy.

More than half of a century later, the field began to broaden when researchers started to observe success using high doses of interleukin 2 (IL-2) to induce remission in metastatic renal cell carcinoma patients, which also came with significant adverse effects.[34] The true revolutionary event that would catapult the field of immuno-oncology into a viable treatment option came following a better understanding of immune surveillance; the process through which innate immune cells eliminate cancer cells. The discovery of immune checkpoints such as CTLA-4 and PD-1 and the development of monoclonal antibodies against these checkpoints (i.e. immune checkpoint inhibitors) led to significant upregulation of immune activation in several stages of the immune cell cycle, leading to anti-tumour responses.[33–35] In a randomized, phase III clinical trial for the treatment of recurrent/metastatic squamous cell carcinoma for the head and neck (R/M HNSCC), patients were treated with nivolumab, an immune checkpoint inhibitor, or with standard treatments such as chemotherapy.[36] A statistically significant prolonged survival was observed in patients treated with nivolumab when compared to patients receiving standard treatments. Additionally, patients receiving nivolumab had an estimated 1 year survival rate of 36.0% as opposed to the 16.6% estimated for those with standard therapy.[36] In 2016, nivolumab and pembrolizumab, 2 anti-PD-1 antibodies, gained FDA approval for the treatment of R/M HNSCC. This was followed shortly thereafter by the FDA approval of pembrolizumab for the

treatment of unresectable recurrent/metastatic HNSCC in 2019.[35] Despite these significant advances forward, immunotherapies continue to remain limited by immune-related adverse events (irAEs) which are seen in 15-90% of patients and causes the patients' immune system to attack healthy tissues.[33,34] As a result, there remains a need for novel therapies that can lead to positive patient outcomes with minimal adverse effects.

1.5 Antibody-Recruiting Molecules

Due to the limitations associated with antibody-based immunotherapies, including the possibility of irAEs, several researchers began to pursue a small molecule-based approach for the treatment of cancer and other pathogens.[37] This approach aims to introduce antibodies to disease-relevant targets through the use of antibody recruiting small molecules (ARMs) which consist of a target binding terminus (TBT) and an antibody binding terminus (ABT). The TBT is used to target the disease-relevant cell or receptor, while the ABT is a hapten which recruits anti-hapten antibodies. The use of small molecules is beneficial because unlike antibody-based treatments, they clear quickly from the blood and are non-immunogenic.[37,38]

The purpose of ARMs is to exploit the immunological memory of the adaptive immune system and elicit a more robust response upon repeated exposure to the same antigen which will in turn lead to cell death.[37] Upon recognition of disease-relevant antigens by T cells and B cells, these cells differentiate into plasma cells which then produce antibodies specific to said antigen. Fc receptors that are present on immune cells are then able to recognize the Fc domain of the anti-hapten antibody and induce cell death through mechanisms such as complement dependent cytotoxicity (CDC), antibody dependent cellular cytotoxicity (ADCC), and antibody dependent phagocytosis (ADCP).[37]

A popular approach when synthesizing ARMs is to use haptens such as L-rhamnose, galactosyl-(1,3)-galactose (α-Gal), and 2,4-dinitrophenyl (DNP) which can be recognized by endogenous antibodies (i.e. antibodies that are naturally occurring in human serum).[37] In humans, IgG1 is the most potent isotype for the induction of Fc-mediated innate immune killing, making DNP and rhamnose, haptens with significant IgG1 anti-hapten titers, of significant interest due to their clinical relevance.[34] While both haptens are promising, DNP is at the forefront of ARM research. In 2011, Dubrovska *et al.* were the first to test the *in vivo* efficacy of their synthesized ARM, which consisted of the immunogenic DNP moiety conjugated to a prostate-specific membrane antigen (PSMA) targeting vector, for its use in treating prostate cancer.[39] It was found that treatment with the ARM supressed the growth of PSMA-positive tumours 4.8-fold compared to control groups treated with the targeting vector alone.[39] Additionally, Chirkin and colleagues developed an ARM for the treatment of pathogenic fungi. The anti-fungal agent calcofluor white was functionalized with DNP for antibody recruitment and their lead compound was found to bind to fungal chitin with high affinity, recruit anti-DNP antibodies, and induce phagocytosis of the fungal cells by human immune cells.[40]

1.6 Combination Therapies

Oftentimes immunotherapy or radiotherapy treatments on their own are insufficient to eliminate large tumours or widespread metastases. However, when used in combination, they have the potential synergize in order to enhance the overall therapeutic effect and may help to overcome the challenges that each face as a standalone therapy.[41]

For years it was believed that radiotherapy was immunosuppressive. However, recent findings suggest that it's also able to stimulate the immune system. Radiation damage to tumours results in the release of a large amount of tumour antigens which are presented to the immune

system. The increased availability of tumour-associated antigens for uptake by antigen presenting cells can then result in a tumour specific attack.[42] RT can also alter the phenotype of cells that weren't killed, which can upregulate tumour associated antigens as well as various markers that make these cells immunostimulatory. A rare but important phenomenon known as the abscopal effect can also occur as a result of RT. This occurs when localized radiotherapy induces a systemic immune response which results in the regression of distant, non-irradiated metastases.[41] However, large metastases are often quite immunosuppressive and are able to avert any immunostimulatory effects that are induced by RT.[41] Previous studies have proposed that combining RT with immunotherapy could lead to synergistic effects in which there is enhanced immune response, inhibited immunosuppression, and altered tumour cell phenotype.[41,42] Furthermore, recent studies have shown that an RT-induced abscopal effect was only seen when treatment was administered in combination with immunotherapy.[41]

Combination radiotherapy and immunotherapy studies in mice were first conducted in the 1960's, where researchers demonstrated that the efficacy of RT in mice bearing mammary carcinoma or fibrosarcoma could be significantly improved if it was preceded by an autologous tumour vaccine.[43] Tumour vaccination involves the *ex vivo* irradiation of a tumour biopsy, which is then administered to the patient prior to undergoing radiation. Tumour antigens vary between tumours and by first priming the patients' immune system for specific TAAs, a positive treatment response is more likely to be observed. This approach was just the beginning of radiation-based combination therapy research and investigations into this therapeutic modality has since continued and evolved to play a key role in cancer treatments today. [44–46]

1.6.1 External Beam Radiotherapy and Immune Checkpoint Inhibitors

Currently, the most prevalent form of combination therapy involving radiation is the use of external beam radiation in conjunction with immune checkpoint inhibitors such as anti-PD-1 and anti-CTLA-4. These combinations have seen significant pre-clinical success, some of which are now being evaluated at the clinical level.[47–50] In 2020, a phase II clinical trial began which assessed the efficacy of pembrolizumab, an anti-PDL1 antibody, and hypofractionated radiotherapy in patients suffering from metastatic triple negative breast cancer, a notoriously difficult to treat malignancy. The combination therapy was found to have modest yet promising results as the treatments were well tolerated and ~33% of patients displayed long-lasting systemic responses.[51] Many of these clinical studies are carried out in small patient populations that are non-responsive or resistant to primary treatment options and as a result, clinical trials with a larger, more diverse subset of patients are needed.

1.6.2 Targeted Radiotherapy and Immune Checkpoint Inhibitors

Despite the promising results seen in combination therapies using external beam radiotherapy, it is often limited to primary tumours and large metastases, leaving smaller metastases unaffected. In order to circumvent this problem, TRT can be used as a means to target all malignancies, including micrometastases, which could enhance anti-tumour activity and lead to better treatment outcomes.[3] In 2018, a pre-clinical study in a melanoma mouse model was carried out showing the efficacy of immune checkpoint inhibitors (ICIs) administered in combination with a lutetium-177 labelled radiopharmaceutical targeting very late antigen-4 (VLA-4). It was found that RT alone had similar results to ICI alone, however, RT+ICI led to high levels of apoptosis as well as prolonged survival.[52] Another noteworthy pre-clinical study demonstrated how suboptimal doses of radiotherapy and immunotherapy are able to synergize

and lead to improved outcomes in a non-Hodgkin's lymphoma mouse model.[53] In this study, animals were administered a dose that was ~50% of the maximum tolerated dose of radiolabelled anti-CD37 antibody and rituximab, either alone or in combination, and their efficacy evaluated. A suboptimal dose was chosen in order to detect and increased therapeutic effect when used in combination and to show the integrity of the treatment method. Results showed that the combination therapy induced a greater anti-tumour effect and prolonged survival compared to control groups and each monotherapy.[53]

Due to the success seen in pre-clinical settings, the use of combination TRT and immunotherapy as a treatment modality has entered into the clinic. In recent years, research investigating the efficacy of [^{177}Lu]Lu- PSMA-617 alongside various immunotherapies for the treatment of prostate cancer has surged and is the subject of several ongoing clinical trials. One example is PRINCE, a phase Ib/II clinical trial currently underway investigating the combination of [^{177}Lu]Lu-PSMA-617 and pembrolizumab in patients with metastatic castration-resistant prostate cancer.[54] Patients received the checkpoint inhibitor pembrolizumab every three weeks for up to two years along with up to six treatments of [^{177}Lu]Lu-PSMA-617 every six weeks. Primary patient endpoints for this trial include PSA response rate and overall safety. While it is too early to make any definitive statements and assess how patients are impacted long-term, interim analysis has showed the treatments are promising with toxicities that are no greater than each monotherapy and 7/9 (78%) patients with measurable disease exhibiting a partial response.[55]

1.6.3 Radiotherapy and Antibody-Recruiting Therapy

The use of ARMs in combination with RT has the potential to be a powerful treatment method but research into this method has only been recently investigated. Similar to

aforementioned combination therapy approaches, RT aims to directly induce tumour cell death while the ARM prompts a systemic anti-tumour immune response, resulting in a synergy between both methods that enhances therapeutic efficacy. To the best of our knowledge, there has only been one pre-clinical example of combination ARM therapy and radiotherapy in the literature, which involved the use of external beam RT with the systemic administration of folate-fluorescein isothiocyanate.[56] This study was shown to be very promising as an abscopal effect was observed in treated animals. Complete regression of both irradiated and non-irradiated metastases was seen in two of six treated animals, while the others had significantly slowed growth in non-irradiated metastases.

Efforts have also been made to produce an ARM that can be used simultaneously for TRT.[57] In 2016, Genady and colleagues developed a single construct for its use in combination radiotherapy and antibody-recruiting therapy. The platform consisted of a triazole moiety for radioiodination, a glutamate-urea-lysine targeting vector for PSMA, and a DNP moiety for antibody recruitment. While the lead compound was able to bind to both PSMA and anti-DNP antibodies, tumour uptake was a mere 3.24 ± 0.06 %ID/g at 6 h post-injection. This result was believed to be due to the presence of a spacer in the compound which inhibited tumour binding but was nonetheless necessary in order to achieve anti-DNP antibody binding. Tumour uptake of another compound lacking this spacer was found to be 9.80 ± 1.55 %ID/g at 6 h post-injection but was also unable to bind anti-DNP antibodies. While this approach seemed to be promising, it also highlights the complexity of ARM compounds, in that it is very challenging to make a molecule that not only has high affinity for the target but also for circulating antibodies. To circumvent this problem, a viable alternative may be the intratumoural administration of the TRT and the ARM.

1.7 Macromolecules in Drug Development: Albumin

Albumin is the most abundant plasma protein in mammals (~60% of total protein content in plasma) and has a molecular weight of ~66.5 kDa.[58,59] Its structure, consisting of 583 amino acid residues, is composed of 3 homologous domains (DI-DIII) and contains 17 disulphide bonds which make albumin stable to moderate temperatures, changes in pH, and denaturing solvents. It is also highly soluble in aqueous solutions due to the presence of charged amino acids such as lysine and glutamic acid.[60] Albumin has several important roles in biological systems such as the transportation of endogenous and exogenous compounds, the maintenance of plasma pH, and controlling the concentrations of Ca^{2+} and Mg^{2+} in mammals.[59–61]

1.7.1 Albumins Role in Drug Development

Albumin is widely used in drug research and development due to its favourable physiological roles as mentioned above as well as its tendency to accumulate in tumour tissues and sites of inflammation.[59,60,62] It is well established that solid tumours have poor lymphatic drainage which can lead to an accumulation of macromolecules in the interstitium; a phenomenon known as the enhanced permeation and retention (EPR) effect.[58,62] Due to the high molecular weight of albumin, it also experiences the EPR effect. In 1986, Matsumara and Maeda demonstrated this occurrence through the intravenous injection of an Evans blue-albumin complex during which there was a progressive accumulation of the complex in the tumour in comparison to other tissues.[63] The same conjugate was also injected directly into both a solid tumour and normal skin during which a rapid clearance rate was observed for normal skin, compared with a very low clearance rate from the tumour.[63]

Due to the significant presence of albumin in biological systems, there are several albumin-binding proteins and receptors in various tissues and cell lines which make them ideal

targets in albumin-based drug design.[62] Albondin/gp60 and secreted protein acidic and rich in cysteine (SPARC) are two common protein receptor targets for the delivery of such compounds. Researchers often target these receptors as they are expressed in the endothelium as well as in malignant cells, which leads to an increased accumulation of the compound in the tumour space thus theoretically increasing its efficacy.[62,64]

With a biological half-life of 19 days, albumin has also gained popularity for its use in extending the circulation half-life of therapeutics.[65] Two albumin binding classes of molecules that are especially popular in TRT research are 4-(p-iodophenyl)butyric acid and Evans blue derivatives.[65] Dumelin *et al.* were first to identify the class of 4-(p-iodophenyl)butyric acid derivatives and found that by conjugating their lead compound to a fluorescein derivative, they were able to extend its circulation time from 4.6 min to 495 min; a 100-fold increase compared to fluorescein on its own.[66] Alternatively, Evans blue may also be used as a means to increase circulation time through its binding to albumin. In a proof-of-concept study, Chen *et al.* developed a theranostic pair of Evans blue molecules conjugated with either NOTA or DOTA chelators (NMEB and DMEB, respectively) which allow for the use of both diagnostic and therapeutic isotopes.[67] These molecules were then conjugated to a cyclic RGD peptide in order to target integrin $\alpha_v\beta_3$ and their efficacy tested *in vivo*. It was found that [^{64}Cu]Cu-NMEB-RGD had tumour uptake 10-fold higher than that of [^{64}Cu]Cu-RGD (16.64 ± 1.99 %ID/g vs 1.06 ±0.03% ID/g at 24 h post-injection) and [^{90}Y]Y-DMEB-RGD led to complete tumour regression and prolonged survival in a human glioma xenograft model.[67]

Owing to its aforementioned characteristics, albumin has become a widely used drug carrier and is being used clinically today. In 2005, Abraxane®, a paclitaxel-albumin nanoparticle, gained FDA approval for the treatment of metastatic breast cancer following the

failure of combination chemotherapy.[68] It has since been used as a first-line treatment for patients with metastatic adenocarcinoma of the pancreas (in addition to gemcitabine) as well as patients with non-small cell lung carcinoma (in combination with carboplatin).[68]

1.8 Intratumoural (I.T) Administration of Ionizing Radiation

Traditionally, therapeutic radiopharmaceuticals are administered intravenously and accumulate at the site of interest using a targeting vector to target the disease at the cellular level.[69] However, the largest obstacle faced when using this method is off-target uptake in healthy tissues, exposing them to potentially high doses of ionizing radiation which may then lead to adverse side effects. To circumvent this issue, researchers explored administering radiopharmaceuticals directly into tumours. This began in the 1960's with the first reports of the use of yttrium-90 radioembolization in patients with hepatic malignancies. Application of yttrium-90 microspheres for the treatment of metastatic colon cancer was evaluated in the early 1970's and it was found that 68% of patients had an objective decrease in tumour size.[25] These promising results led to an increase in microsphere research and by 1999, yttrium-90 glass microspheres gained FDA approval for the treatment of HCC followed by the FDA approval of resin microspheres for colorectal cancer liver metastases in 2002.[25]

The direct infusion of radiopharmaceuticals in a tumour has also been seen in patients with glioblastoma, an aggressive form of brain cancer with a median survival of 10-15 months.[70] Standard first-line cancer treatments such as systemically administered chemotherapy are often ineffective with this form of cancer due to the inability to overcome the blood brain barrier. Recently, publications have detailed the direct infusion of a radiopharmaceutical targeting the neurokinin type 1 receptors (NK1R) that are highly expressed on the glioma cell surface.[70] In a pilot clinical study using a yttrium-90 based radiopharmaceutical, it was found that disease

stabilization or improved neurologic status was seen in 13 of 20 patients with World Health Organization (WHO) stage II-IV gliomas.[70,71]

More recently, advancements in the field of alpha radiotherapy has led to the development of DaRT, a method similar to brachytherapy which involves the placement of radium-224 loaded wires directly into the tumour site.[72–74] The decay of radium-224 releases several short-lived alpha particles which deliver high doses of energy to surrounding tissue by dispersing throughout the tumour via diffusion.[73] In a clinical trial with patients having locally advanced recurrent squamous cell carcinomas (SCC) of the skin, head, and neck, a complete response was seen in 78.6% of lesions and 21.4% of lesions displayed a partial response with >30% reduction in tumour burden. It was also found that overall survival rates 1 year post-implantation were 75% among all patients and 93% among patients with a complete response.[75] Similar to the DaRT approach, the intratumoural injection of a lutetium-177 labelled, albumin-based molecule consisting of *trans*-cyclooctene bovine serum albumin (TCO-BSA) conjugated to a tetrazine small molecule has also emerged in the literature. Preclinical studies in a triple negative breast cancer model have shown that following a multi-dosing approach in which two treatments were administered five days apart were able to slow tumour growth and improve survival outcome. [76]

1.9 Research Overview

The objectives of this thesis are to exploit the favourable characteristics of albumin in order to develop and evaluate novel, intratumourally administered, albumin-based radiotherapy and immunotherapy which may then be used in combination. Due to its low cost as well similarity in structure and function to both mouse and human serum albumin, bovine serum albumin is being used to develop and evaluate each conjugate as a proof-of-concept. An

antibody-recruiting strategy will be pursued as an alternative to traditional immunotherapy in order to potentially avoid the associated adverse effects. The synthesis and characterization of each monotherapy will first be discussed followed by the optimization of each therapy. It is hypothesized that when combined, each monotherapy will synergize to induce tumour cell death while simultaneously stimulating the immune system to evoke an adaptive immune response and increased therapeutic effect.

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Chapter 2 - An Albumin-Based Platform for Intratumoural Radiotherapy Delivery

Disclaimer: The following is in the format of a manuscript by Natalie Mercanti, Kevin Wyszatko, Luis-Rafael Silva, Jacek M. Kwiecien, John Valliant, and Saman Sadeghi.

2.1 Abstract

Introduction: The use of intratumoural injections to deliver radiopharmaceuticals has gained popularity in recent years due to its ability to induce tumoricidal effects while minimizing radiation doses to healthy tissue. Despite its success, ensuring the compound is retained in the tumour continues to be a challenge that can limit therapeutic efficacy. Here, we present a lutetium-177-labelled, albumin-based agent as a promising option for the treatment of triple negative breast cancer (TNBC); a disease which remains difficult to treat due to its aggressive nature and limited therapeutic options.

Methods: [^{177}Lu]Lu-DOTA-BSA was synthesized, characterized, and its *in vivo* profile assessed in a E0771 murine TNBC model. Tissue counting and autoradiography allowed for the measurement of biodistribution of the compound as well as the spatial distribution throughout the tumour. Tumour bearing C57BL/6 mice were intratumourally administered two doses (5 days apart) of 4.44, 5.92, or 7.40 MBq of [^{177}Lu]Lu-DOTA-BSA, unlabeled DOTA-BSA, or PBS. Animals were monitored every 2-3 days for signs of adverse effects and tumour measurements. Tumours were excised 7 days post-treatment for histological analysis.

Results: DOTA-BSA was synthesized and found to possess 3.9 ± 0.4 DOTA chelates per BSA molecule. Radiolabelling of the compound resulted in radiochemical yields of 74 ±2% with radiochemical purity >99%. Stability studies showed a highly stable product as it maintained a radiochemical purity >95% over 7 days. Biodistribution studies and autoradiography revealed significant tumour levels of 52 ± 12 %ID/g and compound retention in the tumour for up to 72 h

post-injection. Multi-dosing therapy survival studies indicate a strong trend of prolonged lifespan, which correlated with increasing doses of therapy ranging from 4.44 MBq to 7.40 MBq delivered intratumorally. Histological analysis of tumours revealed more advanced levels of both apoptosis and necrosis in those treated with 7.40 MBq [^{177}Lu]Lu-DOTA-BSA compared to those treated with PBS.

Conclusions: The intratumoural administration of [^{177}Lu]Lu-DOTA-BSA led to significant tumour retention, minimal non-target uptake, and no adverse side effects at doses up to 7.40 MBq per injection. Therapeutic doses induce severe necrosis and apoptosis in tumour tissues in addition to prolonging survival in treated mice, making [^{177}Lu]Lu-DOTA-BSA a promising radiopharmaceutical for the treatment of TNBC.

2.2 Introduction

Breast cancer has become all too common amongst women worldwide and is the leading cause of cancer related deaths in females.[1,2] Triple negative breast cancer (TNBC) is especially aggressive and accounts for 10-20% of breast cancers.[1] It is often seen in younger women and is associated with high rates of reoccurrence and poor survival outcomes. However, despite the availability of various therapeutic options there is no truly effective treatment for TNBC patients, pointing to a need for improved therapeutic strategies.[1–4]

In recent years, the intratumoural injection of unsealed alpha or beta emitting isotopes has gained significant attention for its ability to suppress tumour proliferation.[5–9] Due to the short penetration depth of the isotopes used, higher doses can be administered while sparing surrounding healthy tissues, leading to the reduction of off-target toxicities that are associated with many targeted radiotherapy treatments. However, ensuring that the compound is retained at the site of administration, without escaping through the tumour vasculature, is an obstacle often

encountered with intratumoural administration strategies.[10] One approach to overcome this challenge is through the use of a macromolecule-based platform. Solid tumours have poor lymphatic drainage, which can lead to accumulation of macromolecules in their interstitia; a phenomenon known as the enhanced permeation and retention (EPR) effect.[11,12] The use of a high molecular weight protein can therefore potentially be used to prolong tumour retention of a radiolabelled derivative, thus promoting therapeutic efficacy. One option is to use albumin which is an abundant plasma protein that has become increasingly common in drug development due to its favourable physiological characteristics as well as its tendency to accumulate in solid tumours. [12–14] Additionally, recent preclinical studies involving the intratumoural injection of a radiolabelled albumin-based compound have shown slowed tumour growth as well as prolonged survival following two doses of treatment in a triple negative breast cancer model.[15] In this report, bovine serum albumin (BSA) was used as a means to anchor the radioisotope in the tumour, enabling slower clearance from the tumour site as well as minimal uptake in non-target tissues. Herein, we describe the development and *in vivo* evaluation of an albumin-based radiotherapeutic in a triple negative metastatic breast cancer model.

2.3 Experimental

2.3.1 Materials and Instrumentation

Chemicals and reagents were purchased from Sigma-Aldrich and Conjuprobe and used without further purification unless otherwise stated. High-performance liquid chromatography was performed on a Waters 1525 Binary HPLC system connected to a Bioscan γ-detector and a 2998 photodiode array detector monitoring at 280 nm. Analytical HPLC separation was performed with an Agilent Zorbax Bio Series GF-250 SEC column using Method A: 0.1 M phosphate

buffer pH 6.8; isocratic elution, 1 mL/min. PD-10 desalting columns packed with Sephadex G-25 resin were purchased from Cytiva and used for product purification.

Radioactive measurements were done using a dose calibrator (Capintec, Remsey, NJ, USA) and a Perkin Elmer Wizard 1470 Automatic Gamma Counter. [^{177}Lu]Lu was produced by the McMaster Nuclear Reactor using the ^{176}Lu(n,γ)^{177}Lu reaction and was provided as a solution of [^{177}Lu]$LuCl_3$ in 0.01 M HCl (2.1 Ci/mg). Radio-TLC was performed using a Bioscan AR-2000 imaging scanner on iTLC-SG glass microfiber chromatography paper (SGI0001, Agilent Technologies) plates and eluted with 0.1 M sodium citrate buffer (pH 5).

2.3.2. Synthesis of DOTA-BSA

DOTA-NHS ester (3.8 mg, 50 eq) in 400 μL of 0.2 M PBS (pH 8.3) was added to bovine serum albumin (10 mg, 1 eq) and stirred at 4ºC for 12 h. The reaction mixture was then purified using a size exclusion PD-10 column and the absorbance of the eluate at 280 nm was measured in order to identify the fractions that contained product. The product-containing fractions were then pooled and concentrated using a 50 kDa Amicon Ultra centrifugal filter. The concentrated product was then washed with water and centrifuged at 4000 rpm for 10 min two times, collected, and lyophilized resulting in a white solid. MALDI-TOF MS was used to determine the degree of chelator conjugation, resulting in 3.9 ± 0.4 DOTAs per BSA molecule.

2.3.3 [^{177}Lu]Lu-DOTA-BSA

To a solution of DOTA-BSA (0.5 mg, 7 nmol) in 0.1 M NaOAc (0.2 mL) was added [^{177}Lu]$LuCl_3$ (0.122 GBq). The reaction mixture was heated at 60ºC for 10 min, quenched with 50 mM EDTA (10 μL), and purified using a size exclusion PD-10 column equilibrated with PBS (pH 7.4). The compound was eluted with 2 mL of PBS, resulting in radiochemical yields of 74 ±2%, a radiochemical purity >99%, and a specific activity of 0.27 GBq/mg.

2.3.4 Animal Studies General

All animal studies were approved by the Animal Research Ethics Board at McMaster University. Mice were maintained under clean conditions with 12 h light/dark cycles and given food and water *ad libitum*. Experimental mice were monitored daily for clinical abnormalities and for the ethical endpoints which include weight loss and decrease in general body condition, tumour mass that impairs normal body function, signs of pain, ulceration of the tumour and a tumour volume ≥ 1500 mm^3.

2.3.5 Biodistribution Study of [^{177}Lu]Lu-DOTA-BSA

Female, 9-week-old, C57Bl/6 mice (Charles River Laboratory - Kingston, NY) were inoculated with 5×10^6 E0771 cells in the left flank. Once the tumours grew to ~100 mm^3 in size, mice were intratumourally injected with [^{177}Lu]Lu-DOTA-BSA (0.22-0.63 MBq). At 24 h and 72 h post-injection (n=3 per time point), mice were anesthetized with 3% isoflurane and euthanized via cervical dislocation. Blood, stomach (with contents), large intestine (with contents), bone (leg bone including knee joint), spleen, urine and bladder, liver, kidneys, thyroid and trachea, lungs, skeletal muscle, adipose, gall bladder, small intestine (with contents), heart, pancreas, and tumour were collected, weighed, and counted in a gamma counter. Decay correction was used to normalize organ activity measurements to time of dose preparation for data calculations with respect to injected dose (i.e. %ID/g). Autoradiography was carried out on excised tumours which were first submerged in optimal cutting temperature (OCT) for 5 min. Embedded tumours were then lowered into the liquid nitrogen vapour phase until completely frozen, sliced, and placed on a phosphor screen (Fujifilm BAS cassette) for 48 h and subsequently imaged (Amersham Typhoon Biomedical Imager).

2.3.6 [^{177}Lu]Lu-DOTA-BSA Multi-dosing Study

Female, 13-15-week-old, C57Bl/6 mice (Charles River Laboratory - Kingston, NY) were tumour inoculated with 5×10^6 E0771 cells in the left flank. Once the tumours grew to ~100 mm^3 in size, mice were intratumourally injected with [^{177}Lu]Lu-DOTA-BSA on days 0 and 5 (4.4 – 7.4 MBq). One cohort (n=25) was followed for survival and tumours measured every 2-3 days. Mice were euthanized as they reached endpoint which was characterized by ulceration of the tumour or a tumour volume of ~1500 mm^3. Another cohort (n=25) was euthanized on day 7 post-treatment, their tumours excised and submitted for histological analysis.

2.3.7 Histology

Tumours were excised on day 7 post-treatment, fixed in formalin for 48 h, and transferred to a solution of 70% ethanol. The samples were then left to decay for 3 months prior to sending for histological processing (STTARR – Toronto, Canada). The paraffin-embedded subcutaneous tumors were stained with hematoxylin and eosin (H&E) and coverslipped. An experienced experimental pathologist blinded to the study, examined the slides under a Nikon Eclipse 50i light microscope. The morphological features of the tumor were identified and scored as follows; (1) the epithelial architecture (scored 0-2, lower = better), (2) local invasion by tumor cells of tissues around the tumor mass (scored 0-2, with 0 = no invasion), (3) intravascular invasion by tumor cells in the tissues around the tumor mass (scored 0-1, with 0 = no intravascular invasion), (4) mitotic index averaged from 3 non-overlapping areas of the tumor mass examined under the 40x magnification, (5) anisokaryosis in the tumor mass (lower score = lower anisokaryosis), (6) absence of borders in tumor cells (scored 0-2, 2 = no discernible cell borders). The morphologic effects potentially attributed to the anti-cancer treatment were; (i)

tumor cell apoptosis in the mass (scored 0-4, with 0 = no apoptosis), (ii) tumor mass necrosis (scored 0-4, with 4 = severe).

2.3.8 Statistical Analysis

Multiple comparisons using two-way ANOVA and post-hoc Tukey tests were used to determine the statistical significance between groups. The log-rank Mantel-Cox test and Gehan-Breslow-Wilcoxon test were used to determine the statistical significance of differences in the Kaplan-Meier survival curve. Null hypotheses were rejected for p-values less than 0.05. Data analysis was carried out using GraphPad Prism 9 (San Diego, CA, USA).

2.4 Results and Discussion

2.4.1 Synthesis and Radiolabelling

Radiolabelled BSA was synthesized and evaluated in a TNBC murine model in order to asses tumour retention and distribution of the compound as well as its tumoricidal effects. BSA was first functionalized with a DOTA chelator in order to allow for its subsequent radiolabelling with lutetium-177. DOTA-NHS ester was incubated with BSA overnight at 4°C and purified via size exclusion chromatography. The conjugation was confirmed through MALDI-MS, which indicated 3.9 ± 0.4 DOTAs per BSA molecule. In order to ensure that DOTA-BSA retained the physical properties of non-functionalized BSA, each compound was analyzed through SEC-HPLC. The compounds yielded an identical analytical trace, with peaks eluting at 7-9 min and 9-10 min which are representative of oligomeric and monomeric BSA, respectively (Figure 2-1).[16]

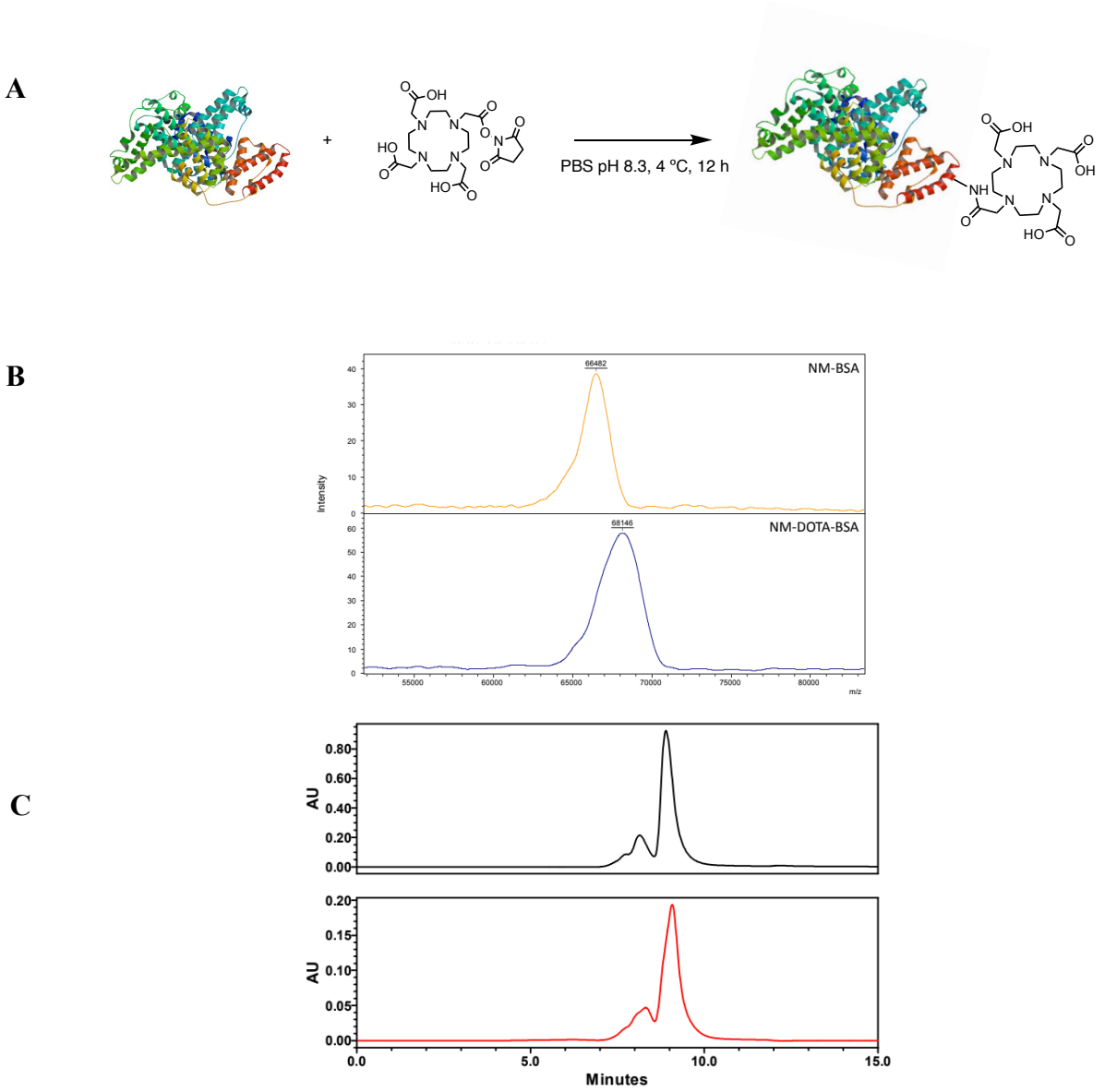


Figure 2-1. Characterization of DOTA-BSA. (A) Synthesis of DOTA-BSA. (B) MALDI-MS of BSA versus DOTA-BSA. (C) SEC-HPLC of BSA (black) and DOTA-BSA (red) at 280 nm (HPLC method A).

The radiolabelled compound was formed by incubating [^{177}Lu]$LuCl_3$ with DOTA-BSA at 60°C for 10 min and the product isolated using size exclusion chromatography. Radiochemical yields of 74 ± 2% were determined by measuring the activity of the reaction mixture before and after purification. The final product was found to have a radiochemical purity >99% as seen through iTLC. (Figure 2-2).

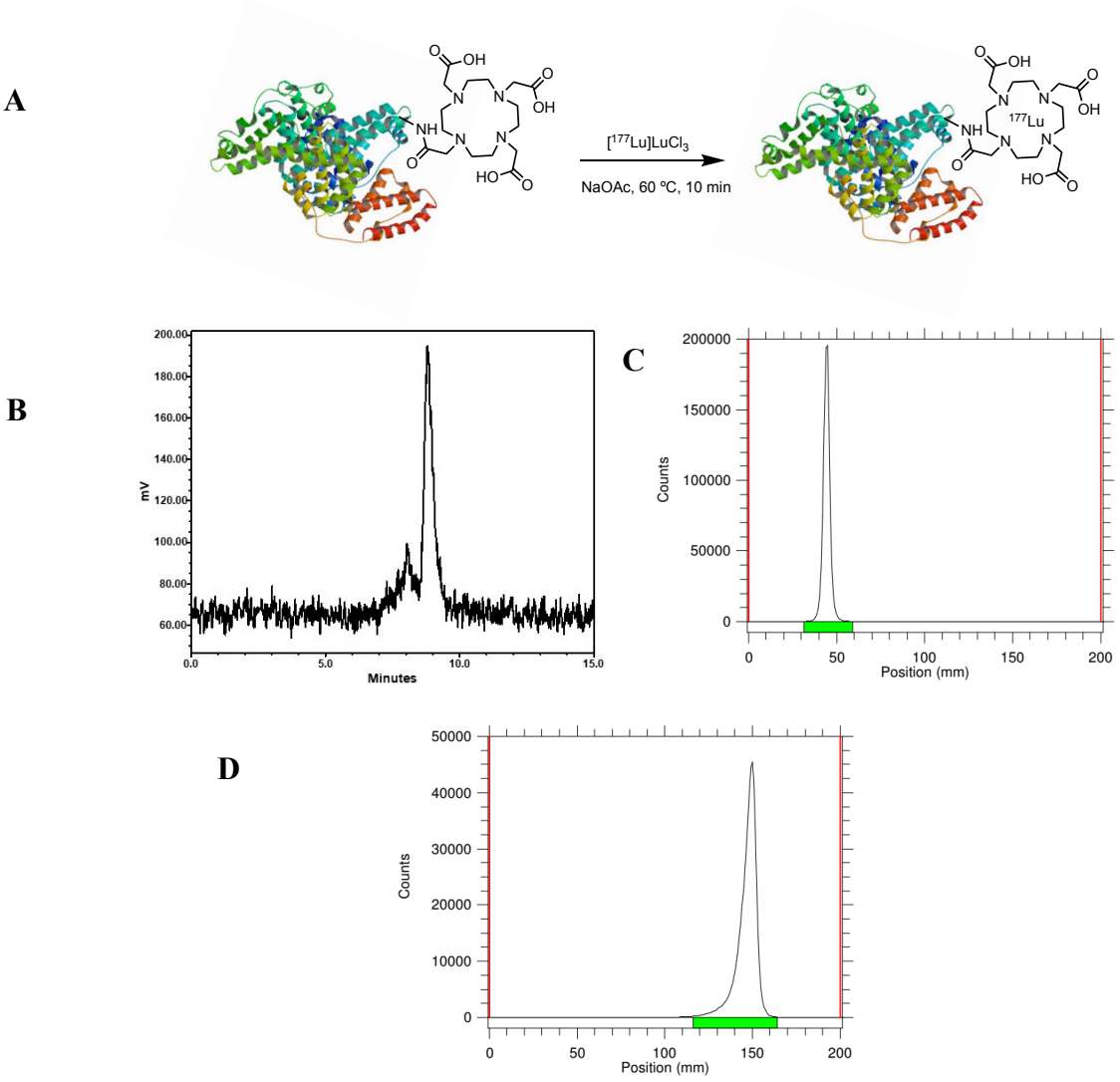
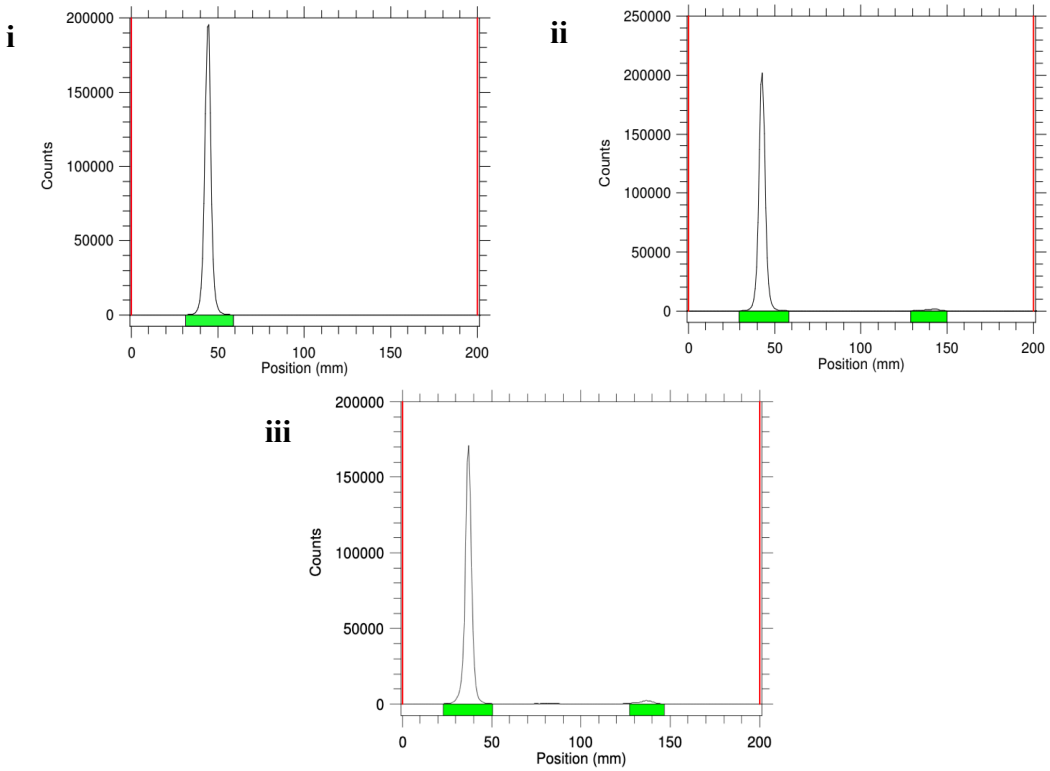


Figure 2-2. Synthesis and characterization of [^{177}Lu]Lu-DOTA-BSA. (A) Synthetic scheme of [^{177}Lu]Lu-DOTA-BSA. (B) Radio-HPLC chromatogram (HPLC method A). (C) iTLC of [^{177}Lu]Lu-DOTA-BSA in 0.1M sodium citrate buffer (pH 5). (D) iTLC of [^{177}Lu]$LuCl_3$ in 0.1M sodium citrate buffer (pH 5).

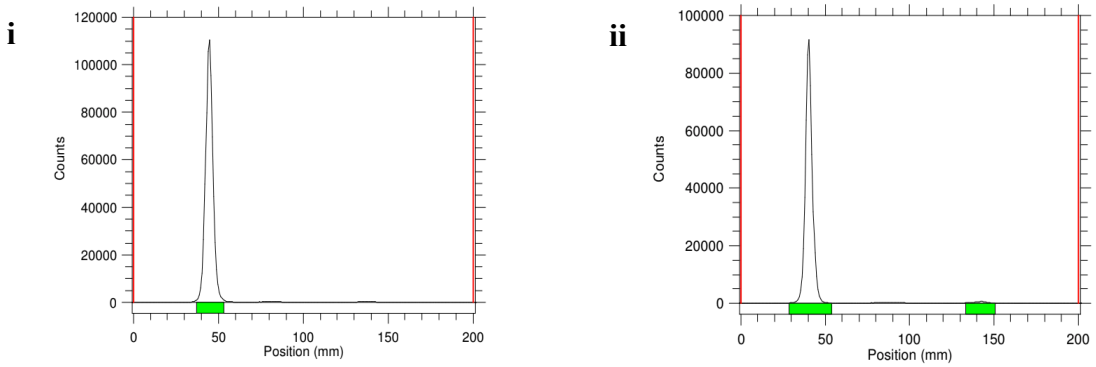
Upon isolating the radiolabelled compound, its stability was then evaluated. An iTLC of the product 0.1M sodium citrate buffer (pH 5) was performed at an activity concentration of 0.32 MBq/mL over the course of 24 hours as well as 7 days post-labelling in order to determine how well the chelate complex remained intact over time. Using 0.1 M sodium citrate buffer (pH 5) as an eluent for iTLC, "free" lutetium-177 would be seen as a peak moving with the solvent front whereas [^{177}Lu]Lu-DOTA-BSA remained on the baseline (Figure 2-2D)Upon analysis, it was

found that the product had a radiochemical purity >95% over 7 days, indicating that it is highly stable. The stability of the compound in mouse plasma was also evaluated in order to determine its behaviour *in vivo* prior to testing. Plasma was added to the product and incubated at 37 ºC for 24 hours, over which time the mixture was analyzed by iTLC intermittently. As with the compound alone, [^{177}Lu]Lu-DOTA-BSA in mouse plasma remained stable over 24 hours with a radiochemical purity >95% (Figure 2-3).

A



B



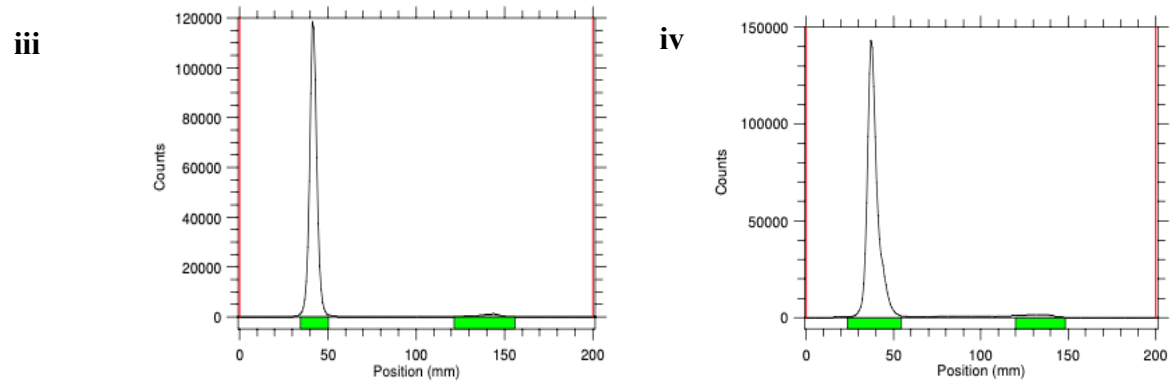


Figure 2-3. (A) iTLC of [^{177}Lu]Lu-DOTA-BSA after 0 h (i), 24 h (ii), and 7 days (iii). (B) iTLC of [^{177}Lu]Lu-DOTA-BSA in mouse plasma at 1 h (i), 3 h (ii), 6 h (iii) and 24 h (iv) post-labelling.

2.4.2 Evaluation of [^{177}Lu]Lu-DOTA-BSA in a Triple Negative Breast Cancer Model

In order to assess the retention of the compound as well as its spatial distribution throughout the tumour, 0.37 MBq [^{177}Lu]Lu-DOTA-BSA in 50 µL PBS was intratumourally administered and biodistribution and autoradiography studies carried out.

Ex vivo tissue counting revealed high tumour to non-tumour ratios indicating minimal leaching and uptake of radioactivity in non-target tissues (Figure 2-4). At 24 and 72 h post-injection, tumour uptake was found to be 52 ± 12 %ID/g and 35 ± 6 %ID/g, respectively. Although the liver and blood were found to have >10%ID/g at 24 h post-injection, by 72 h this decreased to 7.00 ± 1.08 %ID/g and 2.90 ± 0.53 %ID/g respectively, indicating active clearance of lutetium-177 from these tissues.

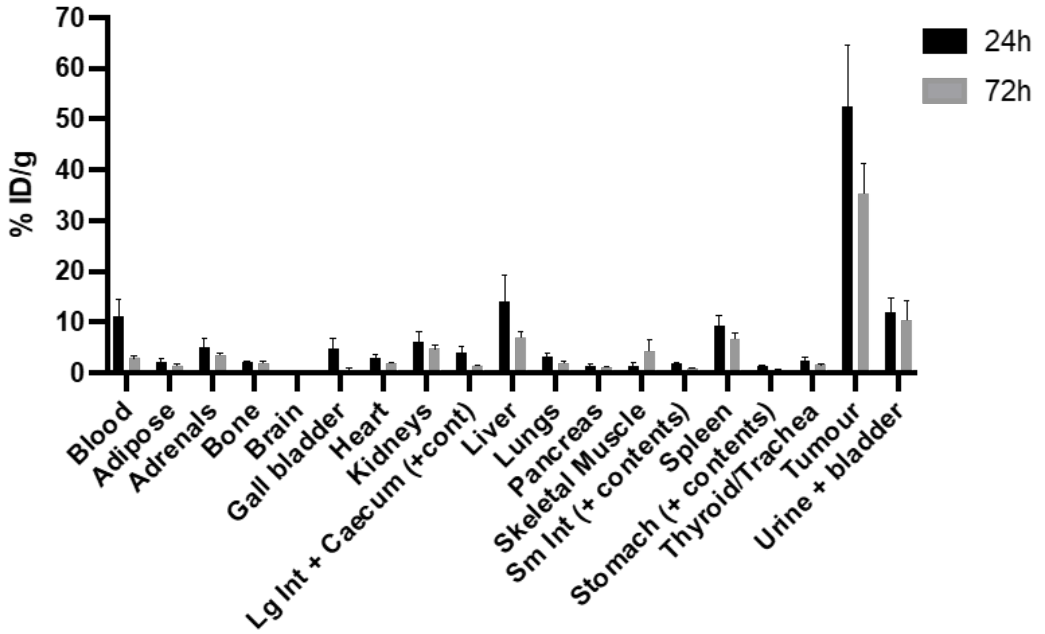


Figure 2-4. Biodistribution of [^{177}Lu]Lu-DOTA-BSA. Graph depicts %ID/g of main organs 24 and 72 h post-administration.

Consistent with biodistribution data, autoradiography illustrated that the compound was retained out to 72 h and the activity largely localized in the periphery of the tumour (Figure 2-5). The lasting retention of the compound is beneficial for therapeutic studies as it allows for ionizing radiation to induce more DNA damage over a longer period of time.

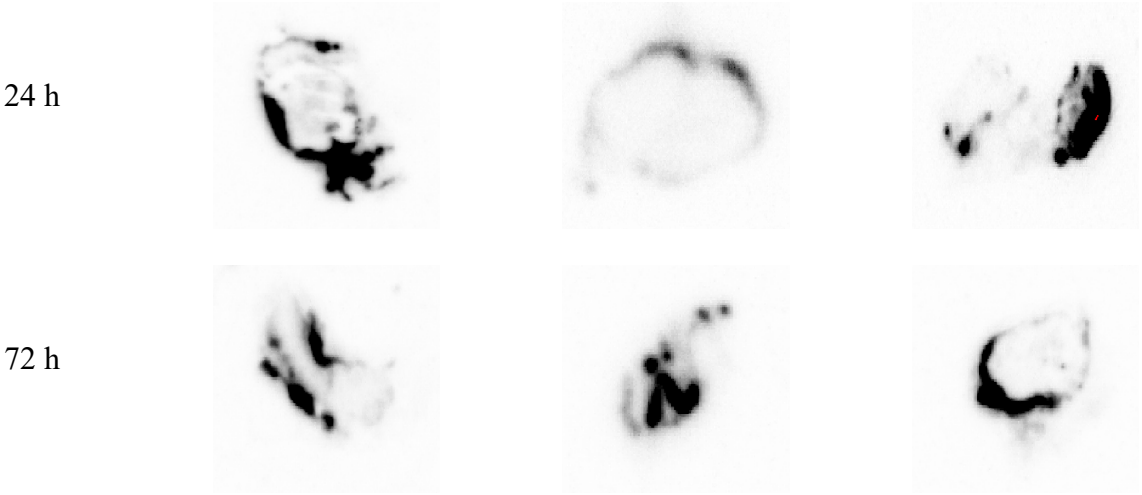


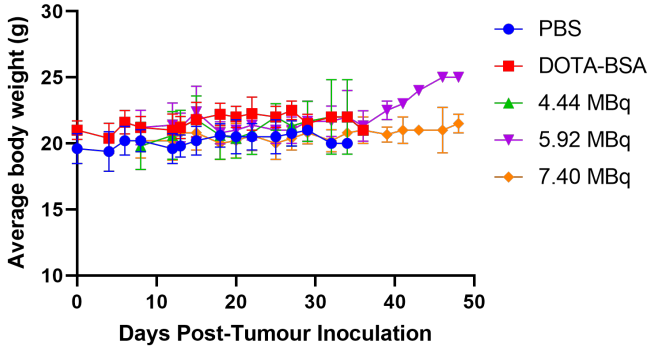
Figure 2-5. Autoradiography images of IT injected [^{177}Lu]Lu-DOTA-BSA 24 h (top) and 72 h (bottom) post-administration. Each image corresponds to the tumour from an individual animal. Dark areas indicate a high concentration of radioactivity.

2.4.3 [^{177}Lu]Lu-DOTA-BSA Multi-Dosing Study

While there is currently no common treatment schedule for all radionuclide therapies, literature precedence has shown that a fractionated approach leads to better therapeutic outcomes.[17] Previous studies have shown that administering two 4.44 MBq doses of a lutetium-177 labeled albumin conjugate [^{177}Lu]Lu-DOTA-Tz-TCO-BSA five days apart led to prolonged survival in a TNBC mouse model.[15] In this report, a fractionated approach was designed, where two doses ranging from 4.4 MBq to 7.4 MBq were administered on days 0 and 5 of treatment. One cohort of animals were monitored for a therapeutic effect and overall well-being while another cohort was sacrificed 7 days post-treatment for the histological analysis of treated tumours.

Over the course of the study, animals were monitored for signs of distress as well as changes in body weight which would be indicative of radiotoxicity. The body weight of the animals is shown in figure 6, with all gaining or maintaining their body weight over the treatment period with no signs of distress. Tumour size measurements indicated a strong correlation with slowing in tumour progression in the cohort receiving [^{177}Lu]Lu-DOTA-BSA (7.40 MBq) (Figure 2-6).

A



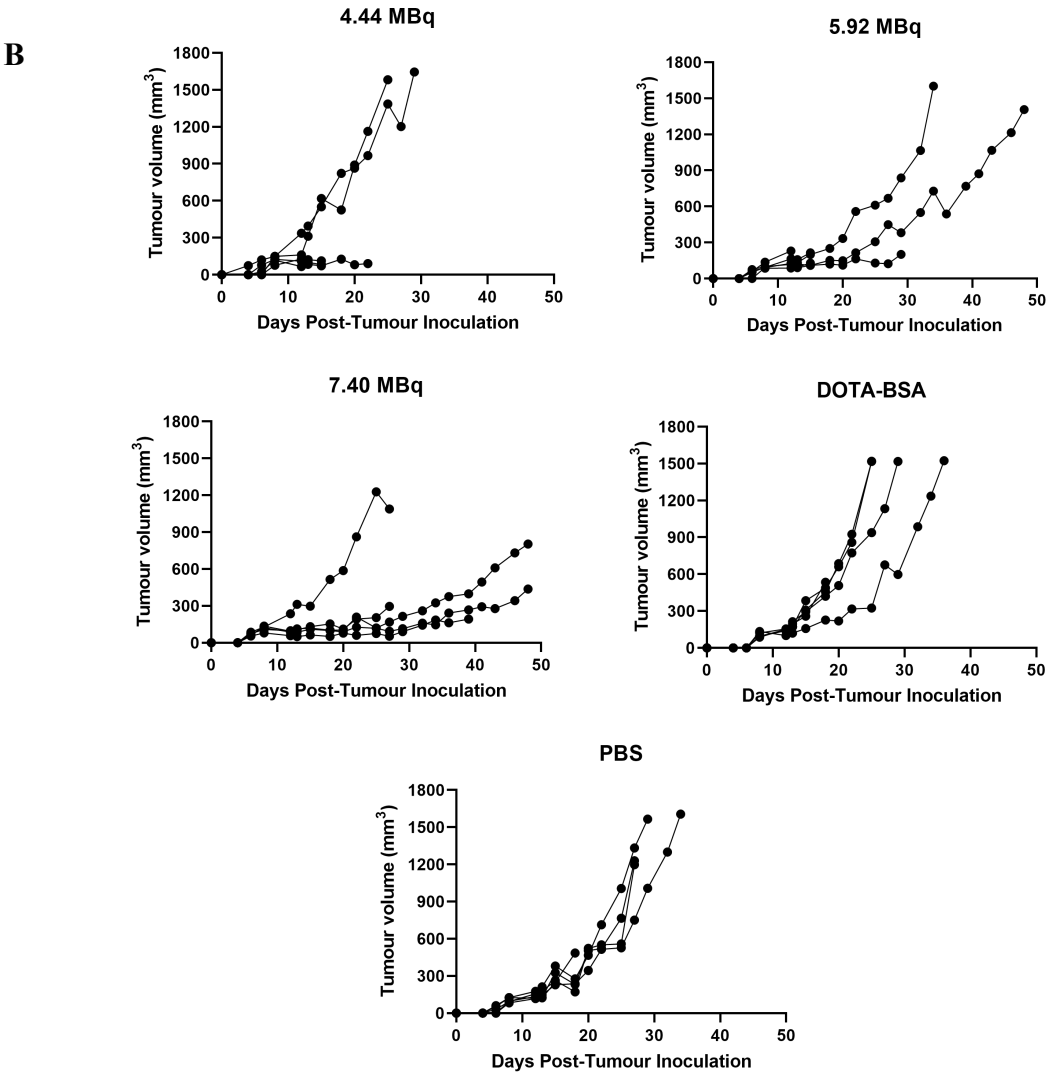


Figure 2-6. [^{177}Lu]Lu-DOTA-BSA Radiotherapy. (A) Average body weight of each treatment group (n=5). (B) Tumour volumes from each treatment group (n=5). Each line represents a single animal in the group.

Statistical analysis revealed that tumour volumes of animals treated with 7.40 MBq [^{177}Lu]Lu-DOTA-BSA were significantly lower than those treated with 4.44 MBq [^{177}Lu]Lu-DOTA-BSA, DOTA-BSA, or PBS from days 20-29, 20-36, and 20-34, respectively. Animals were followed for survival until endpoint was reached (~1500 mm^3 tumour volume) or for up to 40 days post-treatment (Figure 2-7). Consistent with the trend seen in tumour progression,

animals receiving 5.92 MBq or greater of [^{177}Lu]Lu-DOTA-BSA were more likely to have a longer survival time than animals receiving a low dose or unlabelled compound.

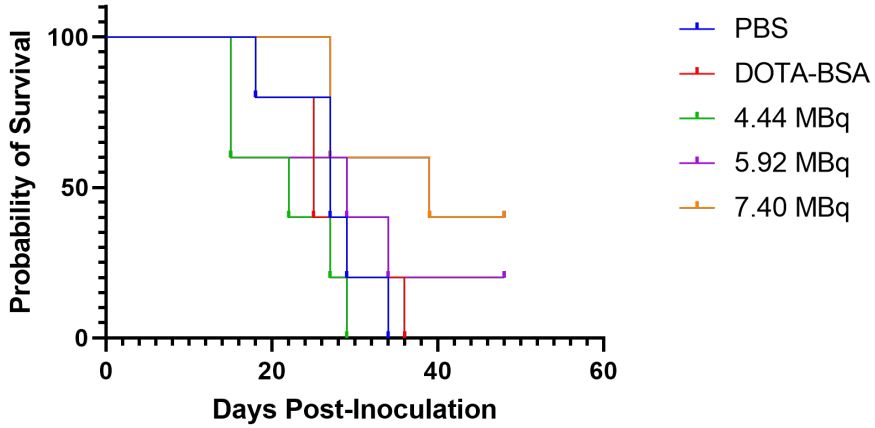


Figure 2-7. Kaplan-Meier survival curve for each treatment group.

To further assess the impact of treatments at the cellular level, histological analysis was carried out on harvested tumours (Figure 2-8). Tissues stained with hematoxylin & eosin (H&E) were evaluated for signs of apoptosis and necrosis using an anti-tumour scoring system from 0-4 (0 = none, 4 = severe). It was found that those treated with PBS had average scores of 2.8 ± 0.45 and 1.6 ± 0.89 for apoptosis and necrosis, respectively, whereas those treated with 7.40 MBq [^{177}Lu]Lu-DOTA-BSA had scores of 3.8 ± 0.45 and 4 ± 0.These findings indicate that high dose treatments are associated with greater destruction to the tumor mass and are also consistent with the trends seen in both tumour progression and survival. Tissues were also investigated for tumour cell invasion outside of the primary mass using a scoring system from 0-2 (0= none, 2= highly invasive). Mice treated with 7.40 MBq [^{177}Lu]Lu-DOTA-BSA had an average score of 0.6 ±0.55 while those treated with PBS had a score of 1.6 ± 0.56, suggesting tumor cells treated with [^{177}Lu]Lu-DOTA-BSA were less able to dissociate from the primary tumour into the surrounding tissues.

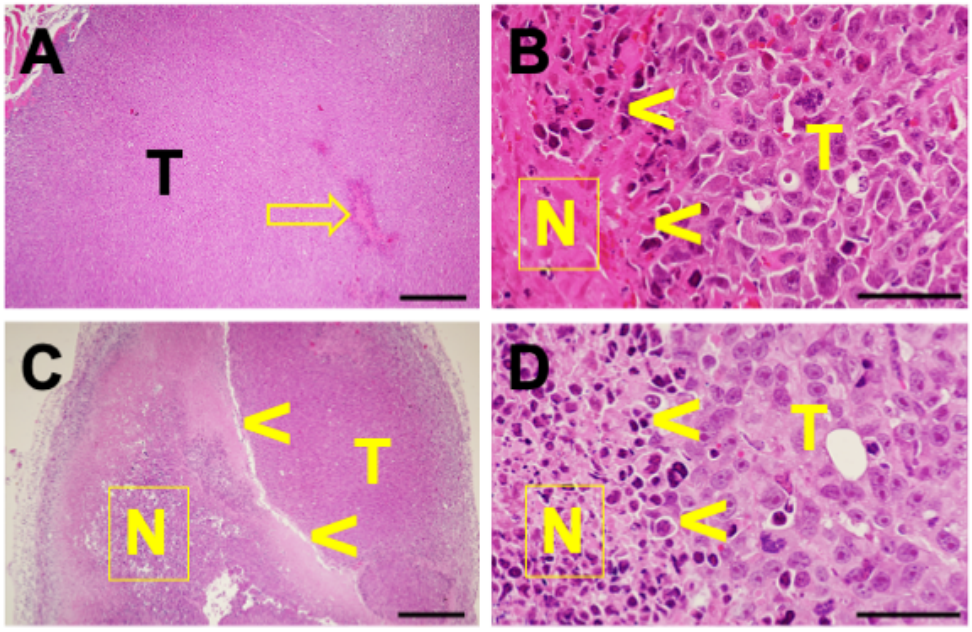


Figure 2-8. Histology of the effect of irradiation treatment on subcutaneous tumors in the mouse model. Microphotographs of the subcutaneous tumor in a mouse treated with PBS (A, B) and with radiation therapy (C, D) show limited areas of coagulation necrosis (open arrow in A; N delineated by arrowheads in B) in the PBS-treated mice and extensive areas of coagulation necrosis in radiation treated ice (N, delineated by arrowheads in C, D) next to the viable tumor (T). H&E, size bars: 500 mm in A, C; 50 mm in B, D.

2.5 Conclusion

We have demonstrated that DOTA-BSA can be prepared and radiolabelled resulting in moderate radiochemical yields and high radiochemical purity, enabling its use in the localized delivery of radiopharmaceuticals. Biodistribution studies and autoradiography revealed high tumour to non-tumour uptake of [^{177}Lu]Lu-DOTA-BSA as well as enhanced tumour retention. A multi-dosing therapy study led to no adverse effects in treated mice and those treated with 5.92 - 7.40 MBq doses were more likely to have a prolonged lifespan. Although therapy studies ceased 40 days post-treatment, 2 of 5 animals from the 7.40 MBq treatment group survived in good health with all tumours exhibiting advanced signs of apoptosis and necrosis, proving this method to be a promising approach for the treatment of TNBC. Future studies can explore additional dosing cycles or the use of other isotopes to further increase therapeutic efficacy and survival.

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Chapter 3 - Preliminary Evaluation of Intratumourally Injected DNP-BSA for Its Use in Antibody Recruiting Therapies

3.1 Introduction

As discussed in Chapter 1, the development of immune checkpoint inhibitor (ICI) immunotherapies and their significant clinical successes in recent years has resulted in immunotherapy becoming a mainstay of cancer treatment.[1–3] This method aims to stimulate the immune system and promote an anti-tumour response by blocking immune checkpoints which are known to downregulate various immune responses.[4,5] Despite the victories seen using ICI immunotherapy, it also comes with limitations including the lack of a prolonged anti-tumour response in the majority of patients and the potential for immune-related adverse events (irAEs) to occur.[6] As a result, alternative immunotherapies are needed in order to enhance tumoricidal effects while also minimizing adverse effects.

One exploratory field in immunology is the development of antibody-recruiting small molecules (ARMs). ARMs are bifunctional molecules consisting of an antibody binding terminus (ABT) and a target binding terminus (TBT). The ABT interacts with anti-hapten antibodies while the TBT recognizes the tumour-associated protein target. The bifunctionality of ARMs allow for its simultaneous association with both antibodies and cell receptors, resulting in the formation of a ternary complex which can elicit antibody dependent immune effector responses.[7,8] This may prove to be an alternative to traditional immunotherapies as they enhance antibody recruitment and binding to tumour cells, subsequently leading to immune mediated clearance.[7]

There are 3 primary mechanisms through which antibodies induce immune-mediated cell death: antibody dependent cellular cytotoxicity (ADCC), complement dependent cytotoxicity (CDC), and antibody dependent cellular phagocytosis (ADCP). ADCC involves the direct

activation of immune cells through the binding of the antibody's crystallizable fragment (F_c) to the F_c receptors on the surface of immune cells, leading to receptor crosslinking and the release of perforin and granzyme which then results in cell death.[7,9] This mechanism also has the potential to lead to long-lasting immunity as it enhances the processing and presentation of specific tumour associated antigens. Next, CDC involves the engagement of plasma complement proteins and begins following cell-surface immobilization of complement proteins such as C1q. This leads to a proteolytic cascade ending in cell lysis or the recruitment of complement-receptor-expressing effector cells, leading to tumour cell clearance.[7] Lastly, ADCP occurs when the Fc regions of antibodies react with the various Fc gamma receptors on macrophages to induce phagocytosis and subsequent death of tumour cells.[10] There is considerably less known about the impact that ADCP has on tumours, compared to ADCC and CDC. This mechanism is thought to induce anti-tumour effects through the phagocytosis of antibody-bound tumour cells, however, it may also act as an immunosuppressive mechanism if apoptotic cells in damaged tissues are phagocytosed.[11]

Traditional antibody-based immunotherapies, such as checkpoint inhibitors, possess certain characteristics which can limit their efficacy. These include the high molecular weight and peptide structure of the antibodies, their "non-self" regions which can induce severe host immune responses, and the fact that only a small subset of patients develop a lasting anti-tumour response.[6,7] The use of ARMs, however, may help to overcome these challenges as they are made with small molecular weight compounds, exploit the use of endogenous antibodies, and have the potential to induce immunity due to the enhancement and presentation of tumour specific antigens. [8] As previously mentioned in Chapter 1, Dubrovska *et al.* were the first to test their DNP-containing ARM *in vivo* for the treatment of prostate cancer and observed that

treatments were able to supress the growth of PSMA-positive tumours 4.8-fold compared to control groups.[12] Additionally, Rullo *et al.* developed an ARM capable of targeting the urokinase-type plasminogen activator receptor (uPAR), a significantly overexpressed receptor in cancer cells.[8] The compound was evaluated in a B16-uPAR mouse model, where it was found that the rate of tumour growth in treated mice was substantially less than the control group and corresponded to a tumour growth inhibition of 90%. It was also found that this compound lead to comparable results to those observed in mice treated with doxorubicin, a first-line chemotherapeutic agent, but without adverse effects such as weight loss. [8]

The most common approach when developing ARMs is to use an ABT which binds to naturally occurring antibodies in humans such as those against rhamnose, galactosyl-(1,3)-galactose (α-Gal), and 2,4-dinitrophenyl (DNP).[13] Research has shown that rhamnose and DNP have significant IgG1 anti-hapten antibody titres which makes them especially relevant as IgG1 is highly effective at inducing Fc-mediated innate immune killing.[13,14] While rhamnose and DNP are both promising haptens, DNP is the most widely used in ARM research. It has been found that approximately 1% of circulating antibodies in humans recognize these nitroarene epitopes, however, the origin of these antibodies is relatively unknown. It has been postulated that potential routes of exposure are through DNP-containing environmental contaminants or the ingestion of proteins containing the nitroaromatic amino acids formed in foods while cooking.[15]

Another consideration when developing an ARM is the target binding terminus. Most often, ARMs are systemically administered and rely on a targeting vector, the TBT, to guide the compound to the site of disease. However, the use of a targeted compound often brings new challenges which include poor pharmacokinetics and difficulty in producing molecules with high affinities toward both the target and the anti-hapten antibody.[16] A method to overcome these

obstacles is the intratumoural administration of the ARM which removes the need for a targeting vector. In recent years, the De Geest group has developed several multivalent antibody recruiting polymers (ARPs) containing a lipid motif at one end which is able to penetrate cell membrane upon IT injection, and DNP moieties for antibody recruitment.[13,17,18] The potential of this intratumoural approach was highlighted as it was demonstrated that their developed ARPs were not only able to recruit anti-DNP antibodies, but also induce phagocytosis in treated cells by human macrophages. Alternatively, the use of a macromolecule such as albumin has the potential to be used to anchor an ARM within the tumour due to its tendency to accumulate in tumour vasculature.[19]

ARMs represent a promising alternative for patients suffering with malignancies that are inadequately treated through traditional immunotherapy and other first-line forms of treatment. Due to their targeted nature and ability to recruit endogenous antibodies, ARMs help to overcome the challenges of traditional therapies, such as off-target effects and rejection of the therapeutic by a host immune response. [7]

3.1.2 Intratumoural Administration of Immunotherapies

While immune checkpoint inhibitors (ICIs) have shown significant clinical success, they do possess limitations, as discussed above, which may be addressed in order to improve patient outcomes. The systemic administration of ICIs can lead to severe immune-related adverse events (irAEs), similar to an autoimmune response, which can be potentially irreversible and life-threatening. Additionally, due to the high cost of ICIs, it may not be a sustainable form of treatment for all healthcare systems or those without health insurance [6]

The intratumoural injection of ICIs in solid tumours has garnered significant attention in recent years as a method to overcome the limitations associated with systemic administration. By

directly injecting the drug into the tumour, highly localized concentrations are achieved which increases the bioavailability of immunostimulatory products.[6] Additionally, due to the reduced drug concentration and the lack of systemic exposure to the drug, the overall chances for irAEs are significantly lessened. The validity of this method has been observed in a phase I trial using IT IL-2 and anti-CTLA-4 for the treatment of patients with advanced melanoma. Both local and abscopal responses were seen in 67% and 89% of patients, respectively, in addition to having an overall response rate of 40%.[20]

Despite the advantages seen through the intratumoural administration of checkpoint inhibitors, their production remains quite costly which may limit widespread use.[6,21] An alternative solution may be the intratumoural administration of an antibody recruiting molecule. Due to the precedence set by the IT administration of ICIs, it is postulated that an IT injected ARM could also lead to positive outcomes while simultaneously overcoming the limitations associated with targeted ARM compounds. Intratumourally injected small molecules without a target binding domain clear quickly from the tumour, preventing any observable immune response or therapeutic effect. To overcome this, there is an opportunity for DNP-BSA, a commercially available albumin conjugate with ~25 DNP moieties per BSA, to be used to induce a therapeutic effect while also enhancing retention of the compound. By coupling a hapten with a macromolecule, there is potential to improve compound retention due to the tendency of high molecular weight proteins to accumulate in tumour vasculature, a phenomenon known as the EPR effect.[22] This is further supported by the results seen in Chapter 2, which demonstrated that intratumourally injected albumin was highly retained within the tumour for up to 72 h post-injection. Additionally, the use of a multivalent compound is advantageous as it increases the

affinity of endogenous antibodies to the target and enhances the efficiency of antibody recruitment which in turn may yield more positive therapeutic outcomes.[13,17]

3.2 Experimental

3.2.1 Materials and Instrumentation

All antibodies and reagents were purchased from Sigma Aldrich, Life Technologies, ThermoFisher, Bethyl Laboratories, Cedarlane, and BD Biosciences and used without further purification. ELISA absorbances measurements were acquired using as Tecan Infinite M10000 plate reader. Flow cytometry was performed using a BD LSRII™ flow cytometer (BD Biosciences, USA).

3.2.2 Animal Studies General

All animal studies were approved by the Animal Research Ethics Board at McMaster University. Mice were maintained under clean conditions in an established animal facility with 12 hour light/dark cycles and given food and water *ad libitum*.

3.2.3 Subcutaneous Immunization with DNP-KLH in FIA

Prior to the first immunization, 6-8-week-old C57Bl/6 mice were retro-orbitally bled and the serum isolated. DNP-KLH (Cedarlane, D8060-09D) in sterile PBS (1 mg/mL) was added to FIA (1:1), vortexed until emulsified, and 100 μL subcutaneously administered to mice every two weeks for a total of 6 weeks. Three to five days following the final immunization, mice were retro-orbitally bled and the serum isolated.

3.2.4 ELISA: Determination of anti-DNP titre in serum

Briefly, DNP-BSA (Life Technologies, A23018) in PBS was diluted to 1 μg/mL with coating buffer (Sigma Aldrich, C3041) and transferred (100 μL/well) to a 96-well plate. The coated plate was then incubated at room temperature for 1 h, followed by three washes with

ELISA wash buffer (Sigma Aldrich, T9039). Blocking buffer (Sigma Aldrich, T6789) was then added to the plate (200 μL/well) and allowed to incubate for 0.5 h, followed by three washes with ELISA wash buffer. Serum dilutions ranging from 1:100 to 1:100 000 or diluent alone were added to the plate (100 μL/well) and left to incubate at room temperature for 1 h. Following three washes with ELISA wash buffer, the secondary antibody goat anti-mouse IgG-AP (1:5000) was added to each well (100 μL) and left to incubate at room temperature for 1 h. The plate was then washed five times with ELISA wash buffer followed by the addition of PNPP substrate (100 μL/well) and its subsequent incubation in the dark for 0.5 h at room temperature. To stop the reaction, 2 M NaOH (50 μL) was added to each well before reading the absorbance of the plate at 405 nm.

3.2.5. Flow Cytometry: Sample Preparation

Blood samples were first collected in Eppendorf tubes containing heparin (10 μL). Blood from each mouse (100 μL) was then transferred into individual tubes for processing. ACK lysing buffer (2 mL) was added to each sample, the mixture vortexed, and then incubated at room temperature for 5 min. Following lysis, Hank's Balanced Salt Solution (2 mL) was added and the mixture centrifuged at 400 rcf for 5 min. The supernatant was discarded followed by the addition of ACK lysing buffer (1 mL) which was allowed to sit for 5 min at room temperature. Hank's Balanced Salt Solution (2 mL) was then added and the mixture was centrifuged at 400 rcf for 5 min. The supernatant was discarded and the remaining liquid (100 μL) transferred to separate tubes along with PBS (100 μL) and centrifuged (400 rcf for 5 min). The supernatant was then removed, resuspended in PBS (200 μL) and centrifuged (400 rcf for 5 min). The supernatant was removed, resuspended in 100 μL FVS (1:500 in PBS; BD Biosciences, 564406), and left to incubate in the dark for 30 min at room temperature. To each tube was added 100 μL PBS

followed by centrifugation (400 rcf for 5 min). The supernatant was discarded, 25 μL Fc block (1:200 in BV staining buffer; BD Biosciences, 553141) added, mixed, and incubated for 30 min in the dark at 4 °C. The antibody master mix (25 μL; see note below) was then added and left to incubate for 30 min in the dark at 4 °C. FACS buffer (150 μL) was then added, mixed by pipetting, and centrifuged (400 rcf for 5 min). The supernatant was removed, PBS (200 μL) added, and the mixture centrifuged (400 rcf for 5 min). Cytofix (ThermoFisher, 00-5523-00) was added (100 μL, 1:3 concentrate:diluent) and left to incubate for 30 min at 4 °C after which FACS buffer (100 μL) was added and the mixture centrifuged (400 rcf for 5 min). The supernatant was removed, resuspended in FACS buffer (200 μL), and centrifuged (400 rcf for 5 min). The supernatant was then removed, resuspended in FACS buffer (200 μL), and added to FACS tubes for analysis.

Note: To make the antibody master mix, CD4 (1:200; BD Biosciences, 561830), CD8 (1:200; BD Biosciences, 563046), B220 (1:200; BD Biosciences, 563894), CD19 (1:200; BD Biosciences, 552854), CD11b (1:200; BD Biosciences, 553311), CD11c (1:100; BD Biosciences, 562782), F4/80 (1:100; BD Biosciences, 743282), Ly6C (1:200; BD Biosciences, 553104), and Ly6G (1:200; BD Biosciences, 560602) were diluted in BV staining buffer.

3.2.6. Pilot ARM Therapy Study

Female, 6-7 week-old, C57Bl/6 mice (Charles River Laboratory, Wilmington, MA, USA) immunized with DNP-KLH in FIA and non-immunized were inoculated with 5×10^6 E0771 cells in the left flank. Immunized and non-immunized mice with tumours ~100 mm^3 in size were separated into three groups (n=5) and administered PBS (control; immunized mice) or DNP-BSA (100 μg, 35 nmol; immunized and non-immunized mice) three times per week for a total of

4 doses. Animals were followed for survival and tumours measured every 2-3 days. Mice were sacrificed when they reached endpoint (ulcerated tumours or tumour volume ≈ 1500 mm^3).

3.2.7 DNP-BSA Tolerability Study

Female, 6-7 week-old, C57Bl/6 mice (Charles River Laboratory, Wilmington, MA, USA) immunized with DNP-KLH in FIA were inoculated with 5×10^6 E0771 cells in the left flank. Mice with tumours ~100 mm^3 in size were separated into five groups (n=5) and administered PBS (control), BSA (control), low dose DNP-BSA (9 nmol), medium dose DNP-BSA (17 nmol), and high dose DNP-BSA (35 nmol) once per week for three weeks. Animal health was monitored over the course of the experiment and tumours measured every 2-3 days. Mice having reached a pre-determined endpoint (tumour volume ~1500 mm^3) were sacrificed and their tumours removed for IHC analysis.

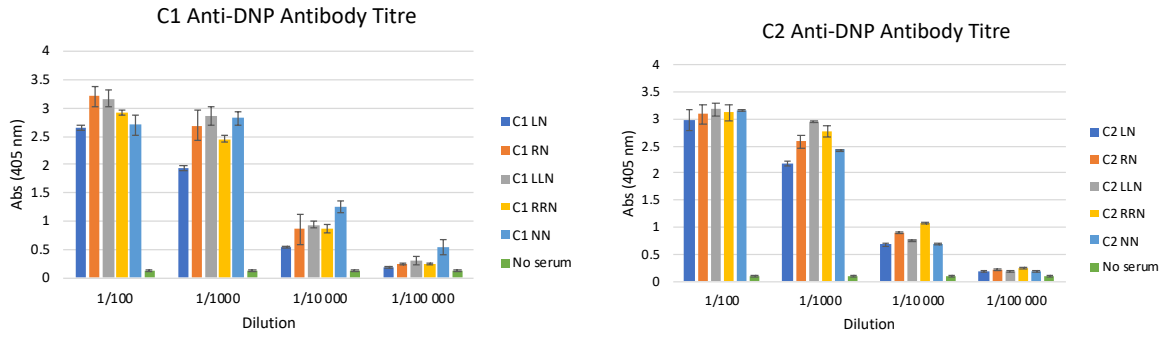
3.3 Results and Discussion

3.3.1 Immunization with DNP-KLH in FIA

While humans are known to have endogenous anti-DNP antibody titres, mice do not, and require immunization prior to DNP-based ARM therapies. Small molecules, such as DNP, are unable to induce a significant immune response due to their low molecular weight and non-immunogenicity. To circumvent this issue, antigens are often coupled to highly immunogenic, high molecular weight carrier proteins such as keyhole limpet hemocyanin (KLH) in order to strengthen the immune response. [23] Previous work has shown that repeated administrations of DNP-KLH, a protein conjugate containing ~400 DNP moieties per KLH, is unable to induce a lasting titre on its own.[24] This finding is also supported in the literature, where it has been shown that while daily subcutaneous immunizations with an antigen can elicit antibody titres, it quickly begins to decrease to background levels once immunizations cease.[25] As a result, Freund's

incomplete adjuvant (FIA) was used to enhance the immune response in order to induce a long lasting antibody titre. Adjuvants are thought to operate through various mechanisms, with a primary mechanism being the "depot" effect in which the adjuvant prevents the antigen from being degraded too quickly by the host and instead slowly releasing the antigen. The steady release of antigen allows for high antibody titres to be produced as it continues to stimulate antibody-producing cells.[26]

Mice were administered DNP-KLH in FIA (1:1) once every 2 weeks for a total of 6 weeks and blood was collected prior to the first immunization as well as 3-5 days after the final immunization.[24] An ELISA was then carried out on the blood samples in order to observe if a significant titre was induced in the animals. Briefly, serum dilutions ranging from 1:1000 to 1:100 000 were added to a coated 96-well plate and left to incubate for 1 hour before washing and adding the alkaline-phosphatase-coupled secondary antibody. Following incubation with the secondary antibody, an enzymatic substrate was added, subsequently quenched, and its absorbance read (Figure 3-1). It was found that animals had an observable anti-DNP antibody titre out to a 1:10 000 dilution and as such, pilot antibody recruiting studies were carried out.



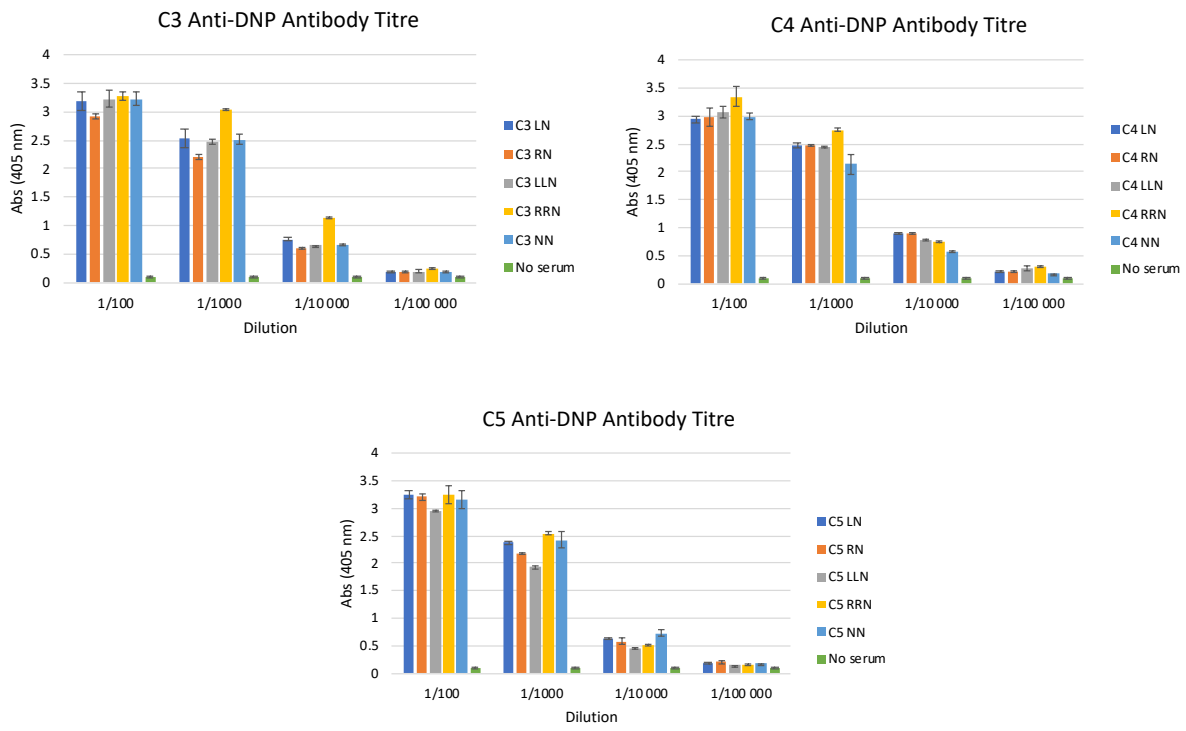


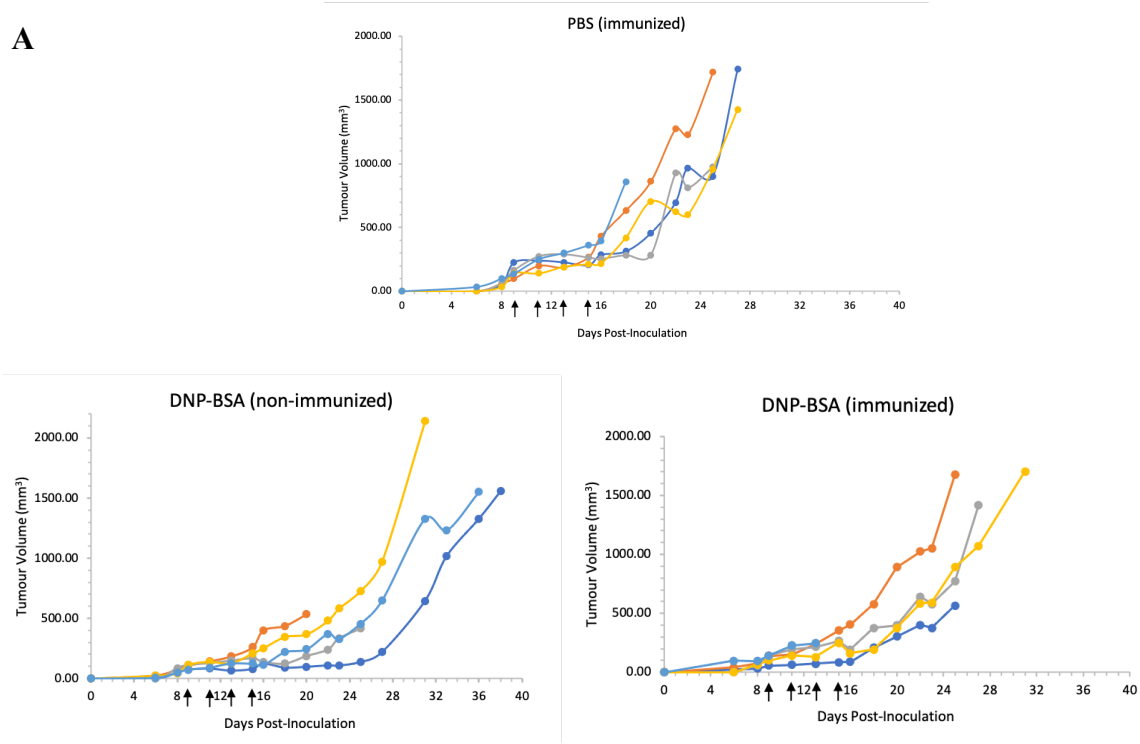
Figure 3-1. Antibody titres from 25 mice immunized with DNP-KLH in FIA. Each graph represents the mice in one cage (CX = cage number; LN – left ear notch, RN – right ear notch, LLN – 2 left ear notches, RRN – 2 right ear notches, NN – no ear notch).

3.3.2 Pilot ARM Therapy Study

While the use of ARMs is promising, these compounds often lack biodistribution data and are commonly administered I.V or I.P in widely varying antigen concentrations.[8,12,27,28] One previous study using DNP-Tz-TCO-BSA, a functionalized albumin molecule using DNP and BSA as an ABT and pseudo TBT respectively, has shown that the intratumoural administration of 1.5-1.9 nmol of DNP three times per week does not lead to a significant survival advantage in treated mice when compared to control mice. [24] Due to the limited *in vivo* precedence of ARMs, a pilot study using a higher concentration of DNP was carried out in order to observe the effect it had on treated animals. DNP-BSA, a commercially available albumin conjugate, was used to prolong the retention time of the compound in the tumour and lead to more positive outcomes.

Both immunized and non-immunized mice were administered 35 nmol of DNP intratumourally every other day once tumours became palpable (~9 days post-inoculation), up to a total of 4 doses. Following the 3rd dose, an immunized mouse was non-responsive and was thus euthanized. Assuming this to be an experimental outlier, animals were given a 4th dose after which it took >1 h for immunized mice to recover, indicating an inability to tolerate repeated doses of DNP-BSA and/or the increased concentration of DNP. Once treatments ceased, the mice were followed for survival and their tumours measured every 2-3 days. Results can be seen in Figure 3-2, where no significant differences in tumour growth or survival were observed in any group.

A



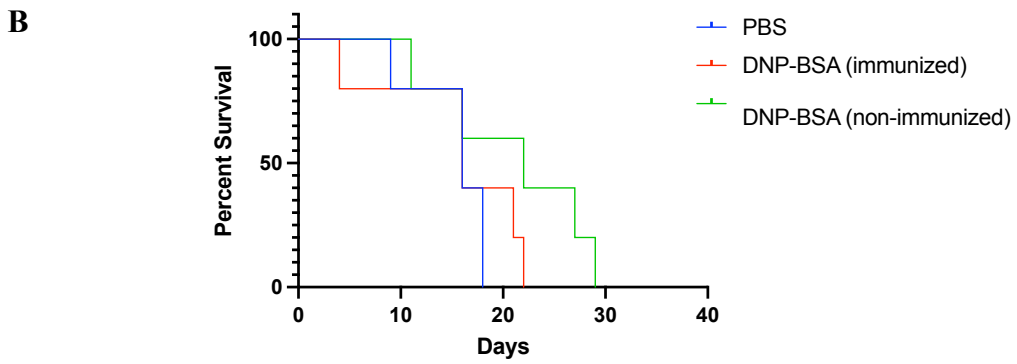
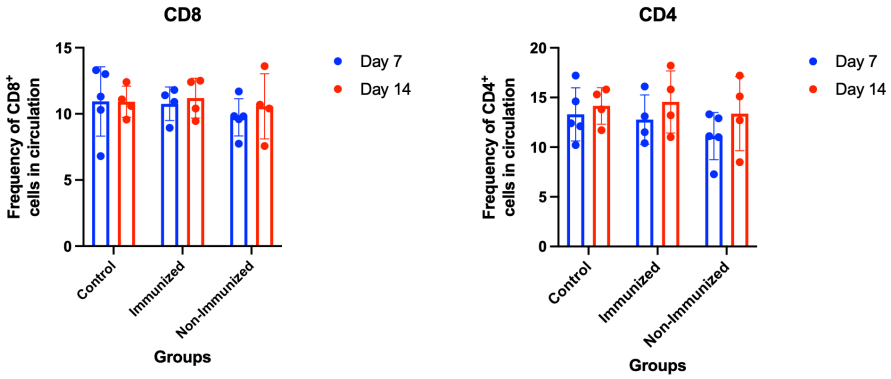


Figure 3-2. Pilot ARM therapy results (n=5). (A) Tumour growth charts of the control (top and bottom left) and treated (bottom right) groups. Arrows signify each administered dose. (B) Kaplan-Meier curve depicting the survival time of each group.

In addition to monitoring the external response of the animals over the course of treatment, the immune response was also assessed using flow cytometry and immunohistochemistry. Mice were bled days 7 and 14 post-first treatment and the samples processed for flow cytometry analysis (Figure 3-3). An adaptive immune response due to an effective ARM therapy would be represented by an increase in T cell and B cell populations over the course of treatment. However, no statistically significant change was observed between any of the groups between weeks 1 and 2 indicating that under the tested conditions, the ARM therapy was not effective at inducing an adaptive immune response nor a therapeutic effect within 14 days post-injection.



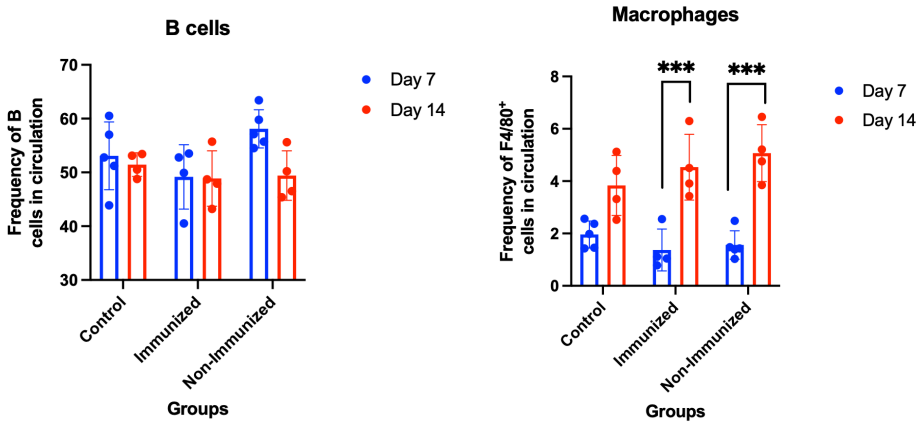


Figure 3-3. Flow cytometry analysis of peripheral blood from treated mice. * $p < 0.05$; *** $p < 0.01$.

A statistically significant increase in macrophage populations in both immunized and non-immunized mice treated with DNP-BSA is observed which may signify that ADCP had occurred. However, because only immunized mice possess sufficient anti-DNP antibody titres to illicit such a response, this cannot be confirmed without further experimentation. While it is possible that the repeated administration of DNP-BSA may lead to antibody production in non-immunized mice followed by an immune response to treatments, without the use of an adjuvant the titre is unlikely to be significant nor long-lasting enough to do so.[25,26] More likely is that the observed increase may be explained as more of an innate response as macrophages are part of the innate immune system and have many different functions. They are known to be one of the most abundant immune cells associated with cancer, with higher populations being correlated to tumour progression as they can be protumourigenic.[29,30] In light of this, the increased macrophage population may be related to tumour advancement in the animals. An alternative possibility for the increase seen is that the macrophages are responding to the presence of immunogenic BSA. Macrophages are not only associated with cancer but also with the recognition and removal of foreign materials and are known to adhere to BSA.[31–33] If the concentration of BSA is too high and is recognized as a foreign body, it is possible that this

cellular increase is the result of macrophage recruitment and adherence to the protein. Macrophages can be separated into 2 subtypes, M1 and M2, which are known to be different in function.[34,35] M1 macrophages are characterized by their ability to induce pathogen and tumour cell elimination whereas M2 macrophages are characterized by tumourigenesis. In our case, the subtype of macrophage recruited was not deduced and we are unable to confirm if one or both reasons play a role in the observed increase without further experimentation. Further investigation into the increased macrophage population is needed in order to determine if this response was tumouricidal or tumourigenic.

In addition to looking at the immune cell population in the peripheral blood, the immune presence in the tumour itself was also investigated. Seven days after the first treatment, 5 mice were sacrificed and their tumours removed for histological analysis. As seen in Figure 3-4, there is no observable difference in $CD4^+$ and $CD8^+$ populations between groups which also aligns with the previously discussed flow cytometry data. The presence of $F4/80^+$ macrophages in the tumour is also consistent with the observed flow cytometry results. While their increase may be explained by the occurrence of ADCP, as previously discussed it may also be explained by an advanced state of disease or an innate response to BSA, resulting in a need for further investigation. Additionally, H&E-stained tissues from each group are consistent in appearance, indicating that no significant tissue damage was achieved as a result of the treatments.

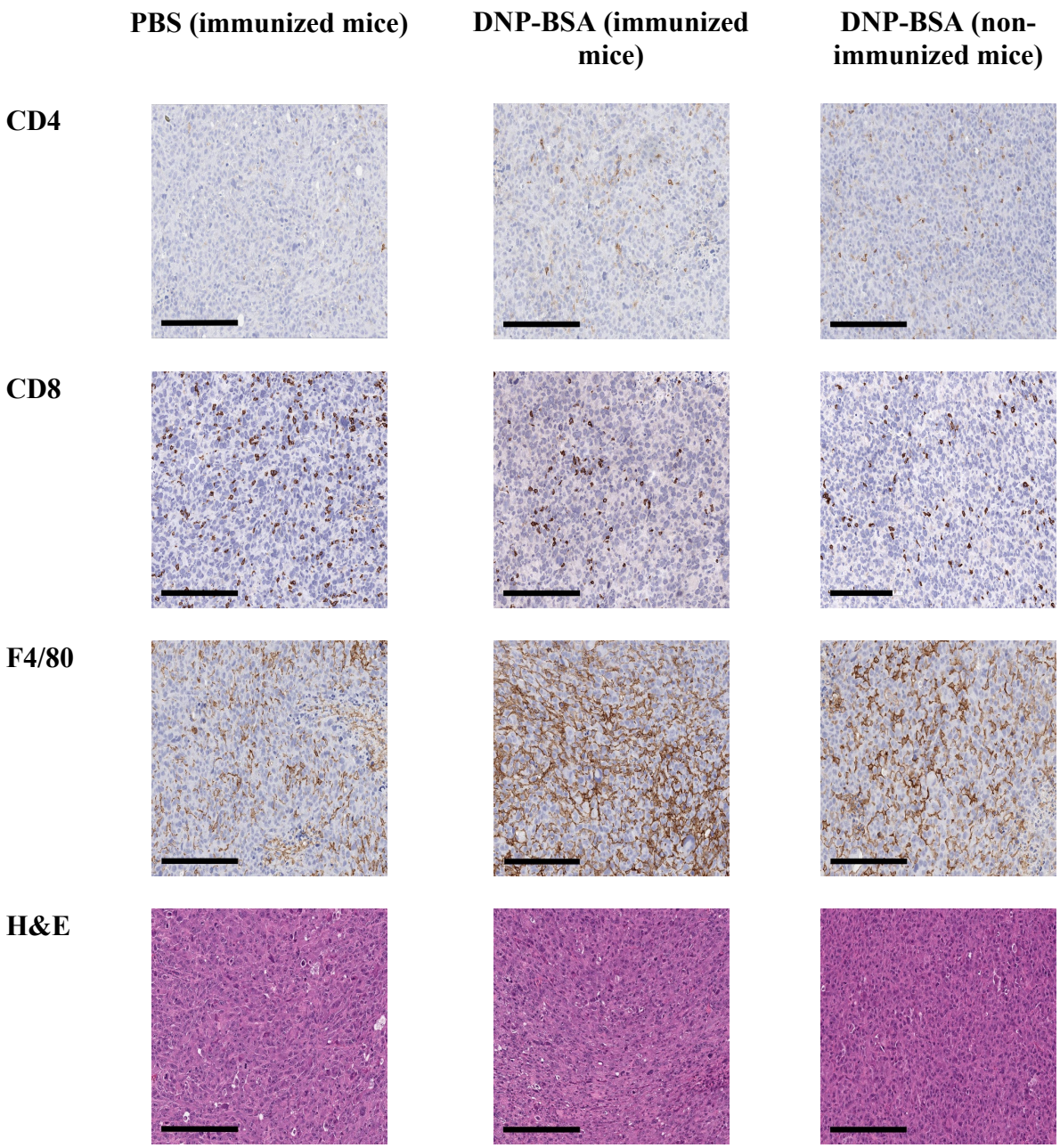


Figure 3-4. IHC analysis of immune cell infiltrates. Tumours were sectioned and stained with CD4, CD8, F4/80, and H&E for pathologic analysis. Each representative image shows a section from an individual tumour. Scale bar = 200 μm.

As previously mentioned, neither flow cytometry nor IHC analysis indicated a significant adaptive immune response in treated versus control mice, which is consistent with the observed tumour growth rate and survival curves. While the literature suggests that a peak immune

response can occur as early as 3 days post-treatment, it has also been reported that it may take as long as 14 days for an adaptive immune response to occur.[36,37] Taking this into consideration, it is possible that the blood and tissue samples were harvested before a significant response had taken place.

3.3.3 DNP-BSA Tolerability Study

Due to the lack of therapeutic efficacy and adverse effects seen in the pilot study, a tolerability study was done in order to determine an appropriate dosing regimen and observe whether an immune response occurs at a later time point. It was thought that the frequency of dosing may be too high and due to evidence that shows BSA compounds are retained in the tumour as long as 120 h, the dose frequency was reduced to once per week.[24] Immunized mice were administered 9 (low), 17 (moderate), or 35 (high) nmol of DNP-BSA once per week for three weeks and were sacrificed as they reached a pre-determined endpoint (tumour volume ~ 1500 mm^3). Body weight amongst animals was maintained throughout the experiment and the low and medium doses were well tolerated. Mice receiving a high dose began to respond in a similar manner as the initial pilot study (Figure 3-5). Following the 2nd and 3rd doses of high dose DNP-BSA, animals were slower to recover (~10 min) with one having to be euthanized. This indicates that even at a lower dosing frequency, animals do not easily tolerate 35 nmol DNP-BSA and as such, lower concentrations should be used for future studies.

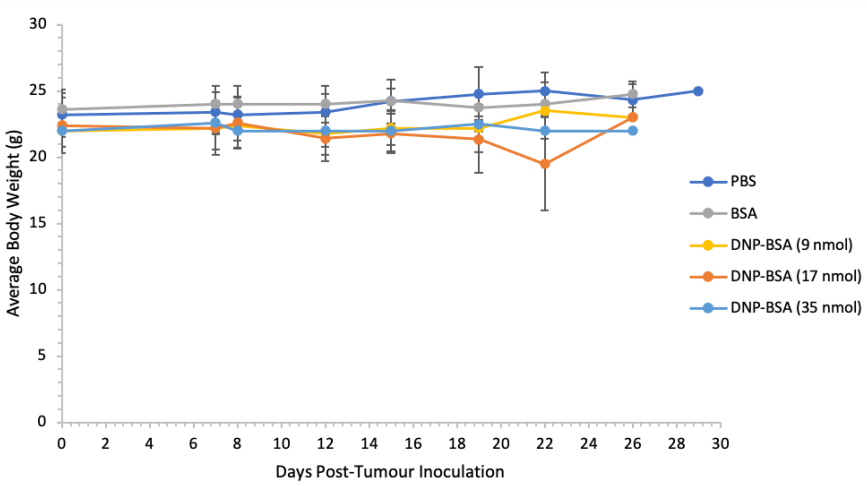
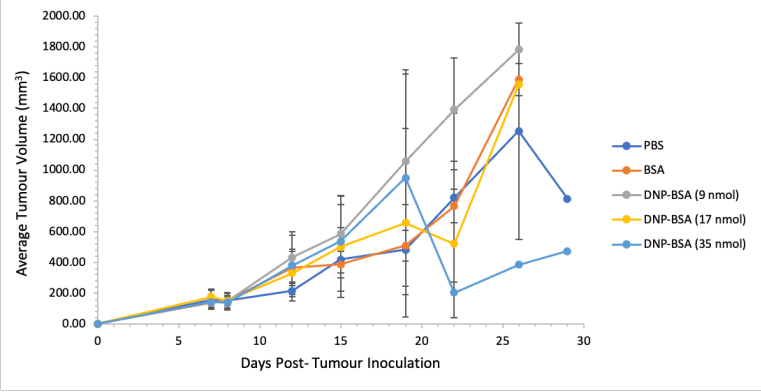


Figure 3-5. DNP-BSA Tolerability Study (n=5). Average body weight of each treatment group over time.

Despite the well tolerated low and moderate doses, a therapeutic effect was not observed in any DNP-BSA treated mice. The tumour growth and lifespan of each cohort were followed over the course of the experiment and monitored for any significant differences between groups. Tumour volumes and survival time of the animals was found to be consistent between groups with all tumours growing at a similar rate and mice reaching endpoint 25-30 days post-tumour implantation (Figure 3-6).



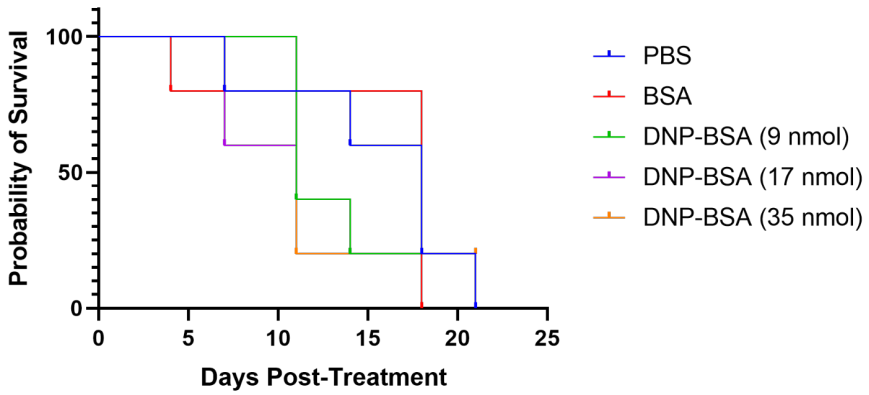


Figure 3-6. DNP-BSA tolerability study (n=5). Top: Average tumour volumes of each treatment group over the course of the experiment. Bottom: Kaplan Meier curve depicting the survival time of each group.

In order to evaluate the impact of the treatments on the tumour at the cellular level, tumours were removed and fixed at endpoint for histological analysis. Tumours were stained for the presence of $CD3^+$ T-cells and $F4/80^+$ macrophages in addition to basic cell morphology. While it is preferable to compare tissues of the same age, the main goal of this study was to observe the animal's health throughout and by removing tumours at a pre-defined endpoint, we are able to gain insight into the intratumoural immune presence in mice at the peak of their disease (Figure 3-7).

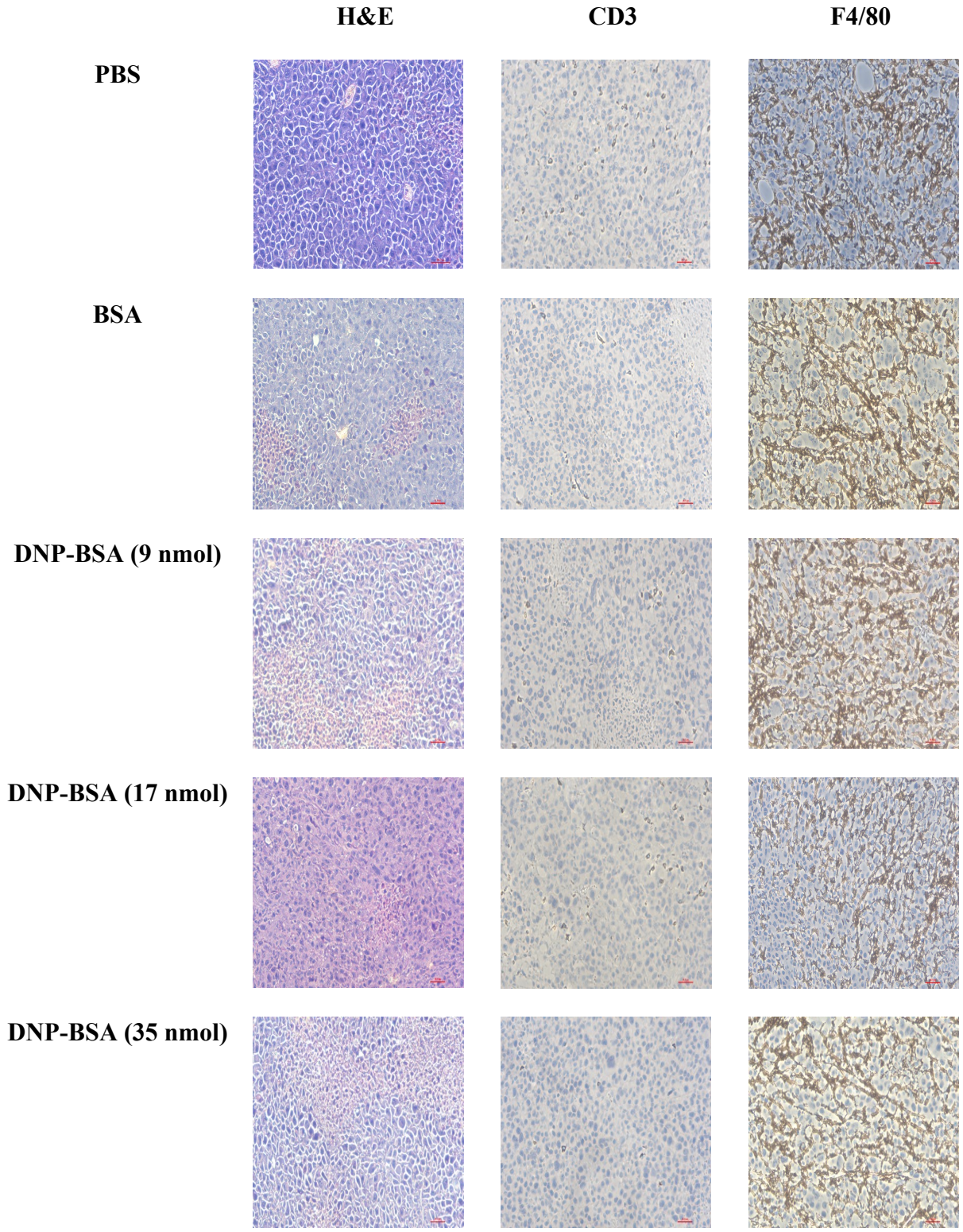


Figure 3-7. IHC analysis of immune cell infiltrates. Tumours were sectioned and stained with CD3, F4/80, and H&E for pathologic analysis. Each image shows a section from an individual tumour at 20× magnification.

Upon analysis, no discernible differences were observed between cohorts and the intratumoural immune presence was more reflective of advanced disease than antibody recruitment. Minimal T-cell recruitment was seen in DNP-BSA treated mice which is indicative that even at later timepoints an adaptive immune response was not triggered by the treatments. The T-cell population and cell morphology in treated mice is also consistent with control mice, further demonstrating that this response is not a result of antibody recruitment and treatments are not inducing significant damage. Unlike in the pilot study, there does not appear to be an increase in F4/80 macrophages in DNP-BSA-treated mice, suggesting their presence is more likely to be due to the advanced stage of disease as results are consistent between treatment and control groups. However, because a reduced dosing frequency was used in this experiment which may have lessened the macrophage response, we cannot exclude the hypotheses regarding ADCP induction as a result of treatments and/or an innate response to BSA without further investigation. The reason for these poor outcomes is unlikely to be due to poor retention of the compound as it was demonstrated in Chapter 2 that intratumourally injected albumin conjugates are retained up to 72 hours post-injection. By frequently administering the compound throughout each ARM therapy, a sufficient amount was present in the tumour over the entirety of the experiment. These results are more likely to be explained by a dosing regimen that is of too low of a frequency, or it may be because the circulating antibodies are unable to recognize the presence of the IT injected compound. The inability of therapeutic antibodies to reach their target is common and is thought to be due to the suboptimal blood circulation and increased interstitial fluid pressure that is often associated with tumour microenvironments.[38,39]

3.4 Conclusion

A pilot antibody recruiting therapy study was carried out in a triple negative breast cancer mouse model and its effects assessed. No notable survival advantage or slowed tumour progression was observed in cohorts treated with DNP-BSA when compared to those treated with PBS. Additionally, treated animals poorly tolerated repeated doses of the antigen indicating that 35 nmol of DNP was above the maximum tolerated dose. Following the pilot study, a tolerability study was then carried out in order to determine a dosing regimen which does not result in adverse effects to the mice. Animals were treated with 9, 17, or 35 nmol concentrations of DNP-BSA once per week for three weeks and the effects observed. It was found that the maximum tolerated dose is 17 nmol of DNP-BSA, as a concentration of 35 nmol was poorly tolerated even when administered once per week. Despite the tolerability of low to moderate doses of DNA-BSA, no therapeutic effect or indication of an adaptive immune response at the cellular level was observed. These results may be due to a suboptimal treatment schedule or the inability of circulating antibodies to recognize the compound. Going forward, moderate to low doses of DNP-BSA may be used to test an appropriate dosing frequency that is able to elicit antibody recruitment. Alternatively, the use of other haptens or binding domains may be explored and their efficacy in inducing an adaptive immune response investigated.

3.5 References

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Chapter 4 - Conclusion and Future Work

4.1 Summary

The aim of this thesis was to evaluate intratumourally injected, albumin-based therapeutics for their use in both radiotherapy and antibody recruiting therapy. Due to the possibility for each therapeutic modality to synergize and elicit enhanced efficacy when used in combination, the goal was to first assess and optimize each monotherapy.

First, DOTA-BSA was synthesized and its radiolabelling optimized prior to being evaluated *in vivo* (Chapter 2). A biodistribution study was performed, which revealed significant tumour uptake and retention up to 72 h post-injection with minimal non-target uptake. Additionally, autoradiography depicted that the compound disperses non-homogeneously in treated tumours. Multi-dosing therapy studies using the lutetium-177 labelled compound demonstrated that mice having received repeated administration of 5.92 MBq – 7.40 MBq were more likely to experience prolonged survival than those treated with 4.44 MBq, PBS, or unlabelled DOTA-BSA. Seven days following the first treatment, tumours were removed and processed for histological analysis. It was found that tumours having been treated with 7.40 MBq doses of [^{177}Lu]Lu-DOTA-BSA exhibited higher levels of both apoptosis and necrosis with average anti-tumour scores of 3.8 ± 0.45 and 4 ± 0, respectively, whereas those treated with PBS had scores of 2.8 ± 0.45 and 1.6 ± 0.89, respectively.

Using commercially available DNP-BSA, an antibody-recruiting therapy was also investigated (Chapter 3). Due to the limited precedence of ARMs in the literature, a pilot study was first carried out to assess the animal's response to treatments. A cohort of animals were first immunized with DNP-KLH in FIA to produce a significant anti-DNP antibody titre which was then confirmed through an ELISA. Both immunized and non-immunized mice were then treated

with 35 nmol DNP-BSA three times per week, a dosing schedule which ultimately led to adverse effects in the animals and resulted in a premature end to the experiment. The immune presence in circulating blood was also investigated over the course of the pilot study. Animals were bled 7 and 14 days post-treatment and the PMBCs evaluated through flow cytometry. No significant differences were seen in the presence of T cells ($CD4^+$ and $CD8^+$) or B cells ($B220^+$) and while a significant increase in $F4/80^+$ macrophages was seen in DNP-BSA treated mice, this was not likely to be a positive response to treatment. Next, a tolerability study was done in order to determine the maximum tolerated dose concentrations of DNP-BSA without any adverse effects when administered at a lower frequency. Mice were treated once per week with 9, 17, or 35 nmol of DNP-BSA and it was found that those treated with moderate to low concentrations did not exhibit adverse effects. Histological analysis was done on the tumours from both studies in order to assess the intratumoural immune response and determine whether an adaptive immune response occurred as a result of the treatments. Tumour tissues were stained for the presence of T cells ($CD3^+$, $CD4^+$, $CD8^+$), macrophages ($F4/80^+$) and basic cell morphology (H&E). Unfortunately, no significant differences were observed between groups in either study, indicating an adaptive response had not occurred.

4.2 Future Directions

4.2.1 Combination Radiotherapy and Antibody Recruiting Therapy

As discussed in Chapter 3, a lack of antigen recognition by circulating antibodies may be a possible explanation for the unfavourable outcomes seen in preliminary antibody recruiting therapy studies. The tumour microenvironment often has heightened interstitial fluid pressure as well as a poor vasculature which lessens blood circulation, factors which are both thought to limit the penetration depth of antibodies within a tumour.[1,2] A method to overcome this obstacle

is through the use of a combination therapy approach with [^{177}Lu]Lu-DOTA-BSA (Chapter 2), as researchers have shown that radiation is able to enhance antibody penetration into a tumour.[3–5] In a study done by Johansson *et al*., tumour bearing mice were intraperitoneally administered an iodine-131-labelled monoclonal antibody before, during, or after external beam radiation therapy (EBRT) or alone. Results showed that mice who receieved EBRT prior to the radiolabelled antibody had the highest percent injected dose per gram in tumour tissue when compared to the other treatment groups. Due to this precedence, it's believed that by first irradiating the tumour, the tumour microenvironment will be "broken up" allowing for an increased number of penetrating antibodies and thus an increased immune presence at the site. Additionally, not only will this method give the opportunity to increase antibody penetration, it also allows for synergies to occur between each therapy which may lead to an enhanced therapeutic effect.

Combination therapies have come to the forefront of cancer treatment due to their enhanced efficacy compared to traditional monotherapy approaches.[6] One popular method of combination therapy is EBRT alongside checkpoint inhibitor immunotherapies which have been shown to slow tumour growth, induce immune changes within the tumour microenvironment, and even induce abscopal effects.[7–9] While this approach has proved to be successful, there is still room for further investigation and improvement. The use of internal, targeted radiotherapy may give the opportunity for enhanced tumouricidal activity as this approach delivers a continuous dose of radiation over longer periods of time as opposed to EBRT, where doses are delivered intermittently. Additionally, immune checkpoint inhibitors are limited by immune related adverse events (irAEs) which are often difficult to predict and treat.[10,11] To overcome these challenges and perhaps enhance therapeutic efficacy, the combination of each monotherapy outlined in chapters 2 and 3 may serve as an alternative approach to treatment.

Prior to starting the combination therapy, an *in vivo* study investigating the dosing schedule of the ARM monotherapy should be carried out. Based on the results observed in Chapter 3, a DNP-BSA concentration of 17 nmol should be used as this was the highest dose that did not elicit adverse effects in treated animals. Since neither a therapeutic effect nor adaptive immune response was seen when this dose was administered once per week, a more frequent dosing schedule should be investigated. Following the optimization of the treatment regimen, the monotherapies may be applied to a combination therapy approach with the first dose being [^{177}Lu]Lu-DOTA-BSA followed by DNP-BSA three days later in order to enhance antibody penetration into the tumour (Figure 4-1). The frequency at which DNP-BSA is administered throughout the combination therapy should be chosen following the optimization of the antibody recruiting monotherapy.

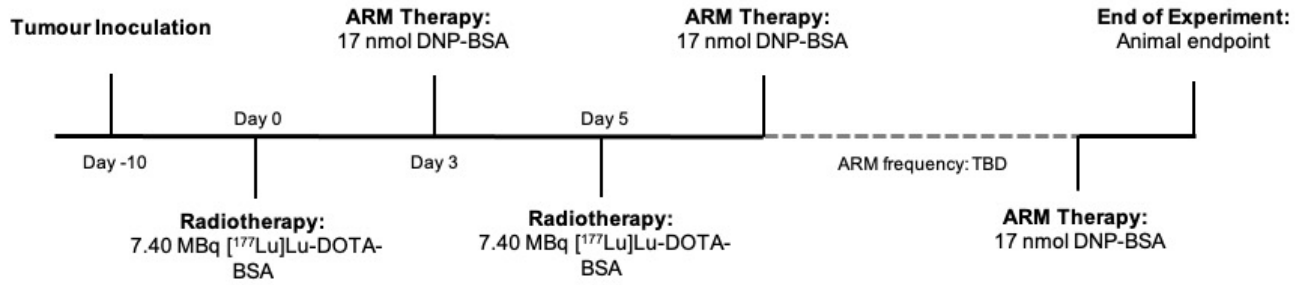

Figure 4-1. Proposed combination radiotherapy and antibody recruiting therapy treatment schedule.

Alternatively, the use of an alternative ARM strategy may also be investigated as results detailed in Chapter 3 suggest that the current DNP-BSA construct may be unable to elicit antibody recruitment. Targeted ARMs continue to be of interest in the development of new contructs, however, their limitations remain which has caused several researchers to begin to evaluate non-targeted approaches. De Coen *et al.* have recently developed an antibody recruiting glycopolymer (ARGP) consisting of a rhamonse backbone for antibody recruiting and a

lipophilic motif for penetration into the cell membrane.[12] This method may overcome the suggested lack of antibody recogition following treatment with DNP-BSA as the lipophilic end of the ARGP inserts into the cell surface while the rhamnose moieties remain exposed at the surface and were found to bind IgG in human serum. Additionally, Uvyn *et al*. developed a similar ARM construct consisting of a lipid motif for cell membrane penetration and DNP moieties for antibody recruitment.[13] This study demonstrated a greater than 4 log increase in binding avidity when the polymer contained multiple units of DNP as opposed to a multivalent compound as well as the ability to recruit anti-DNP antibodies. Their developed ARP was also found to induce antibody dependent phagocytosis to a similar extent as the monoclonal antibody Cetuximab *in vitro.* These results suggest that using an ARM designed to have its ABT present on the cell surface reduces the chances of compound internalisation and promotes enhanced antibody recruitment to the site, thus providing an opportunity for improved therapeutic efficacy.

4.2.2 Investigating the Immune Response to ARM Therapy

Macrophages are abundant in the immune system and are well known to play both pro-tumourigenic and pro-tumouricidal roles.[14] These cells can generally be seperated into M1 and M2 subtypes which are known for pathogen/cancer cell elimation and tumour promotion, respectively.[15,16] In instances of cancer, macrophages play a key role in aiding tumour progression in ways such as stimulating angiogenesis and inducing immunosuppression in order to avoid tumour cell death. Additionally, trends have been observed in that a high density of tumour associated macrophages is also associated with poor patient prognosis. On the other hand, these cells are also necessary for basic functions including the elimination of pathogens through phagocytosis and maintaining homeostasis.[17,18] In light of this information, the reason for the increased macrophage population observed in the pilot antibody recruiting study in

Chapter 3 becomes difficult to deduce without further experimentation. It may be explained by the induction of ADCP as a response to treatments or it may be due to the advanced stage of disease and administration of BSA. To first determine if the observed macrophage increase is a positive response to treatment, DNP-BSA should be evaluated for its ability to induce ADCP through the use of cell-based assays. A positive outcome would confirm the formation of a ternary complex resulting in phagocytosis, which would then prompt the need for further investigation as to why a therapeutic effect was not observed in Chapter 3. In the case of a weak ADCP response having been induced, there is potential for it to be enhanced through the manipulation of tumour associated macrophages as the M1 subtype has been shown to induce a more efficent ADCP response.[19,20] Macrophages present in the tumour microenvironment may also be characterized in order to investigate the possibility that the observed increase is a pro-tumorigenic response. A majority of M1 macrophages would suggest an anti-tumour response while a majority of M2 macrophages would be indicative of tumour progression.

4.2.3 Multi-dosing Radiotherapy

Although the results seen in Chapter 2 are promising, the treatment regimen can be improved upon in order to induce a complete response in tumour-bearing animals versus tumour growth suppression. This may be done by increasing the number of administered doses, increasing the amount of activity given, or by using different isotopes such as yttrium-90 or actinium-225.

A fractionated approach should continue to be pursued due to its clinical relevance and the evidence that smaller, more frequent doses are well tolerated and also better able to enhance therapeutic efficacy and anti-tumour immunity.[21–24] In the work reported, mice treated with two 7.4 MBq doses of [^{177}Lu]Lu-DOTA-BSA experienced slowed tumour growth in comparison to

other treatment groups. However, growth resumed 17-22 days following the first treatment. Given that these doses were well tolerated, future studies that involve the administration of an equivalent third dose on day 10 of treatment may be carried out. This may prevent tumour regrowth and lead to a curative outcome.

Alternatively, higher doses of radioactivity may be administered for each treatment. Animals in the pilot radiotherapy study did not experience any adverse effects that would be a sign of radiotoxicity, which opens the possibility for the administration of higher doses to be well tolerated by treated mice. A maximum tolerated dose (MTD) study would first need to be carried out in order to assess the MTD of [^{177}Lu]Lu-DOTA-BSA. Doses ranging from 8 – 18.5 MBq should be investigated as literature precedence for lutetium-177 based radiopharmaceuticals suggests mice can tolerate up to 18.5 MBq with no signs of toxicity and only temporary weight loss.[25]

Lastly, the use of an alpha emitting isotope is also a promising method to enhance therapeutic efficacy. While the use of beta emitting isotopes has shown significant pre-clinical and clinical success, they are limited in their ability to induce cell death at lower doses which often lead to sub-lethal and repairable single stranded DNA breaks, and tend to lose their efficacy in hypoxic tumour microenvironments due to their strong dependence on oxygen to illicit cell damage.[26–28] Alpha particles are superior to beta particles in that they have a significantly higher linear energy transfer, deposit more than 500 times more energy per unit path length, and induce double stranded DNA breaks which result in cell death.[27,29] Isotopes such as actinium-225 or thorium-227 would be especially useful as they are clinically relevant and may also be chelated by DOTA, eliminating the need for different BSA-chelate conjugates to be synthesized.[27] While studies have shown that alpha therapy leads to limited instances of

radiotoxicity and is often well tolerated, the exposure of healthy tissues to such high energy particles is always a risk to be considered.[30–32] By using the platform established in Chapter 2, we are able to reduce the threat of off-target toxicity, a concern for the I.V administration of alpha emitting isotopes, by delivering treatments through an intratumoural injection. Due to the significantly higher energy of alpha particles, it would be prudent to take a less aggressive approach to treatment by first performing a dose escalation study.

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Appendix I

Chapter 2 - Supplementary Information

A.1 Histological Analysis of Excised Tumours Post-Therapy

The intratumoural immune presence following therapy was investigated through histological analysis (Figure S2-1). Tumours excised 7 days post-treatment were stained for the presence of $CD4^+$ and $CD8^+$ T cells as well as $F4/80^+$ macrophages. Minimal differences were observed between treatment and control groups which may be due to the immunosuppressive nature of the tumour microenvironment which in turn counteracts anti-tumour therapeutic effects.[1] The presence of intratumoural macrophages, which have been found to have radioresistant properties in C57Bl/6 mice, may explain the lack of $CD8^+$ T-cells which are key for an anti-tumour effect.[2–4] Macrophages have been known to play a role in inhibiting T-cell function through the depletion of arginine and cysteine which are needed for T cell activation and proliferation respectively, as well as producing reactive oxygen species which destroy T cell receptors.[5] A TUNEL assay was also carried out in order to evaluate the amount of DNA fragmentation that was induced as a result of the treatments. Minimal differences were observed between treated tumours and control groups, an outcome which may be a result of the initiation of DNA repair pathways by tumour cells.[6]

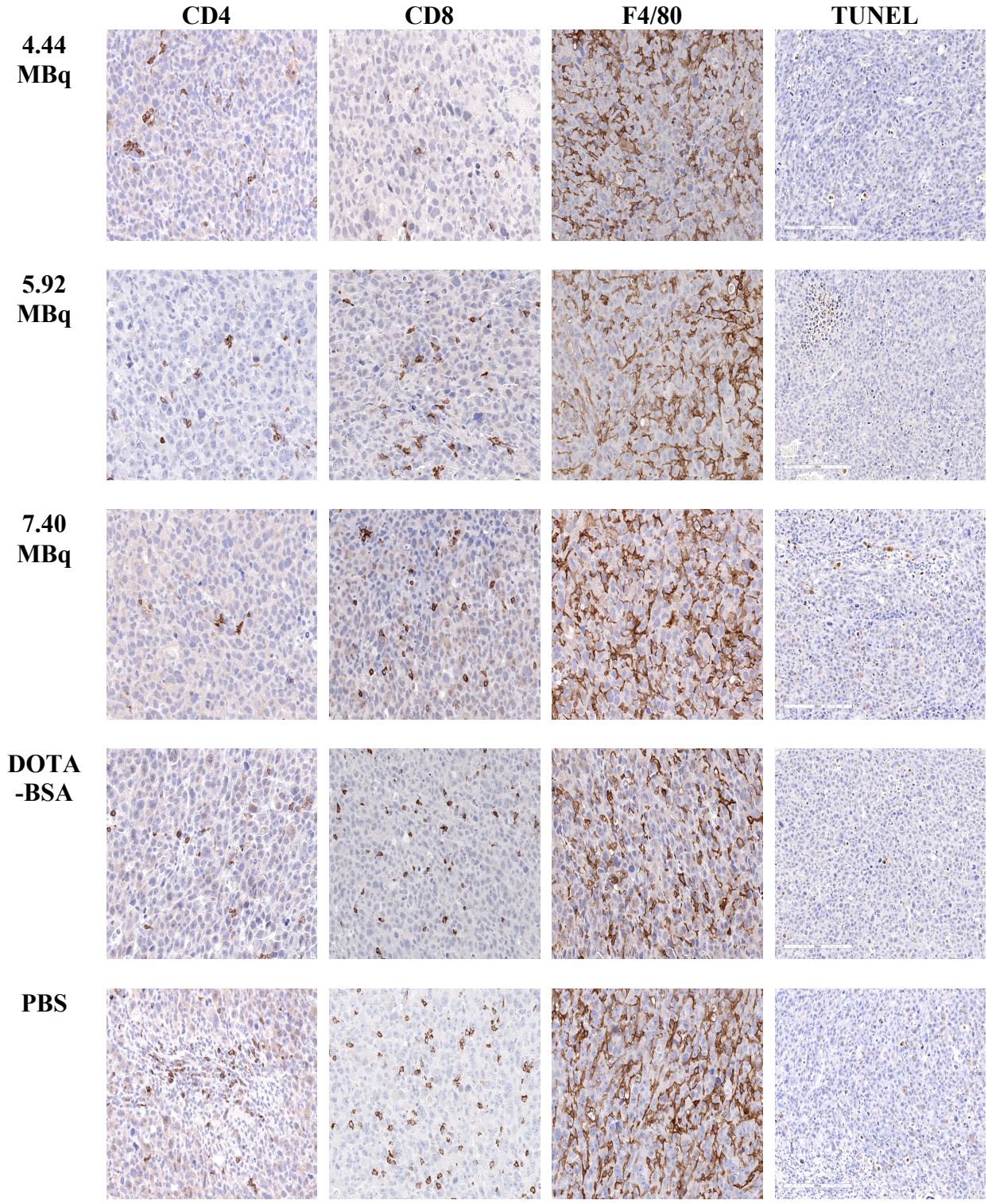

Figure S2-1. IHC analysis of immune cell infiltrates. Tumours were sectioned and stained for CD4, CD8, and F4/80. A TUNEL assay was performed to investigate DNA fragmentation. Each image shows a section from an individual tumour at 20× magnification.

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