SUBSTANCE P RADIOLABELED WITH ^{68}GA AS A NOVEL-IMAGING AGENT FOR INCREASED NEUROKININ-1 RECEPTOR AVAILABILITY IN CHRONIC PAIN DISORDERS

by

Janine Suthiram

Submitted in fulfilment in accordance with the requirements for the

degree of

DOCTOR OF PHILOSOPHY

in the subject

MEDICAL NUCLEAR SCIENCE

at the

UNIVERSITY OF PRETORIA

PROMOTER: Prof M.M. Sathekge

CO-PROMOTERS: Prof J.R. Zeevaart
Dr T. Ebenhan

December 2019

DECLARATION

I, **Janine Suthiram (student number: 14465932)**, declare that the dissertation, "*Substance P radiolabeled with ^{68}Ga as a novel-imaging agent for increased Neurokinin-1 Receptor availability in chronic pain disorders,*" which I hereby submit in accordance with the requirements for the degree Doctor of Philosophy in the subject Medical Nuclear Science at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at another university. All sources cited or quoted in this dissertation are indicated and acknowledged with a comprehensive list of references.

.....

Janine Suthiram

17 December 2019

DEDICATION

“I am He who will sustain you, I have made you; I will care for you. I will carry you and I will rescue you.”
Isaiah 46:4

For my God, my husband and my parents, all of whom I love deeply, whole heartedly and without reservation, this is dedicated to you!

ACKNOWLEDGEMENTS

"You are worthy of it all, for from You are ALL things and to You are ALL things, you deserve the glory!" (Lyrics from Worthy of it All by David Brymer)

First and foremost all praise, glory and honour is due to my Lord and Saviour Jesus Christ for granting me the courage to persevere through the difficult times, for filling my heart with joy and gratitude for the good times and most importantly for His incomprehensible peace in the midst of the storms. You are truly the God of the impossible – without You I would be nothing!

To Zahir, my best friend, my dream come true, my incredible husband! Babe you are my whole world and then some and I will forever be grateful for the day you walked into my life. That "chance" meeting, that in time proved to be God ordained, has turned into this extraordinary life that we're living. We are so blessed! Thank you for managing our home and everything else in our lives so that I could focus on my studies. You have always been selfless in your actions and those actions speak volumes of your love for me. Thanks for building up my confidence when it was broken and for the much needed words of encouragement. Thank you for always supporting my dreams, for reminding me that I am stronger than I think, and for each day that you courageously and unashamedly live out what it means to be brave, to walk in faith and to trust God. I couldn't ask for a better partner to be on this journey with. You are my inspiration and I love you beyond measure!

To my amazing parents, Reena and Daya Naidoo, who have supported me unconditionally in every stage of my life. You will never know just how much you mean to me! I will never forget the many days that you have wiped my tears whilst reminding me of the good plans and purposes that God has for me! I will carry those words always. For the countless hours of prayers, words of wisdom and unwavering love, what can I say other than, I love you immensely and feel so blessed to call you mum and dad. Thank you for teaching me to strive for perfection and nothing less. Zahir,

mum and dad – I couldn't have asked for a better support system. Without your love and faith, none of this would be possible!

I would like to express my heartfelt appreciation and deepest gratitude to the following people for their contribution to this work:

- Prof Mike Sathekge – thank you for your guidance and assistance even though your time was limited. Your love for your patients and the advancement of medicine is truly inspiring! I admire your humility and your ability to relate to everyone you encounter. I will never forget your enthusiasm and delight when I would share my good results with you, it always motivated me to give my very best. I count it a privilege to be your student!
- Prof Jan Rijn Zeevaart – thank you for affording me the opportunity to carry out this interesting research as well as for your steadfast support and understanding. Before signing up, I had doubts if I could do this but you were the first colleague to express faith in my ability. I will never forget that! I know at times it has been challenging to keep everything afloat but know that I am grateful for the time you made to contribute to my project, offer a lending hand or just to remind me that I will get through it. You never doubted me and for that I am thankful!
- Dr Thomas Ebenhan, more like Prof! Thank you, thank you, thank you, a thousand times over! Not just for the countless hours spent in providing a challenging project that never disappointed but more so for your mentorship and awesome friendship. Your guidance and unwavering support has proven absolutely invaluable and has gotten me through the really tough times. The immense amount of hours that you have given to this project is both valued and appreciated. You have generously imparted a wealth of knowledge to me, and for this I am grateful. Thank you for pushing me out of my work comfort zones and trusting me to deliver. I admire the passion, dedication and enthusiasm you have for this field and your continuous efforts to make an impact. Here's to future unimaginable work hours, scientific conversations into the wee hours of the morning and making an imprint in the science world!
- Dr Claire Newton for pulling the proverbial "rabbit out of the hat" when it came to meeting those crazy deadlines. Despite instrument breakdowns and being on maternity leave, you assured me that we would make it and we did! Thank you for

going the extra mile for me and the project. I know you have many students that require your time, thank you for prioritising this project when I needed it the most. Thank you for so generously sharing your expertise with me and for your time and effort in teaching me to interpret the data. I look forward to many more collaborative efforts!

- Dr Duduetsang Saku for your tenacity and perseverance in helping me meet my obligations to funders. You truly went above and beyond to seek out expertise that could positively impact the project outcomes and to set up fruitful collaborations. Not to mention listening intently to all my project hurdles and then pushing me to not give up. I am proud that I get to work with a strong women like you!
- Dr Hester Oosthuizen – for your time and effort in providing constructive criticism with regards to scientific writing at very short notice I might add. Thank you for your assistance even whilst you were ill and on holiday. I am so grateful to have met you and worked with you!
- Ms Delene van Wyk for your time and expertise as a radiographer! Your meticulous labelling and recording of data was of great help when my memory failed. Thank you for your diligence and for being the calm one during somewhat chaotic study days. Your reassuring smile always put me at ease.
- The Radiochemistry group at Necsa, especially Ms Judith Wagener & Dr Carl Wagener for your willingness to help, it is much appreciated. Thank you for lending a hand on study days, allowing me to vent my frustrations and for offering innovative ideas. Many thanks to Ms Biljana Marjanovic-Painter for the long hours in the laboratory that in the end consisted of many laughs and triumphs. Thank you for your time and patience in going through the data and results with me as many times as needed. Thank you Ms Mariaan Miles & Ms Dielda Mienie for all the coffee deliveries, the support, the tears, the good times and the laughs. A big thank you to Ms Jillene Visser for proofreading at very short notice. You are a wonderful addition to our team. Your quirky sense of humour brightens up my day!
- Many thanks to the Nuclear Medicine Department at the University of Pretoria for hosting me during my study days and nights. Many thanks to Ms Danka Erasmus for assisting with the theranostic experimental work. Special thanks to Dr Janke Kleynhans for reviewing some of my work and providing valuable feedback.

❖ Last but not least many thanks to Necsa for affording me the time and financial support to engage in these studies.

EXECUTIVE SUMMARY

The World Health Organization estimates that up to 33% of the world's population suffers from chronic pain originating from an underlying musculoskeletal disorder. The prevalence of this class of diseases is on the rise with a high estimated economic impact world-wide, also in South Africa. The clinical challenge associated with chronic pain is a lack of understanding of the core pathology of the disease mechanisms involved coupled with mostly non-specific diagnostic tools and ineffective chronic pain treatment. The emerging neuropeptide Substance P (SP) targets the neurokinin-1 receptor (NK1R) and is intimately involved in the processes of inflammation and chronic pain. This is evident from histology reports of elevated levels of SP and NK1R in chronically painful tendon tissue.

Molecular imaging modalities such as Positron Emission Tomography/Computed Tomography (PET/CT) have the ability to visualise and provide a quantitative measurement *in vivo* of the function of cellular and biological processes. Visualisation of the normal NK1R expression *in vivo* as well as its pathological expression on non-neural cells and tissue cells could improve understanding of the behaviour of the receptor. In this study the tracer biodistribution, pharmacokinetic and targeted *in vivo* PET/CT imaging of [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP was evaluated for the assessment of NK1R expression in healthy dogs and in dogs with suspected osteoarthritic conditions.

The radiolabelling approach used ^{68}Ga-activity from a tin-dioxide-based ^{68}Ge/^{68}Ga generator, supporting optimal radiosynthesis of [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP by way of varying parameters such as ^{68}Ga-peptide acidity (pH) of radiolabelling solution, peptide concentration, reaction time, the benefit of a heated step, purification and the introduction of freeze dried aliquots of the peptide. Formulation was carried out to ensure physiological pH, sterility, low salt and reduced ethanol content. The potential to adapt the radiosynthesis proposed for [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP to its therapeutic counterpart [^{213}Bi]Bi-DOTA-[Thi^{8},Met(O_2)^{11}]SP was determined. The modification of the peptide sequence warranted *in vitro* assessment of the SP analogue testing DOTA-[Thi^{8},Met(O_2)^{11}]SP mode of action towards the tachykinin receptor family and its dose dependent agonist/antagonist

behaviour at NK1R in particular. Healthy and diseased (suspected osteoarthritis) outbreed dogs were the animal of choice because their physiology is known to be closer to humans. Furthermore, a larger animal model is a more suitable fit to a clinical PET/CT camera. Ethical approval was obtained from the Animal Ethics Committee at the University of Pretoria. Animals that met the inclusion criteria were injected with the radiotracer and each animal was allowed three static whole-body PET/CT scans at 30, 60 and 120 min post injection. Blood samples were obtained at certain time points and the bladder of the animal voided prior to each image. Time-activity curves facilitated the calculation of the pharmacological half-life and urinary elimination rate of the radiopharmaceutical.

Radiosynthesis, optimisation, and a safe for administration formulation of [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP (0.05 mg/28 nmol, pH 6.5, <5% ethanol) was achieved by way of a robust, repeatable method (95 ºC, 15 min, C18 purification) that used freeze-dried aliquots of the peptide. The optimised parameters were improved when compared with other ^{68}Ga-peptide preparations suitable for pre-clinical or clinical application. Reliably high radiolabelling efficiencies were achieved (>90%) with colloids <6% and uncomplexed ^{68}Ga <3% (n > 3). Excellent radiochemical purity >99% could be achieved following C18-based solid phase extraction. [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP was prepared with high specific activity (13.5 ± 3.9 MBq/nmol) providing sufficient yields to serve multiple doses for pre-clinical imaging studies.

[^{213}Bi]Bi-DOTA-[Thi^{8},Met(O_2)^{11}]SP was prepared using a simple, robust radiolabelling method similar to that employed for [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP. The theranostic pair of [^{68}Ga]Ga-/[^{213}Bi]Bi-DOTA-[Thi^{8},Met(O_2)^{11}]SP may not have application in chronic pain disorders, however the safe to administer formulation may be of benefit for other applications. The optimised radiosynthesis of [^{213}Bi]Bi-DOTA-[Thi^{8},Met(O_2)^{11}]SP achieved a RCP of 65.7%, 91.2% and 97.5% (using 0.05 mg, 222-259 MBq, n = 3) after 5, 10 and 15 min incubation, respectively. A simpler radiolabelling method applicable to [^{68}Ga]Ga-/[^{213}Bi]Bi-DOTA-[Thi^{8},Met(O_2)^{11}]SP was successfully established.

[^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP was found to have similar efficacy and potency as native SP but greater selectivity for the NK1R *in vitro.* The DOTA conjugated peptide functions as an agonist at the receptor with no antagonistic behavior. Activation of NK1R may result in undesired pharmacological effects which must be carefully

considered when attempting translation into the clinical setting. The radiotracer was unable to elicit significant activation of any of the other tachykinin receptors at high concentrations and also did not have any antagonistic behaviour at these receptors.

Favourable pharmacokinetics and biodistribution was determined in outbreed dogs for [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP which was deemed suitable for imaging of osteoarthritic pain. The pharmacological half-life was determined to be around 15 min. Excretion was predominantly renal with acceptable, transient liver uptake. Areas synonymous with osteoarthritis including the legs, paws, hips and shoulders presented with unilateral uptake of the tracer. This was observed in bone and soft tissue. Elevated and persistent uptake in characteristic NK1R-dense tissues such as the gut mucosa could be visualised.

[^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP-PET/CT imaging may be a powerful tool to detect NK1R-mediated tissue pain. However further investigations should focus on correlating the NK1R expression in non-neuronal tissues with the accumulation of [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP *in vivo*.

LANGUAGE EDITOR

I, Hester Oosthuizen, hereby declare that I edited the dissertation by Janine Suthiram titled, "*Substance P radiolabeled with ^{68}Ga as a novel-imaging agent for increased Neurokinin-1 Receptor availability in chronic pain disorders".*

LIST OF ABBREVIATIONS

AEC	Animal ethics committee
^{213}Bi	Bismuth-213
CT	Computerised tomography
DAG	Diacylglycerol
DMEM	Dulbecco's Modified Eagle's Medium
DNIC	Diffuse noxious inhibitory control
DOTA	1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid
DOTA-TATE	Tetraazacyclododecanetetraacetic acid–DPhe1-Tyr3-octreotate
DTPA	Di-ethylene-tri-amine-pentaacetic acid
EC_{50}	Half maximal effective concentration
E_{max}	Maximum response
^{18}F	Fluorine-18
^{68}Ga	Gallium-68
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
^{68}Ge	Germanium-68
GLP	Good laboratory practice
GMP	Good manufacturing practice
GPCR	G protein-coupled receptors
GTP	guanosine triphosphate
HCl	Hydrochloric acid
HPLC	High-performance liquid chromatography
ITLC-SG	Instant thin layer chromatography
i.v.	Intravenously
LE	Labelling efficiency
LPS	Lipopolysaccharide
min	minute
MS	Mass spectrometry

MRI	Magnetic resonance imaging
NK1R	Neurokinin-1 receptor
NK2R	Neurokinin-2 receptor
NK3R	Neurokinin-3 receptor
NKA	Neurokinin A
NKB	Neurokinin B
NODAGA	1,4,7-triazacyclononane,1-glutaric acid-4,7-acetic acid
NOTA	1,4,7-triazacyclononane-1,4,7-triacetic acid
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pEC_{50}	Potency
PIP2	Phosphatidylinositol (4,5)-bisphosphate
PLC	Phospholipase C
PKC	Phosphokinase C
PET	Positron emission tomography
p.i.	post injection
PRRT	Peptide receptor radionuclide therapy
PSMA-11	Glu-NH-CO-NH-Lys(Ahx)-HBED-CC
PSMA-617	2-[4-[2-[[4-[[(2*S*)-1-[[(5*S*)-5-carboxy-5-[[(1*S*)-1,3-dicarboxypropyl]carbamoylamino]pentyl]amino]-3-naphthalen-2-yl-1-oxopropan-2-yl]carbamoyl]cyclohexyl]methylamino]-2-oxoethyl]-7,10-bis(carboxylatomethyl)-1,4,7,10-tetrazacyclododec-1-yl]acetate
RCP	Radiochemical purity
ROI	Region of interest
RT	Retention time
S1R	Sigma-1 receptor
SD	Standard deviation
SEM	Standard error of mean
SPECT	Single-photon emission computed tomography
SP	Substance P
SPE	Solid phase extraction

SUV	Standardised uptake value
TFA	Trifluoroacetic acid
^{99m}Tc	Technetium-99m
TLC	Thin Layer Chromatography
TM	Transmembrane spanning
T/NT	Target/non-target ratio
UP	University of Pretoria
UV	Ultraviolet
WHO	World Health Organization

TABLE OF CONTENTS

CHAPTER 2: LITERATURE REVIEW

CHAPTER 3/PAPER 1: RADIOSYNTHESIS, OPTIMISATION AND FORMULATION OF GALLIUM-68-1,4,7,10-TETRAAZACYCLODODECANE-1,4,7,10-TETRAACETIC ACID-[Thi^{8}, Met(O$_2$)^{11}]-SUBSTANCE P - A POTENTIAL NEUROKININ-1 RECEPTOR IMAGING AGENT

CHAPTER 5/PAPER 3: IN VITRO EVALUATION OF THE TACHYKININ RECEPTOR ACTIVITY AND SELECTIVITY OF DOTA-[THI^{8}, MET$(O_2)^{11}$]-SUBSTANCE P

CHAPTER 6/PAPER 4: TRACER BIODISTRIBUTION, PHARMACOKINETIC AND TARGETED IN VIVO IMAGING OF CHRONIC PAIN DISORDERS IN DOGS USING GALLIUM-68 RADIOLABELED DOTA-[THI^{8}, MET$(O_2)^{11}$]-SUBSTANCE P-PET/CT

LIST OF FIGURES

CHAPTER 1:

CHAPTER 2:

CHAPTER 3/PAPER 1:

CHAPTER 4/PAPER 2:

CHAPTER 5/PAPER 3:

CHAPTER 6/PAPER 4:

LIST OF TABLES

CHAPTER 1:

CHAPTER 2:

CHAPTER 3/PAPER 1:

CHAPTER 4/PAPER 2:

CHAPTER 5/PAPER 3:

CHAPTER 6/PAPER 4:

1 INTRODUCTION AND AIM

1.1 INTRODUCTION

Musculoskeletal disorders that give rise to chronic pain is a frequently occurring health problem with a high estimated economic impact world-wide and in South Africa.[1] Chronic pain has been associated with several pathophysiological mechanisms including increased amounts of Substance P (SP) and neurokinin-1 receptor (NK1R) expression that have been documented in histological samples of chronically painful tendon tissue.[2] The role of SP in nociceptive pain signalling has been demonstrated in animal studies. However, data obtained from investigations in humans does not fully substantiate these findings.[3] Furthermore, the systemic blockade of NK1R in humans has not shown any convincing analgesic effect, despite there being promising results in rodents.[4] This infers that the visualisation of both regular (healthy) distribution and pathological expression of NK1R on non-neural cells such as immune cells (macrophages, mast cells, and lymphocytes) and tissue cells (tenocytes, fibroblasts, endothelial cells, and synovial cells) would contribute greatly towards an improved understanding of the role of the receptor.

Various imaging techniques have been used to assess pain processing, including functional magnetic resonance imaging,[5] proton spectroscopy,[6,7,8] Voxel-based morphometry,[9] diffusion tensor imaging,[10] arterial spin labelling,[11] single-photon emission computed tomography (SPECT) and positron emission tomography (PET). Most PET imaging studies of the NK1R used ligands that were functionalized with a fluorine-18[12,13] and carbon-11[14,15] radiolabel and have been aimed at evaluating the use of NK1 antagonists for the treatment of pain disorders. These studies have yielded valuable data regarding the distribution of the NK1R in the biological system, but they focused mainly on the brain and other regions of the central nervous system such as the spinal cord. More in-depth knowledge of NK receptor interactions could aid in the development of targeting strategies to disrupt disease-relevant subcellular signalling of the NK receptor family. This in turn could contribute to the development

of the next generation of NK receptor antagonists[16] that have the potential to be effective therapeutic agents.[17] Therefore the aim of this study was to develop a positron emission tomography/computed tomography (PET/CT) imaging agent that can be used to further investigate the SP/NK1R pathway and its involvement in chronic pain disorders.

The use of nuclear imaging as a diagnostic tool has grown over the years with significant advances being made in the development of both SPECT- and PET-based radiopharmaceuticals. PET/CT is considered the superior imaging system although it is not always readily available or affordable. When compared to SPECT, PET imaging offers improved image quality, greater interpretive certainty, higher diagnostic accuracy, lower patient dosimetry, and shorter imaging protocols.[18] The introduction of the commercial gallium-68/germanium-68 (^{68}Ga/^{68}Ge) generator allows for direct access to a short-lived PET radionuclide on-site at a hospital or a PET imaging facility, for a period of up to one year, without the need for a cyclotron in close proximity. This has made ^{68}Ga-based radiotracers more attractive for researchers developing PET-radiopharmaceuticals.[19] If readily available, molecular imaging and nuclear medicine technology could play an invaluable role in gathering medical information, provided by a three-dimensional, high-resolution scan of the entire body, which would otherwise be unavailable.

PET/CT using the emerging radiopharmaceutical, gallium-68-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid-[Thi^{8}, Met(O_2)^{11}]-Substance P ([^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP), might prove to be a useful non-invasive technique to study the involvement of the NK1R in conditions characterised by chronic osteoarthritic pain symptoms. The neuropeptide SP was identified as the biomarker of interest because it is involved in pain perception and has a high affinity for the NK1R.

To our knowledge, the application of the naive [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP for the visualisation of the NK1R distribution in chronic pain disorders is a novel use of the radiotracer. The radiolabelling of DOTA-[Thi^{8},Met(O_2)^{11}]SP with ^{68}Ga has been investigated, including translation of the methodology to the alpha-emitting therapeutic radioisotope bismuth-213 (^{213}Bi). Cellular uptake of the DOTA-conjugated

ligand has been evaluated in a NK1R transfected cell line followed by a preliminary *in vivo* investigation in dogs presenting with acute or chronic osteoarthritic pain symptoms to determine the biodistribution and pharmacokinetic profile of [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP.

1.2 THE RESEARCH PROBLEM

The SP/NK1R pathway is one of the most studied neurotransmitter pathways in the central nervous system. Nuclear imaging provides the unique opportunity to use pharmacologically active compounds to discover possible applications for NK1R mediated pathologies with a longitudinal approach. SP has been implicated as playing a role in inflammation and chronic pain and therefore has the potential to visualize possible NK1R involvement in chronic pain disorders. A better understanding of the SP involvement towards the receptor availability and function *in vivo* is necessary.

1.3 RELEVANCE & MOTIVATION FOR THE STUDY

Tachykinins are one of the most studied families of neuropeptides due to their involvement in important physiological processes and diseases, including chronic pain, inflammation,[20] cancer,[21] and infection.[16] Recent reports of the involvement of tachykinins in fibrosis and pruritis has reignited the interest of the scientific community in the study of tachykinins.[16] One of the major challenges that hamper the development of effective drugs for the treatment of chronic pain is the lack of understanding of the mechanisms that convert acute tissue insult to chronic pain. The same can be said for pain conditions in which the underlying pathology is not apparent.[22]

Molecular imaging and nuclear medicine procedures such as [^{68}Ga]Ga-DOTA-[Thi^{8}, Met(O_2)^{11}]SP-PET/CT are non-invasive and painless yielding rapid results for the clinician. These procedures are intended to form an integral part of patient care identifying abnormalities very early in the progression of a disease before medical problems are apparent using conventional diagnostic tests. This allows for a disease to be treated at a less extensive stage, thus improving its overall prognosis. This may also alleviate the need for unnecessary treatment or the use of extensive diagnostic tests.

1.4 STUDY AIMS AND OBJECTIVES

The main aim of this study was to produce a safe-to-administer radiotracer prototype ([^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP) for the detection of the increased presence of NK1 receptors related to chronic pain *in vivo*. [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP-PET/CT imaging was intended to compare tracer biodistribution and NK1R targeting in healthy dogs and dogs with suspected osteoarthritis characterised by chronic pain symptoms.

1.4.1 OBJECTIVE 1

Develop an optimised radiosynthesis for [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP that results in a safe-to-administer formulation of the prototype radiopharmaceutical to study NK1R availability in healthy dogs and dogs with suspected chronic pain.

Hypothesis: The purified [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP will have a radiochemical purity of >95 %.

1.4.2 OBJECTIVE 2

To demonstrate that DOTA-[Thi^{8},Met(O_2)^{11}]SP still effectively targets the NK receptor family.

Hypothesis: [DOTA-[Thi^{8},Met(O_2)^{11}]SP will have a high affinity and selectivity (comparable to native Substance P) for NK1R.

1.4.3 OBJECTIVE 3

To study the tracer affinity in areas of increased NK1R expression related to chronic pain; [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP imaging in healthy dogs compared to dogs with suspected chronic pain.

Hypothesis: There will be expected biodistribution in the liver, lung, heart, small and large intestine, spleen, kidney, and bladder while there will be elevated uptake in painful foci or tissue (for both soft tissue and bone).

1.5 RESEARCH DESIGN

The principle research design of this study (**Figure 1.1**) follows a classical approach to radiopharmaceutical development. The lead compound is synthesised, radiolabelled and subjected to quality control in the laboratory. The radiolabelled compound is then assessed *in vitro* in a suitable cell line for target sensitivity and affinity as well as dose response. This is followed by pre-clinical *in vivo* assessment of the potential radiotracer through biodistribution studies in suitable animal models.

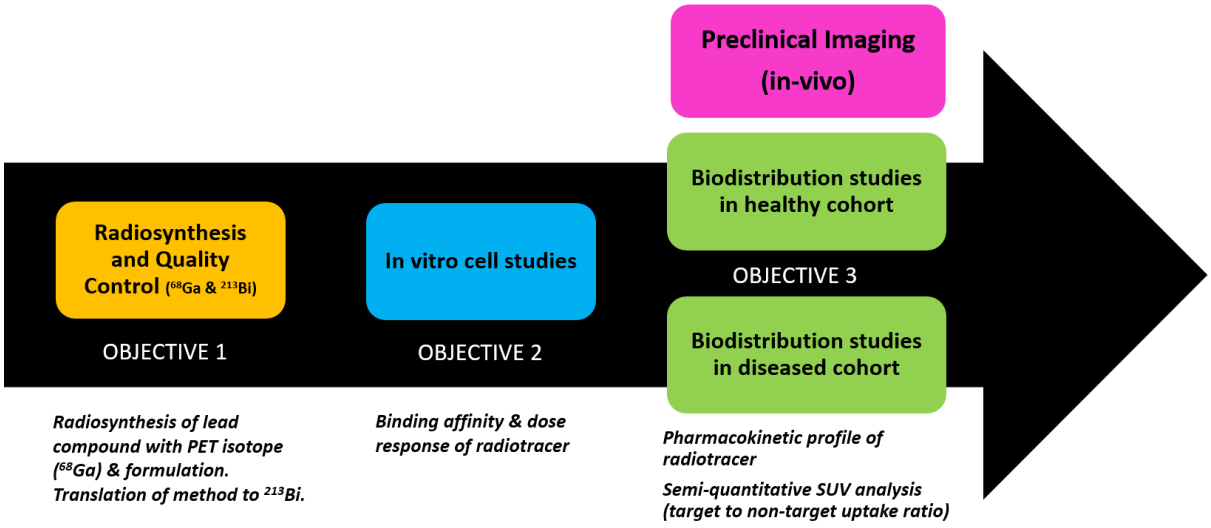


Figure 1.1: Strategic development of a novel PET tracer for the imaging of NK1R expression related to chronic pain disorders.

1.6 RESEARCH METHODOLOGIES

1.6.1 OBJECTIVE 1: [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP RADIOPHARMACEUTICAL PROTOTYPE DEVELOPMENT AND FORMULATION

1.6.1.1 Evaluate radiolabelling parameters to develop an optimised radiolabelling protocol

- Labelling pH: SP retains its biological activity at low pH's (3-4) and is unstable in aqueous medium.

- Temperature of labelling reaction: DOTA conjugated peptides are effectively radiolabelled at higher temperatures. The labelling efficiency at temperatures of 60, 90 and 95 ˚C was determined.
- Time required for effective labelling (up to 20 min to minimise loss of radioactivity due to half-life).
- Peptide concentration: The lowest possible concentration of peptide that would result in a high labelling efficiency was determined.
- Modified reaction procedure:
    a. Pre-heating and buffering the ^{68}Ga-activity prior to radiolabelling,
    b. The impact of adding the peptide directly to the ^{68}Ga versus dissolving it in the buffer first.

1.6.1.2. Develop a safe-to-administer prototype radiopharmaceutical

- Purification of the crude radiolabelled product using solid-phase extraction.
- High pressure liquid chromatography (HPLC) and instant thin-layer chromatography (ITLC) analyses of both the crude and purified radiolabelled product.
- Thermodynamic stability testing of the radiolabelled product for up to 2 h following incubation at 37 ˚C and subsequent HPLC analysis.
- Adapting the finalised radiolabelling procedure (including steps for physiological pH adjustment and sterile filtration prior to injection) to meet Good Laboratory Practice (GLP) requirements for the pre-clinical imaging study.

1.6.1.3. Adapt the radiolabelling methodology to ^{213}Bi

Investigating the ability to adapt the optimised radiolabelling procedure for [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP to [^{213}Bi]Bi-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP. The use of DOTA-[Thi^{8},Met(O$_2$)^{11}]SP as the molecular targeting vector radiolabelled with ^{68}Ga for diagnostic imaging and ^{213}Bi for therapy may be considered a theranostic pair.

1.6.2 OBJECTIVE 2: ASSESSMENT OF DOTA-[Thi^{8},Met(O$_2$)^{11}]SP INTERACTION WITH THE NK1R

1.6.2.1 Endogenous ligand dose responses

Evaluate the potency and selectivity of DOTA-[Thi^{8},Met(O$_2$)^{11}]SP for NK1R in HEK 293-T cells (a 'blank' background into which different tachykinin receptors could be introduced) to verify that the amino acid modifications on DOTA-[Thi^{8},Met(O$_2$)^{11}]SP have not inadvertently altered the receptor specificity of this SP analogue.

1.6.3 OBJECTIVE 3: PERFORMANCE OF [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP-PET/CT FOR DETECTION OF NK1R MEDIATED PAIN DISORDERS

- Determine pharmacokinetic tracer behaviour and image-guided biodistribution in healthy dogs.
- *Ex vivo* blood and urine sampling to determine pharmacological half-life and tracer recovery of [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP.
- Non-invasive PET/CT imaging using [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP to localize chronic pain foci in dogs with chronic osteoarthritic pain symptoms.

1.7 ETHICAL & SAFETY CONSIDERATIONS

This study used a vertebrate animal model of domestic canines (large outbreed, male/ female, young adolescent and mature) with suspected acute or chronic osteoarthritic pain symptoms as well as healthy dogs. The animals were recruited and assessed for suitability against the study's inclusion/exclusion criteria (**Appendix 1A**) by a referring veterinarian (Onderstepoort Veterinary Academic Hospital (OVAH) and Pretoria East Veterinary Clinic).

1.7.1 ETHICAL APPROVAL

The study protocol was submitted to the Animal Ethics Committee (AEC) at the University of Pretoria for review and was subsequently approved (Project Number: V089-14). The study was conducted according to the Ethics Committee requirements in accordance with generally accepted GLP guidelines. Progress reports were

compiled and submitted to the AEC annually. Any amendments to the study were also reported to the committee.

1.7.2 INFORMED CONSENT PROCESS AND DOCUMENTATION

The referring veterinarian was given responsibility for obtaining each animal owners informed consent prior to enrolling a dog in the study. The animal owner was given the opportunity to read the information leaflet and ask any questions prior to signing the informed consent form (**Appendix 1B**). The animal owner was entitled to withdraw the informed consent for their dog at any time during the study. The animal owner was notified about the importance of adhering to the study schedule (**Appendix 1C**) and the requirements regarding fasting and pregnancy of female dogs.

The original consent form was stored in the investigator's study file or animal notes. At the time of enrolment into the study, a copy of the signed consent form and information sheet was given to the animal owner to keep.

1.7.3 SAFETY CONSIDERATIONS

1.7.3.1 Possible side effects and discomfort involved in the procedure

SP has the following biochemical/physiological actions: potent vasodilator and hypotensive agent; induces salivation; increases capillary permeability; induces mast cell degranulation. The amount of SP analogue that was administered in this study is low therefore the risk for these side effects was minimal.

The heart rate, blood pressure, body temperature, oxygen saturation (SpO_2), respiration rate, aspired carbon dioxide, electrocardiography, and anaesthetic plane was monitored closely for each animal during the procedure.

Every effort was made to treat the animal as a patient would normally be treated in the Nuclear Medicine Department. Most steps in the protocol were expected to cause the animal only mild discomfort (**Table 1.1**). Nuclear medicine is a non-invasive imaging technique and therefore was not expected to induce any illness or pain in the animals.

Table 1.1: Aspects of the animal study that could cause discomfort to the animal with the expected level of discomfort.

Source of discomfort	Level of discomfort
Transportation	+
Anaesthesia	+
Urinary catheterisation	+
Femoral catheterisation	+
Injection of the radiotracer	++
Radiation dose (from radiotracer & imaging equipment)	+
Toxicity of the radiotracer	+
Recovery from anaesthesia	++

[+] *Mild discomfort: no significant impediment to the animal's well-being or general condition or a short-term mild form of pain, suffering or distress.*
[++] *Moderate discomfort: moderate impairment of the animal's well-being or general condition or long-lasting mild pain, suffering or distress or short-term moderate pain, suffering or distress*
[+++] *Severe discomfort: severe impairment of the animal's well-being or general condition or severe pain, suffering or distress or long-lasting moderate pain, suffering or distress*[23]

1.7.3.2 Radiation protection

Radiopharmaceuticals emit ionising radiation. However, the dose of radiation given to the animals in the study was of tracer level and less than the dose used routinely for imaging studies in humans in a nuclear medicine ward. The radiopharmaceutical was prepared behind a lead shield and handled using a lead pot or lead syringe holder. Radiation and contamination monitoring was performed in accordance with the Department of Health regulations applicable in the Nuclear Medicine Department. All radioactive waste materials and solutions were disposed of in separate demarcated waste containers. Biological waste (urine and blood samples) was left to decay in a shielded area, classified as non-radiological and incinerated at OVAH as per normal procedure.

1.8 REFERENCES

1. Winkelstein B. Mechanisms of central sensitization, neuroimmunology and injury biomechanics in persistent pain: implications for musculoskeletal disorders. J Electromyogr Kinesiol [Internet]. 2004 [cited 2019 Sep 2];14:87-93. Available from: https://www.sciencedirect.com/science/article/abs/pii/S1050641103001433?via%3Dihub doi: 10.1016/j.jelekin.2003.09.017

2. Andersson G, Danielson P, Alfredson H, Forsgren, S. Presence of Substance P and the neurokinin-1 receptor in tenocytes of the human Achilles tendon. Regul Peptides [Internet]. 2008 [cited 2019 Sep 1];150(1-3):81-7. Available from https://www.sciencedirect.com/science/article/abs/pii/S0167011508000359?via%3Dihub doi: 10.1016/j.regpep.2008.02.005

3. Babenko VV, Graven-Nielsen T, Svensson P, Drewes AM, Jensen TS, Arendt-Nielsen L. Experimental human muscle pain induced by intramuscular injections of bradykinin, serotonin, and substance P. Eur J Pain [Internet]. 1999 [cited 2019 Aug 30];3:93-102. Available from https://onlinelibrary.wiley.com/doi/abs/10.1053/eujp.1998.0103?sid=nlm%3Apubmed doi: 10.1053/eujp.1998.0103

4. Hill, R. NK1 (substance P) receptor antagonists – why are they not analgesic in humans? Trends Pharmacol Sci [Internet]. 2000 [cited 2019 Sep 15];21:244-6. Available from https://www.cell.com/trends/pharmacological-sciences/fulltext/S0165-6147(00)01502-9?_returnURL=https%3A%2F%2Flinkinghub.elsevier.com%2Fretrieve%2Fpii%2FS0165614700015029%3Fshowall%3Dtrue doi: 10.1016/s0165-6147(00)01502-9

5. Gracely RH, Petzke F, Wolf JM, Clauw DJ. Functional magnetic resonance imaging evidence of augmented pain processing in fibromyalgia. Arthritis Rheumatol [Internet]. 2002 [cited 2019 Jan 10];46:1333-43. Available from https://onlinelibrary.wiley.com/doi/10.1002/art.10225 doi: 10.1002/art.10225

6. Harris RE, Sundgren PC, Pang Y, Hsu M, Petrou M, Kim SH, et al. Dynamic levels of glutamate within the insula are associated with improvements in multiple pain dominions in fibromyalgia. Arthritis Rheumatol [Internet]. 2008 [cited 2019 Sep 15];58(3):9037. Available from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2827610/ doi: 10.1002/art.23223

7. Harris RE, Sundgren PC, Craig AD, Kirshenbaum E, Sen A, Napadow V, et al. Elevated insular glutamate in fibromyalgia is associated with experimental pain. Arthritis Rheumatol [Internet]. 2009 [cited 2019 Sep 20];60(10):3146-52. Available from https://onlinelibrary.wiley.com/doi/full/10.1002/art.24849 doi: 10.1002/art.24849

8. Fayed N, Garcia-Campayo J, Magallon R, Andres-Bergareche H, Luciano JV, Andres E, et al. Localised ^{1}H-NMR spectroscopy in patients with fibromyalgia: a controlled study of changes in cerebral glutamate/glutamine, inositol, choline, and N-acetylaspartate. Arthritis Res Ther [Internet]. 2010 [cited 2019 Sep 8];12(4):R134. Available from: https://arthritis-research.biomedcentral.com/articles/10.1186/ar3072 doi: 10.1186/ar3072

9. Bellato E, Marini E, Castoldi F, Barbasetti N, Mattei L, Bonasia DE, et al. Fibromyalgia syndrome: etiology, pathogenesis, diagnosis, and treatment. Pain Res Treat [Internet]. 2012 [cited 2019 Sep 22];2012:426130. Available from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3503476 doi: 10.1155/2012/426130

10. Sundgren PC, Petrou M, Harris RE, Fan X, Foerster B, Mehrotra N, et al. Diffusion weighted and diffusion tensor imaging in fibromyalgia patients: a prospective study of whole brain diffusivity, apparent diffusion coefficient, and fraction anisotropy in different regions of the brain and correlation with symptom severity. Acad Radiol [Internet]. 2007 [cited 2019 Sep 2];14(7):839-46. Available from: https://www.sciencedirect.com/science/article/pii/S1076633207001808 doi: 10.1016/j.acra.2007.03.015

11. Nebel MB, Gracely RH. Neuroimaging of fibromyalgia. Rheum Dis Clin North Am [Internet]. 2009 [cited 2019 Sep 4];35(2):313-27. Available from: https://www.rheumatic.theclinics.com/article/S0889-857X(09)00044-1/abstract doi: 10.1016/j.rdc.2009.06.004

12. Hargreaves R. Imaging Substance P receptors (NK-1) in the living human brain using positron emission tomography. J Clin Psychiatry [Internet]. 2002 [cited 2019 Sep 4];63(Suppl 11):18-24. Available from: https://www.psychiatrist.com/jcp/article/pages/2002/v63s11/v63s1104.aspx

13. Hietala J, Nyman MJ, Eskola O, Laakso A, Grönroos T, Oikonen V, et al. Visualisation and quantification of neurokinin-1 (NK-1) receptors in the human brain. Mol Imaging Biol. 2005 Jul-Aug;7(4):262-72.

14. Bergstrom M, Fasth KJ, Kilpatrick G, Ward P, Cable KM, Wipperman MD, et al. Brain uptake and receptor binding of two [^{11}C] labelled selective high affinity NK1-antagonists, GR203040 and GR205171 – PET studies in rhesus monkey. Neuropharmacology [Internet]. 2000 [cited 2019 Oct 1];39(4):664-70. Available from: https://www.sciencedirect.com/science/article/abs/pii/S0028390899001823?via%3Dihub doi: 10.1016/s0028-3908(99)00182-3

15. Wolfensberger SPA, van Berckel BNM, Airaksinen AJ, Maruyama K, Lubberink M, Boellaard R, et al. First evaluation of [^{11}C]R116301 as an *in vivo* tracer of NK1 receptors in man. Mol Imaging Biol. 2009 Mar;11:241-5.

16. Steinhoff MS, von Mentzer B, Geppetti P, Pothoulakis C, Bunnett NW. Tachykinins and their receptors: Contributions to physiological control and the mechanisms of disease. Physiol Rev [Internet]. 2014 [cited 2019 Oct 11];94(1):265-301. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3929113/ doi: 10.1152/physrev.00031.2013

17. Martinez AN, Phillip MT. Substance P and Antagonists of the Neurokinin-1 Receptor in Neuroinflammation Associated with Infectious and Neurodegenerative

Diseases of the Central Nervous System. J Neurol Neuromedicine [Internet]. 2016 Jul [cited 2019 Oct11];1(2):29–36. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4943220/

18. Bateman, TM. Advantages and disadvantages of PET and SPECT in a busy clinical practice. J Nucl Cardiol [Internet]. 2012 [cited 2019 Sep 30];19:S3-11. Available from: https://link.springer.com/article/10.1007%2Fs12350-011-9490-9 doi: 10.1007/s12350-011-9490-9

19. Jalilian, AR. An overview on Ga-68 radiopharmaceuticals for positron emission tomography applications. Iran J Nucl Med [Internet]. 2016 Jan [cited 2019 Oct 1];24(1):1-10. Available from: http://irjnm.tums.ac.ir/article_15753.html

20. Canning, BJ. Potential role of tachykinins in inflammatory diseases. J Allergy Clin Immunol [Internet]. 1997 [cited 2019 Sep 24];99(5):579-82. Available from: https://www.jacionline.org/article/S0091-6749(97)70015-0/fulltext doi: 10.1016/S0091-6749(97)70015-0

21. Palma, C. Tachykinins and their receptors in human malignancies. Curr Drug Targets [Internet]. 2006 [cited 2019 Oct 10];7(8):1043-52. Available from: http://www.eurekaselect.com/56449/article doi: 10.2174/138945006778019282

22. Gold MS, Gebhart GF. Nociceptor sensitization in pain pathogenesis. Nat Med [Internet]. 2010 [cited 2019 Sep 18];16(11):1248-57. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5022111/ doi: 10.1038/nm.2235

23. Instantie voor Dierenwelzijn Utrecht. Optimal animal welfare and focus on alternatives. [Internet]. European Directive 2010/63/EU Official Journal of the European Union: Annex VIII Severity classification of procedures; 2010 [cited 2019 Sep 22]. Available from https://www.ivd-utrecht.nl/en/

2 LITERATURE REVIEW

2.1 SUBSTANCE P AND THE NEUROKININ-1 RECEPTOR

Substance P (SP) was identified for the first time in 1931 when it was isolated as a crude extract from the brain and gut of horses.[1] The substance was found to lower blood pressure and stimulate smooth muscle *in vitro* and retained its activity when evaporated to a dry powder. A few years later Gaddum and Shild[2] named the unidentified substance, exactly what it had been all the while colloquially referred to in the laboratory, as SP (P for Powder). It was in 1953 that the pioneering work of Fred Lembeck[3] provided strong evidence that SP is in fact a sensory neurotransmitter associated with pain transmission due to elevated concentrations of the substance located in the dorsal root of the spinal cord.[4]

Twenty years later, Susan Leeman and colleagues were able to isolate a peptide in pure form from the hypothalamus of bovine and identified it as SP based on multiple biological and chemical criteria.[5] A few months later the same group of researchers published the amino acid sequence of SP as H – Arg – Pro – Lys – Pro – Gln – Gln – Phe – Phe – Gly – Leu – Met – NH_2, classifying it as an undecapeptide having an eleven amino acid sequence with a terminal amide group[6] (**Figure 2.1**).

At the same time they published the very first *in vitro* synthesis[7] which led to the development of a radioimmunoassay[8] using iodine-125 and over the following years more evidence was gathered to support SP's classification as a neurotransmitter.[9,10,11,12] During this time, interest in SP related research gained significant momentum as evident by the steep increase in the number of publications between 1970 and 1980.[13]

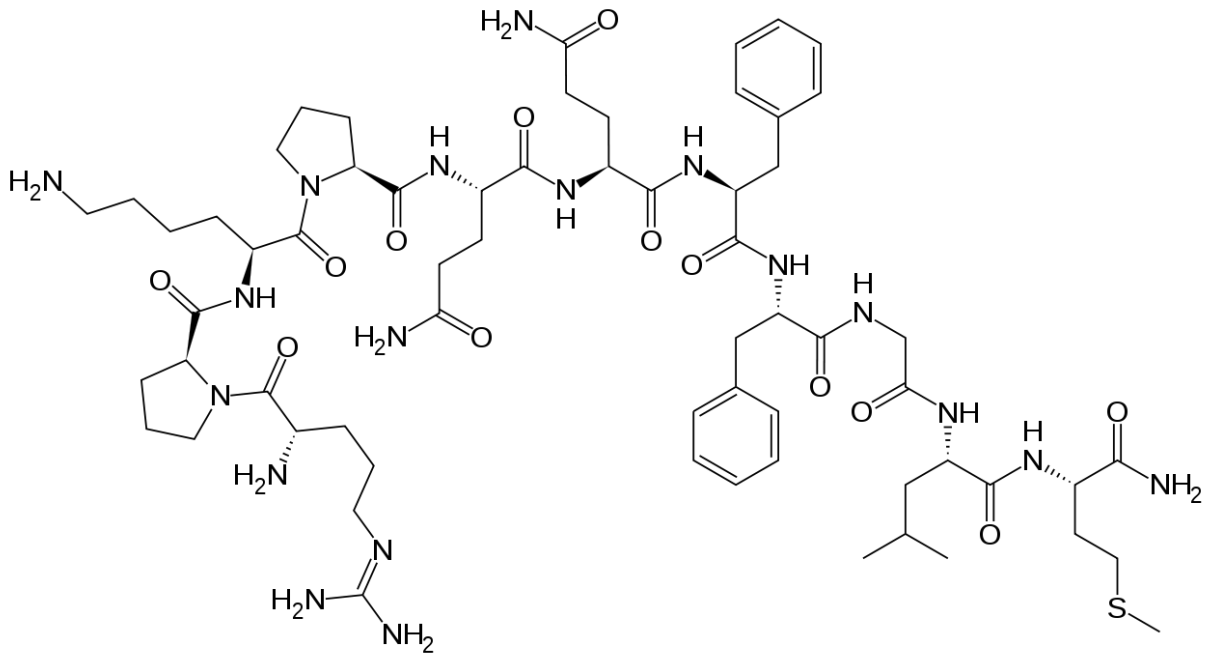


Figure 2.1: Structure of Substance P with a molar mass of 1347.6 g.mol^{-1}.

SP is originally synthesised as a larger protein in the ribosome and is converted by enzyme action to the active eleven amino acid residue which is widely distributed in the central and peripheral nervous systems of the body.[14] Its release is induced by stressful stimuli and the intensity and frequency of the stimulation has a direct correlation to the amount of SP released.[15] More frequent and intense stimuli will result in the diffusion of the peptide further from the site of release subsequently activating approximately 3 to 5 times more neurokinin-1 receptor (NK1R) expressing neurons.[15]

SP belongs to a group of neuropeptides referred to as tachykinins which can be found in mammals and other animal species. There are three mammalian tachykinins, namely Substance P (SP), neurokinin A (NKA), and neurokinin B (NKB), all of which bear a similar C-terminal sequence.[16] They regulate a diverse range of physiological processes and are expressed throughout the nervous and immune systems.[17] In 1987, an SP related receptor was cloned[18] and not long after three distinct neurokinin receptors (NKR) were identified – NK1R, NK2R, NK3R, and later NK4R. These receptors consist of seven hydrophobic transmembrane domains that are coupled to

G-proteins.[4] SP binds preferentially to the NK1R and NKA and NKB are the preferred ligands for the NK2R and the NK3R, respectively. The NK4R has been identified in muscle and binds preferentially to NKA.[19] The NK1R has a relative affinity of 100- and 500-fold less for NKA and NKB respectively when compared to its affinity for SP.[20] However, under certain conditions, such as high ligand concentration or receptor availability, the tachykinins are not highly selective for any given receptor and can therefore bind to all three receptors.[4,14]

The NK1R is a glycoprotein with an extracellular amino-terminus and intracellular carboxyl tail (**Figure 2.2**). It is made up of 407 amino acid residues amounting to a relative molar mass of 46 kDa.[21] The second and third domains of the receptor, which are membrane-spanning, are where agonist/antagonist binding takes place. When SP binds to NK1R rapid endocytosis and internalisation of the receptor takes place followed by rapid recycling to the cell membrane.[15]

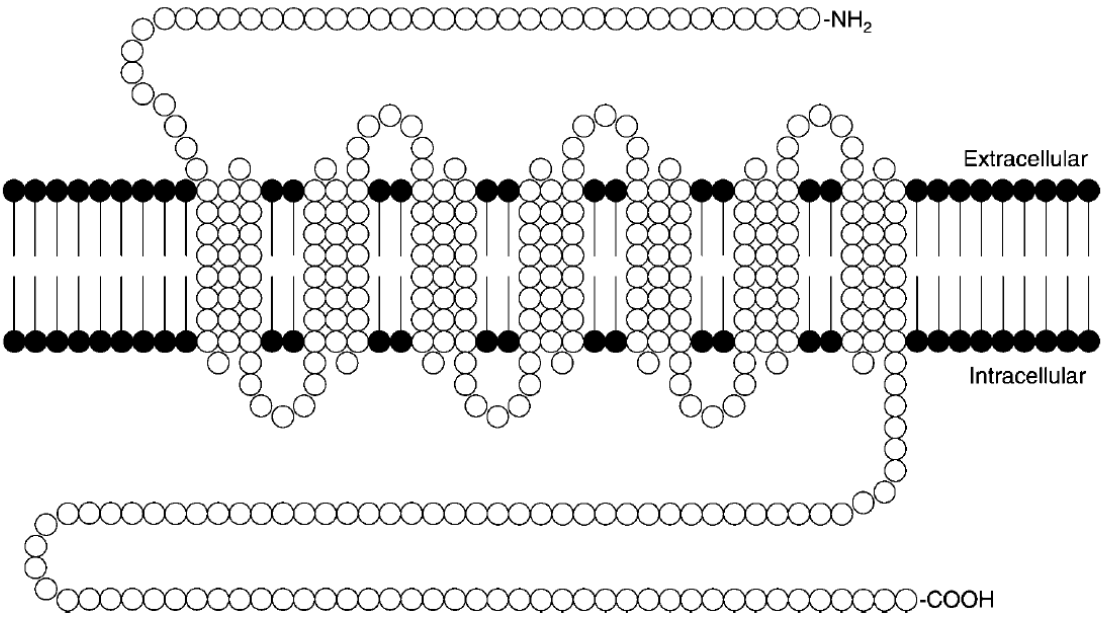


Figure 2.2: Schematic illustration of the neurokinin-1 receptor.[14]

In 1991, the first non-peptide NK1R antagonist (CP96-345) was synthesised[22] and it facilitated the evaluation of the binding of SP versus non-peptide antagonists. The results of the study revealed that the small hydrophobic CP96-345 was binding in a pocket deeply between the transmembrane segments compared to SP which was

binding at the extracellular loops of the receptor.[23] Studies have suggested that the amino acid residue of proline in position 4 of SP is responsible for the selective interaction of the peptide with the NK1R.[24] In order to further understand the role of this neuropeptide and its preferred receptor in the mammalian system, the following section deals with the wide expression of SP and the NK1R on different cells.

2.2 THE EXPRESSION OF SUBSTANCE P AND THE NEUROKININ-1 RECEPTOR

Since its discovery, the SP/NK1R pathway has been studied extensively towards its role in nociceptive pain responses and neurogenic inflammation.[25] The NK1R is expressed in the central and peripheral nervous system of mammals.[26] High levels of SP and the NK1R are observed in areas of the brain involved in regulation of affective behaviour and stress response. Unlike other receptors for neurotransmitters, NK1 receptors are only expressed by about 5% of neurons in the central nervous system.[15] Studies have indicated that high levels of SP are not always accompanied by a similarly high co-expression of the NK1R.[27,28,29] In terms of the central nervous system, the distribution of the NK1R generally corresponds to the distribution of SP. In some regions there is a mismatch, such as the substantia nigra and lateral interpeduncular nuclei. This can be due to technical limitations in the staining techniques used[27,28] or possibly that in those areas the SP binds to the closely related NK2R and NK3R; however, there is no experimental evidence for the latter.

Even though SP is a peptide that is of neuronal origin, it can also be produced by inflammatory cells such as macrophages, eosinophils, lymphocytes, and dendritic cells.[14] This data was obtained from rodent studies.[30,31,32] The SP/NK1R pathway is a potent stimulator of pro-inflammatory cytokines to the extent that it has been suggested that all inflammatory processes may have a requirement for SP.[33]

Literature evidence supports the ability of tachykinin receptors to modify the response of many different inflammatory cells including mast cells, granulocytes, monocytes/macrophages, and lymphocytes. However, the classification of the receptors mediating these effects is not conclusively known.[28] In neurogenic inflammation, SP has been shown to stimulate the recruitment of granulocytes

(neutrophils and eosinophils) at loci of inflammation. Using agonists and antagonists, studies of the skin of the guinea-pig,[34] rat trachea[35] and mouse air-pouch[36] have indicated that the NK1R mediates granulocyte accumulation. NK's are not generally expressed on mast cells but under the influence of interleukin-4 and/or stem cell factor, bone marrow-derived mast cells have been shown to express the NK1R.[37]

SP has been found to stimulate human monocytes by the production of inflammatory cytokines. Studies have indicated that antagonists of SP have the ability to inhibit the effects of SP on human monocytes/macrophages and therefore hypothesised that there must be a specific receptor for the peptide that is expressed on these cells.[38] High affinity binding of SP to a receptor on monocytes/macrophages was detected but it was not possible to determine if the receptor belonged to the NKR family.[39] Well characterized NK1Rs are expressed on the human B lymphoblastic cell line IM-9.[40,41,42] Other studies have however been unsuccessful in proving the presence of SP receptors on lymphocytes.[43,44] This could be due to the fact that in addition to classical NK1Rs, other SP signalling pathways that involve non-NKRs may exist in leukocytes.[28] There could even be a receptor independent pathway present similar to that seen in SP induced mast cell degranulation.[44]

Fibroblasts are one of the main cellular components of inflammatory responses. The migration of fibroblasts from neighbouring connective tissue into sites of inflammation is mediated by various chemotactic signals. A study involving human skin fibroblasts indicated that SP does in fact have a significant chemotactic response on human fibroblasts and that this response is mediated by an NK1R.[45] SP has been found to occur in perivascular and free unmyelinated nerve fibres in human synovial tissue. In a study involving Bolton Hunter iodine-125-labelled SP (^{125}I-BH-SP) the presence of binding sites on vascular endothelial cells of human synovial blood vessels was indicated. The detected binding sites showed characteristics comparable with that of the NK1R.[46] In the synovium, NK1Rs are expressed on both macrophage-like cells and fibroblast-like cells. In a study investigating the formation of osteoclasts via synovial fibroblastic cells, SP was found to stimulate the proliferation of these synovial cells through NK1R mediation.[47]

The human tenocytes of the achilles tendon also express SP and the NK1R.[48] This has implicated SP as having a role in hypercellularity and angiogenesis in tendinosis.[49]

SP and the NK1R have been implicated in a number of different conditions involving the aforementioned cell types, including cancer[50], but for the purpose of this study the focus will be musculoskeletal conditions that are characterised by chronic pain.

2.3 THE INVOLVEMENT OF SUBSTANCE P AND THE NEUROKININ-1 RECEPTOR IN CHRONIC PAIN DISORDERS

Several diverse preclinical studies have indicated that SP may play a role in a variety of disorders including pain,[51,52] emesis,[53] anxiety,[54] inflammatory pain,[55] and hyperalgesia.[56] The neuropeptide has been particularly implicated in pain perception due to the fact that it transmits information about tissue damage from peripheral receptors to the central nervous system so that it can be converted to the sensation of pain. In the 1980s there were several studies investigating the link between SP and chronic widespread pain.[57,58,59]

2.3.1 COMPLEXITY OF PAIN

Pain is the term that is used to describe an unpleasant sensory and emotional experience. This experience is commonly associated with potential or actual tissue damage.[60] Injury to tissues and nerves triggers the release of chemicals that result in an inflammatory reaction which is accompanied by pain.[61] The pain signal sent to the brain is carried by thin unmyelinated nerves called nociceptors that synapse with neurons in the dorsal horn of the spinal cord which transmits the signal via the spinothalamic tract to the cerebral cortex. It is here that the pain is perceived, localised, and interpreted. In most instances the pain is intended to be beneficial to the recovery process but under certain conditions the pain stimulus or perception remains and becomes chronic, even after the injury has healed. Chronic pain affects millions of people worldwide and is often challenging to treat.

Generally, there are two distinct common types of pain - nociceptive and clinical/pathologic pain.[62] Nociceptive pain has a high threshold and limited duration

and can be described as pain experienced acutely in response to a noxious or intense stimulus.[63] This type of pain can also be termed physiological since it is a key component of the human body's normal defence mechanism.

Nociceptors are sensory receptors in the skin, muscle, joints, and viscera that convey pain to the central nervous system by selectively responding to stimuli that are noxious or have the potential to damage tissue. They have the ability to become sensitised meaning that their excitability can be increased.[64] In chronic pain disorders, ongoing disease states or tissue damage result in sensitization of primary afferent and spinal cord neurons.[65] This sensitisation results in a reduced threshold and increased magnitude of a response to both noxious and non-noxious stimuli.[65] Clinical/pathologic pain is as a consequence of the development of abnormal sensitivity in the somatosensory system. Clinical pain can be acute or chronic in nature. The acute type would typically result from inflammation or soft tissue injury and the resulting pain response is a biologically valuable function, that being to protect. This is not to be confused with nociceptive pain because in acute clinical pain the tissue damage has already taken place and cannot be prevented. Therefore the protective function only enables undisturbed healing and repair of the damage.[63] This category of pain can be further divided into neuropathic pain that is specifically initiated by a primary lesion or disease in the somatosensory nervous system and inflammatory pain that is as a result of sensitization of the nociceptive pathway elicited by the release of a variety of mediators at the site of tissue inflammation.

The current working definition of pain takes into account the various dimensions involved when experiencing pain – sensory dimensions (quality of pain, location and intensity), affective dimensions (emotions) and cognitive dimensions (thoughts).[66] Every individual's pain makeup is unique in that the level of involvement of the aforementioned dimensions varies. The resulting expression of pain is therefore markedly influenced. In reality pathological processes do not occur in isolation which means that different mechanisms or types of pain may be found in one patient.

Painful musculoskeletal disorders are very common and have a high estimated economic impact world-wide[67] and in South Africa. The World Health Organization has indicated that the burden from this class of diseases is not only visible but also on the rise in both the developed and developing world.[68] For instance, the cost of

lower back pain is estimated to be between 40 and 50 billion US dollars annually.[69] Musculoskeletal disorders can affect muscle, bones and joints in all major areas of the body. Common disorders in which musculoskeletal pain is present includes but is not limited to tendinosis, fibromyalgia, rheumatoid arthritis, osteoarthritis, gout, and lower back pain.

For the purpose of this study, only a few of the more common disorders where the SP/NK1R pathway is implicated but requires further evaluation to better understand the pathophysiology of the disorders, will be discussed in detail.

2.3.2 TENDINOSIS

Over the years the role of SP in tendons has gained interest.[70] Even though most studies have focused on the healing processes that take place following tendon rupture, SP has been implicated in chronic tendon pain accompanied by structural tissue changes referred to as tendinosis.[71] In a study carried out in 2008, SP was identified in the tenocytes of the human Achilles tendon and the NK1R was found to be widely distributed in blood vessel walls, nerve fascicles, and tenocytes of the tendon tissue. SP and NK1R expression was observed prominently in tendons that exhibited hypercellularity and neovascularisation which are both characteristic features of tendinosis.[71] When compared to normal tendon tissue, increased levels of SP was observed in tendinosis.[49] A study carried out in 2011 demonstrated that the repeated administration of exogenous SP to the paratendinous tissue and one week of Achilles tendon overuse in rabbits resulted in histopathological changes similar to that observed in human Achilles tendinosis. This suggests that SP plays a role in tendinosis. In a more recent study[49] it was determined that SP, through stimulation of the NK1R, has the ability to reduce cachexin-induced apoptosis of human tenocytes that has been observed in the biopsies of tendinosis tissue.[72] This confirms that SP is a potent regulator of cell turnover in tendon tissue and is able to stimulate hypercellularity through varying mechanisms. Further evaluation is necessary to determine if this can be of clinical significance since the effects of SP and NK1R may be potentially blocked using antagonists.

2.3.3 ARTHRITIS

The secretion of neuropeptides such as SP into the joint cavity has implicated the nervous system in the etiology and pathogenesis of joint diseases.[47] SP is able to stimulate the production of collagenase and the prostaglandin E_2 in synovial cells[73] and has a local vasodilatory function in human synovial tissue. It is also able to mediate the extravasation of plasma proteins into the synovial cavity.[74] For these reasons, SP has been considered a useful marker for the severity of arthritis.

Chronic pain and bone destruction are commonly associated with arthritis which is a joint disorder that involves inflammation of one or more joints. Studies have indicated that the level of SP in arthritic joint fluid increases[75] and that intra-articular injection of SP results in an increased severity of experimental arthritis.[76] Furthermore, SP can be released from peripheral nerve endings into joints. The NK1R gene is expressed in the synovium of both rheumatoid arthritis and osteoarthritis.[77] In studies[78,79] involving the use of an NK1R antagonist, anti-inflammatory effects were observed in experimental arthritis providing evidence that SP may be involved in the pathogenesis of rat arthritis. The animal arthritis model is complicated and as a result it is difficult to demonstrate the effect of SP alone. Therefore, a study in 2005 investigated the effects of SP on synovial fibroblastic cells via the NK1R. The results indicated that SP stimulated the proliferation of synovial fibroblastic cells mediated by the NK1R. This release of SP from the peripheral nerve endings during chronic arthritis may be one of the risk factors for arthritis. Further elucidation of the SP/NK1R pathway in synovial cells may provide a better understanding of arthritis.

2.3.4 FIBROMYALGIA

Fibromyalgia was already described as early as the nineteenth century by Gowers[80] who used the term “fibrositis”. In the 1950s, fibrositis was described as a pain syndrome in the absence of any organic disease. In 1977, Smythe and Moldofsky[81] coined the new term “fibromyalgia” and proposed diagnostic criteria that could be used to identify patients with the fibromyalgia syndrome. In 1990 the American College of Rheumatology published a report[82] that aimed to redefine the diagnostic criteria for fibromyalgia by using the recognition of the disease’s characteristics. They evaluated patients in 16 different centres across the United States and Canada and

proposed that positive diagnosis would consist of a self-report history of widespread pain (above and below the waist and on both sides of the body together with axial skeletal pain) lasting more than three months with the demonstration of at least 11 out of 18 specific bilateral tender points (**Figure 2.3**) on examination by digital palpation applying approximately 4 kg of pressure.[83] By 2010 this diagnostic criteria was updated[84] to include fatigue, non-refreshing sleep and other somatic complaints. Usually extensive laboratory testing is not used. However, physicians will sometimes run a complete blood cell count, comprehensive metabolic panel, thyroid function test, erythrocyte sedimentation rate test, and C-reactive protein level[85] determination to aid in diagnosis.

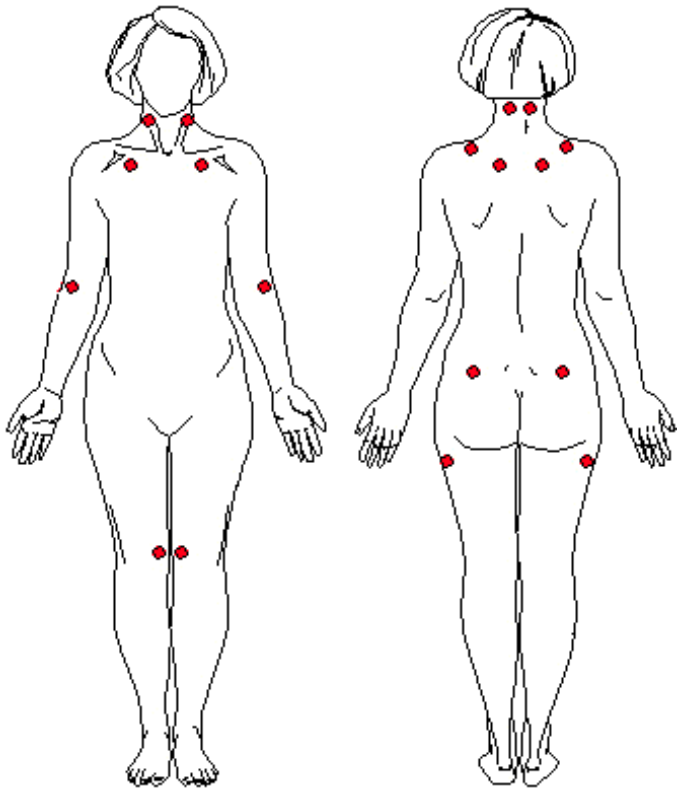


Figure 2.3: Illustration of tender point localisations.[82]

The lack of an absolute diagnostic criterion that can be applied collectively to patients often means that doctors will settle for a fibromyalgia diagnosis following elimination of other conditions.[86] To prevent misdiagnosis physicians must carefully consider many other potential diagnoses such as mental health disorders, hypothyroidism, rheumatoid arthritis, adrenal dysfunction, and multiple myeloma.[87] In general, almost

every patient complains of pain, fatigue, and sleep disturbance.[88,89] The features of the pain must be further investigated as well as additional symptoms that may appear unrelated to fibromyalgia such as irritable bowel syndrome, headaches, restless legs syndrome, irritable bladder syndrome, Raynaud's phenomenon, weight fluctuations, morning stiffness, heat and cold intolerance, and cognitive disturbances.[90]

Diagnosis of fibromyalgia is still predominantly based on the criteria established by the American College of Rheumatology and the amendments that were made in 2010. However there has been much criticism of the use of this criterion over the years due to studies that have failed to show a correlation between tender point counts and clinical pain.[91] In an attempt to capture the entire spectrum of problems related to fibromyalgia, the Fibromyalgia Impact Questionnaire was developed in the 1980s. It was later modified to reflect ongoing experience with the system. It has shown promise in the clinical setting however there are some problems with the system, including gender bias and ambiguity.[92] Despite all the information that has been gathered over the years regarding fibromyalgia, it still remains a very multifaceted condition that results in persistent and debilitating pain that has a severe impact on a patient's ability to work and carry out every day activities.[90] The awareness and understanding of this condition has improved significantly over the years; yet there are still up to 75 % of patients remaining undiagnosed.[93] It is therefore evident that a diagnostic tool that could support the tender point analysis would prove extremely beneficial to physicians faced with patients that have a myriad of symptoms.

It has long been thought that SP is involved in the chronic widespread pain that is associated with fibromyalgia. The amplification of pain impulses from the periphery (such as skin and muscle) to the spinal cord and brain is referred to as central sensitisation. This implies that the level of pain that is experienced is not directly proportional to the pain stimulus. Pain amplification is seen most frequently in the spinal cord at the synapse where major chemicals such as glutamate and SP are released. The activity of this synapse is affected by the descending system. Certain medications for the treatment of fibromyalgia have proven to be effective due to their impact on the descending system. Examples of effective treatments include antidepressants that are able to reduce pain by increasing the inhibitory effect of the

descending system or anti-epileptic medication which reduces the release of glutamate and therefore pain.

A study determining the levels of SP in the cerebrospinal fluid indicated a significant difference in the concentrations between fibromyalgia patients and healthy controls (mean 36 fmol/ml versus mean 12 fmol/ml).[94] Elevated levels of SP and glutamate and the low level of major metabolites of serotonin, norepinephrine and dopamine[95] supports the idea that central amplification is responsible for the abnormal pain transmission and perception in fibromyalgia patients.[96] Similar results were observed in other studies[97,98,99] except where assays involving serum resulted in inconsistent results.[100] Treatments that made use of SP/NK1R antagonists were exploited but the drugs did not perform as expected.[101]

The application of intense painful stimulus for a period of two to five minutes results in the production of generalised whole-body analgesia. This may occur by activation of the endogenous analgesic systems which is designated by the process of Diffuse Noxious Inhibitory Control (DNIC).[102] This process occurs in healthy subjects and is a model that is used to quantify central pain sensitization in chronic pain patients. It hypothesises that strong pain that is sustained in one place will decrease pain responsiveness in several other places in the body. In fibromyalgia patients however, several studies have indicated that DNIC is either attenuated or is absent.[103,104] This is also the case for patients that suffer from other chronic pain conditions. Although this phenomenon is not observed in all fibromyalgia patients it is more common in patients than in healthy controls. In a study by Lautenbacher and Rollman,[104] fibromyalgia patients exhibited deficient pain modulation when compared with matched pain-free control subjects but they could not definitively establish whether the pain reduction observed in the healthy patients was due to the DNIC effect or another mechanism. Investigation of DNIC in fibromyalgia patients can positively affect the treatment approach. Patients who have an absent DNIC can be treated with the aim of restoring DNIC therefore determining if DNIC is absent or present in patients may aid in tailoring the treatment regime.

Elevated levels of SP have been found in other chronic pain conditions such as daily headache and co-morbidities reported with fibromyalgia such as irritable bowel syndrome, sleep disturbance and mood change including depression. There is also

an overlap between patients with chronic widespread pain and post-traumatic stress disorder (PTSD) whereby elevated levels of SP were found in PTSD sufferers. Elevated SP levels in fibromyalgia are not seen in patients diagnosed with chronic fatigue syndrome suggesting a difference between the conditions. The wide variation in the levels of SP could be due to the fact that multiple stressors are involved.[105] Another study revealed that fibromyalgia patients exhibit significant cellular changes in their skin.[106] Interestingly, SP is released in the skin and has even been associated with flare, itch[107] and psoriasis.[108]

From the literature it is clear that SP is involved in many of the processes that lead to the symptoms associated with fibromyalgia as well as other conditions characterised by chronic pain. Therefore, further evaluation of the SP/NK1R pathway with the use of nuclear imaging could prove valuable. Image guided biodistribution studies, in an animal model, of a suitable radiotracer with a specificity for NK1R, would allow for identification of localised uptake in applicable pathologies. In the plethora of pain, this study focused on soft tissue imaging to identify areas of elevated localised uptake of the radiotracer.

2.4 NUCLEAR MEDICINE

2.4.1 THE ASTONISHING WORLD OF RADIOPHARMACEUTICALS

Nuclear medicine procedures use radioisotopes that are combined with other chemical compounds or pharmaceuticals. These radioactive compounds are known as radiopharmaceuticals (also known as radiotracers, radiodiagnostic agents or tracers[109]) and are used for the treatment and diagnosis of diseases. Once they are administered to a patient, they have the ability to localize in specific organs or cellular receptors.

Radiopharmaceuticals can be divided into two classes, based on their medical application: diagnostics and therapeutics. For the purpose of this study, only the application of diagnostics is discussed. Diagnostic radiopharmaceuticals aid in the diagnosis or monitoring of a disease or manifestation of a disease in humans.[110] Therefore the radionuclide component of the radiopharmaceutical must have properties that allow it to be easily detected by an appropriate imaging system such as Single-Photon Emission Computed Tomography (SPECT) or Positron Emission

Tomography (PET). The biological component in both instances is usually a small organic or inorganic compound with definite composition or even a macromolecule such as monoclonal antibodies or proteins.[111]

The functioning of a radiopharmaceutical is determined by the characteristics of the two functional components from which it is formed, that is, the radionuclide and the targeting component. The radiation emitted by the radioisotope penetrates the surrounding tissue and is detected by a radiation detector outside the body thus allowing external location and tracking of the radiotracer's radioactivity within the biological system such as the human body. The morphologic structure or the physiologic function of the organ is then assessed.[109] This provides a unique advantage because the disease state can be assessed or treated based on the cellular function and physiology as opposed to just relying on the anatomical information.

The process of SPECT involves reconstruction of the information obtained from gamma detectors to produce a three-dimensional image of the distribution of the radionuclide in the patient. This information is then used to make a quick, accurate diagnosis of a patient's illness. PET functions in a similar manner[112] except that the scanner (a ring of detectors) detects a pair of gamma rays travelling at a 180° angle from each other that are emitted by a positron-emitting radionuclide, such as gallium-68 or fluorine-18 or carbon-11, upon annihilation (i.e. colliding with an electron).

2.4.2 DESIGNING AN EFFECTIVE IMAGING AGENT

Diagnostic radiopharmaceuticals are used to image the functioning of specific organs such as the heart, liver and thyroid, examine blood flow to the brain and assess bone growth. Diagnostic radiopharmaceuticals have also been used to confirm other diagnostic procedures as well as assess the effects of surgery and treatment. The technique exploits the way the body handles substances differently when a disease or pathology is present.

The challenge and focus of radiopharmaceutical development has always been on designing a radioactive drug that selectively targets a specific site in the human body with little to no accompanying uptake in non-targeted organs and tissue. This requires an in-depth investigation of the target, target molecule and its metabolism and the

labelling site of the molecule. Designing an optimal radiopharmaceutical can be a complicated process of carefully considering the physical and chemical factors that influence the tracer's delivery to the target site and subsequent *in vivo* metabolism.

An example of one of the approaches that can be followed to design a receptor-based target-specific radiopharmaceutical is illustrated in **Figure 2.4**. The biologically active moiety (BAM) is the targeting molecule which is based on the intended target and is responsible for transporting the radioactivity to the target area. The chelator strongly coordinates to the metal centre/radionuclide and is covalently attached to the targeting molecule either directly or through a linker molecule. The linker is often used to modify the pharmacokinetics of the complex and can be a simple hydrocarbon chain or a more complex molecule.[111]

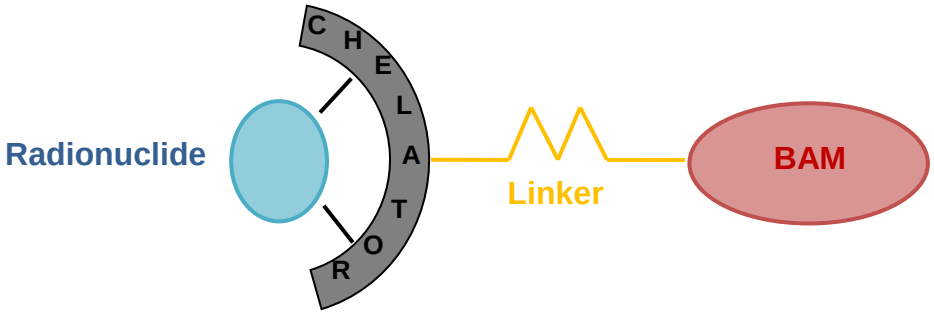


Figure 2.4: Schematic representation of a receptor-based target-specific radiopharmaceutical.[111]

A diagnostic radiopharmaceutical should ideally:

- possess physical characteristics detectable by nuclear medical imaging apparatus;
- provide a minimal radiation dose to the patient;
- contain either a small organic or inorganic compound with definite composition or macromolecule such as monoclonal antibodies or proteins[111] that determines organ specificity and the metabolism of the actual radiopharmaceutical;
- exhibit a nontoxic behaviour or metabolism to comply with requirements regarding safety for human administration;[109] and

- possess a targeting mechanism that facilitates high accumulation in the area of interest.

With regards to PET-based imaging, which is the focus of this study, currently the most common PET radioisotopes are fluorine-18 (^{18}F), carbon-11 (^{11}C), and gallium-68 (^{68}Ga). Oxygen-15 (^{15}O), nitrogen-13 (^{13}N), rubidium-82 (^{82}Rb) and copper-64 (^{64}Cu) are also PET isotopes of interest (**Table 2.1**). Of all these radioisotopes, ^{18}F has been regarded as the workhorse of PET clinics.[113]

Table 2.1: Radionuclides used in PET nuclear medicine and their properties.[114, 115, 116.117]

Radionuclide	Type of decay	Principal photon emission (keV)	Physical half-life (h)
^{82}Ru*	Positron emission	511	0.02
^{64}Cu	β^+	1320	12.7
^{68}Ga*	B^+ and electron capture	1900	1.13
^{177}Lu	β^-	208, 113	159.4
^{15}O	Positron emission	511	0.03
^{11}C	Positron emission	511	0.33
^{13}N	Positron emission	511	0.17
^{18}F	Positron emission	511	1.83

*Refers to radionuclides that are available in a generator format

2.4.2.1 Gallium-68: A suitable medicinal radioisotope

^{68}Ga was first introduced to clinical medicine in the early 1960s, even before [^{18}F]-fluorodeoxyglucose was used.[118] It was very quickly overtaken by more favourable agents such as technetium-99m and fluorine-18. One of the reasons for this was the fact that ^{68}Ga was obtained from a generator in the form of a complex with ethylenediaminetetraacetic acid (EDTA) and the complex had to be destroyed before forming the radiopharmaceutical. Radionuclide generators are self-contained systems that hold a mixture of a mother and daughter radionuclide pair in equilibrium. When “milked” or eluted, the generator is designed to yield the daughter radionuclide

which has been formed from the decay of the mother radionuclide.[113] In 2008, a method[119] to purify the eluate from the Ga generator was introduced and this was applied to the production of ^{68}Ga-DOTATOC which is currently one of the most widely used ^{68}Ga-PET radiopharmaceuticals.

The first published clinical work with a ^{68}Ga-based radiopharmaceutical was reported in 2001.[120] In 2005 a group of Russian radiochemists[121] developed and made available a new type of germanium-68/gallium-68 generator (^{68}Ge/^{68}Ga) which allowed for acidic elution (hydrochloric acid) of the generator to yield a cationic ^{68}Ga which provided greater complexation options.[122] With these advances, the PET radionuclide ^{68}Ga has gained significant research and clinical interest from the start of the 21st century.

There are several properties of ^{68}Ga making it attractive as a radiometal-isotope for imaging purposes. The 68 min half-life of ^{68}Ga matches the pharmacokinetics of many peptides and other small molecules. The resulting Ga-68 labelled radiopharmaceuticals have rapid blood clearance, quick diffusion, and target localization. The short physical half-life also allows for improved dosimetry and repeat imaging.[123] More importantly, it can be produced cost efficiently using a ^{68}Ge/^{68}Ga generator making it on-site accessible within nuclear medicine departments. The longer half-life of the parent isotope ^{68}Ge (270 days) means that the generator can be used for up to 1 year (9 months under GMP conditions) or even longer. The ability to elute the generator at relatively short (2-3 h) pre-defined intervals, means that more than one patient can be imaged in a day. Several versions of the ^{68}Ge/^{68}Ga generator are now commercially available providing motivation for the development of new gallium-based radiopharmaceuticals (**Figure 2.5**). The $^{68}Ga^{3+}$ cation has the ability to form stable complexes with a variety of nitrogen and oxygen donor ligands, making the radionuclide very versatile. The most frequently used PET isotope, ^{18}F, is cyclotron produced which is costly and difficult to obtain if there is no cyclotron in close proximity to the hospital. Furthermore, the expense associated with establishing and maintaining cyclotron facilities has in some ways limited the availability of PET imaging.[124] Even though ^{68}Ga has a much lower positron yield and higher endpoint positron energy emission,[125] its ready availability through the generator system makes it an attractive alternative to its superior counterpart ^{18}F.

TiO_2
column matrix
Cyclotron Company
Russia, 1996

TiO_2
column matrix
Eckert & Ziegler
Germany, 2008

SnO_2
column matrix
iThemba Laboratory
South Africa, 2008

SnO_2-based resin
column matrix
Isotope Technologies
Garching
Germany, 2010

Figure 2.5: The different types of commercially available generators according to the column matrix, manufacturers and year that they were placed on the market.[113]

Once the radionuclide (or label) is determined it is imperative to investigate whether the label can be successfully attached to the chosen biological molecule. The chemical properties of both components must be determined in order to assess their compatibility.

2.4.2.2 Substance P: A suitable biomarker for pain imaging

In order to develop an appropriate imaging agent, the choice of biomarker is critical since this molecule will direct the radioactive tag that allows for imaging, to the correct location. In terms of determining NK1R availability, the first choice for a ligand for the imaging agent would be SP since in the biological system it has a high affinity and selectivity for the receptor. The availability of artificially synthesized SP makes it possible to introduce exogenously a radiolabelled version of the peptide. It is likely that there will be competition between the endogenous and the exogenously introduced SP but this principle has been applied and proven before in the imaging of somatostatin receptors with ^{68}Ga-DOTATATE.[126]

Over the years, several studies have focused on synthesizing metabolically stable analogues of SP. Reversing the direction of a single peptide bond in the C terminal hexapeptide analogue of SP (retro-inverso modification) yields either a full agonist of SP or an analogue that is completely devoid of biological activity.[127] This depends on which amide bond in the peptide sequence is modified implying that the C-terminal could play a role in ensuring the peptide has resistance to proteolytic degradation. The analogue [pGlu^6 psi(NH-CO)(RS)-Phe^7]SP-(6-11) did not possess any biological activity. Retro-inverso modification particularly at amide bond Phe^8-Gly^9 results in [pGlu^6,Phe^8 psi(NH-CO)Gly^9]SP-(6-11), a biologically active analogue that demonstrated excellent resistance to proteolytic degradation when evaluated in rat parotid and hypothalamic slice systems. In another study, SP was labelled with five different fluorescent probes and their ligand binding, receptor activation and fluorescent labelling properties were determined.[128] Studies have shown that Lys^3 and Phe^8 of SP interacts with the NH_2-terminal extracellular tail and second extracellular loop of the SP receptor respectively in mice.[129] This indicates the importance of any alterations at these positions such as changes in charge that ultimately change the overall charge of the parent compound. The addition of one of the fluorescent probes (with multiple negative charges) that significantly changed the overall charge of the peptide resulted in inhibition of proper ligand-receptor interaction between the fluorescent SP and the receptor. One positively charged probe which changed the overall peptide charge did not greatly influence the parent compounds activity. The smaller, neutral probes were however the best option as they had no impact on the normal functioning of SP at its receptor.

In 2005, a research group in France[130] evaluated the effect of simultaneously incorporating proline/proline-amino acid chimeras in positions 9, 10 and/or 11 of SP. The affinity and activity of these derivatives on the two NK1R binding sites was assessed to determine structural requirements for high-affinity binding to the receptor. The results of the study confirmed that the C-terminal component is crucial and should exist in an extended confirmation close to a polyproline II structure whilst bound to the receptor. In addition, the peptide backbone flexibility around the hinge-point residue Gly^9 plays a vital role in subtly positioning crucial side chains.

^{68}Ga-labelled SP derivatives have been reported a few times in literature thus far. In 2004 a patent was filed (Europe and USA) regarding the use of SP whereby it was radiolabelled with various isotopes including gallium-68 for application in brain imaging, particularly glioblastoma multiforme.[131,132] The patent has since lapsed and also does not cover the application of ^{68}Ga-labelled SP for imaging of chronic pain disorders or evaluation of NK1R behaviour in osteoarthritic conditions characterised by chronic pain symptoms. In the patent study, ^{90}Y-DOTAGA-SP was given to patients in therapeutic doses and no adverse reactions were indicated. In 2008, a group from Brazil reported a similar radiolabelling procedure of SP using lutetium-177 for the evaluation of pancreatic tumour cell uptake in nude mice.[133] The results aimed to give an indication of the specificity of the radiolabelled compound to NK1R that are typically overexpressed in malignant brain tumours. In 2013, a group of Polish researchers presented a poster at the European Association of Nuclear Medicine conference in Lyon titled, "Routine preparation of ^{68}Ga-DOTA-SP doses for locoregional administration follow up of [^{213}Bi]Bi-DOTA-SP in the course of glioma therapy" (where DOTA = 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid).[134] The work formed part of the IAEA co-ordinated research project No. 16476 entitled "Development of ^{68}Ga based PET-Radiopharmaceuticals for Management of Cancer and other Chronic Diseases". In that study, [^{68}Ga]Ga-DOTA-SP was administered to patients in combination with [^{213}Bi]Bi-DOTA-SP as targeted alpha-radionuclide therapy of functionally critically located gliomas. The administration was however by intracavitary injection and not by intravenous bolus administration because SP cannot traverse the blood-brain barrier. The analogue of SP that was utilised was DOTA-[Thi^{8},Met(O_2)^{11}]SP with the following structure bearing modifications to the amino acid residues in the 8th and 11th position:

DOTA - Arg - Pro - Lys - Pro - Gln - Gln - Phe – **Thi** - Gly - Leu - **Met(O_2)**-NH_2

Since the initial report of the radiotracer, clinical trials have progressed and great successes have been reported on the prolonged survival of patients treated with the therapeutic agent.[135,136] It is this analogue of SP, DOTA-[Thi^{8},Met(O_2)^{11}]SP that was utilised for the purpose of this study.

Bismuth-213 (t ½ = 45.7 min) is a mixed alpha/beta emitting radionuclide[137] and one of the radionuclides that has been proposed as the theranostic partner to ^{68}Ga. The

term theranostics is essentially derived from combining therapeutics and diagnostics and refers to the concept of using the combination of a diagnostic to identify disease and a second radioactive drug to treat the identified disease. The end result of a personalised approach is improved patient care.[138] The diagnostic can also be used to assess follow up treatment or to stage reoccurrence. The aim of the theranostic approach is to apply targeted therapy that is based on a very precise diagnostic test. In the glioma study, [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP is the diagnostic and [^{213}Bi]Bi-DOTA-[Thi^{8},Met(O_2)^{11}]SP the therapeutic partner, and they are co-administered to the patient. Therapeutic nuclear medicine is slowly moving from the use of beta emitters to alpha emitters because of the improved therapy effectiveness. The high energy deposited in a short range in tissue (~80 µm) is what makes alpha-emitters so effective at destroying cancer cells. The short range of ^{213}Bi also ensures that the radiation dose to surrounding non-target tissue is minimal.[139] The introduction of a clinically approved ^{225}Ac/^{213}Bi generator[140] has allowed for the labelling of antibodies and peptides. In this study, the optimised radiolabelling methodology developed for [^{68}Ga]Ga-DOTA-[Thi^{8}, Met(O_2)^{11}]SP will be applied to ^{213}Bi to produce [^{213}Bi]Bi-DOTA-[Thi^{8}, Met(O_2)^{11}]SP.

2.4.2.3 DOTA: A suitable chelator

Chelators are fundamental components of radiopharmaceuticals as they bind the radiometal ion in a tight stable coordination complex. An effective chelator can impart favourable *in vivo* pharmacokinetics and improve the targeting properties of a radiopharmaceutical.[141] For ^{68}Ga based radiopharmaceuticals, the chelator must be able to bind the Ga^{3+} ion under mild aqueous conditions to yield a complex with adequate kinetic stability *in vivo*.

The first clinically relevant chelated complex of ^{68}Ga, [^{68}Ga]Ga-ethylenediaminetetraacetic acid (EDTA), was created when a first generation of the ^{68}Ge/^{68}Ga generator was eluted into an aqueous solution of EDTA.[142] It was only decades later that the bifunctional chelator DOTA was discovered. The aminocarboxylate macrocycle results in ^{68}Ga-DOTA bioconjugates that are stable *in vitro* in serum for almost two months.[143]

Typical radiolabelling conditions for DOTA conjugates entails heating to 95 °C, for 30 min at pH 4. The high temperature needed to facilitate the macrocycle enveloping the

radiometal is often unsuitable for many proteins of interest and the lengthy time needed for the radiolabelling process allows for decay of the radioactivity.[144] Even though DOTA is the current gold standard of aza-macrocyclic chelators for ^{68}Ga, these drawbacks have prompted research into alternate chelators (**Figure 2.6**) that can function at ambient temperature with minimal radiolabelling time needed for complexation.

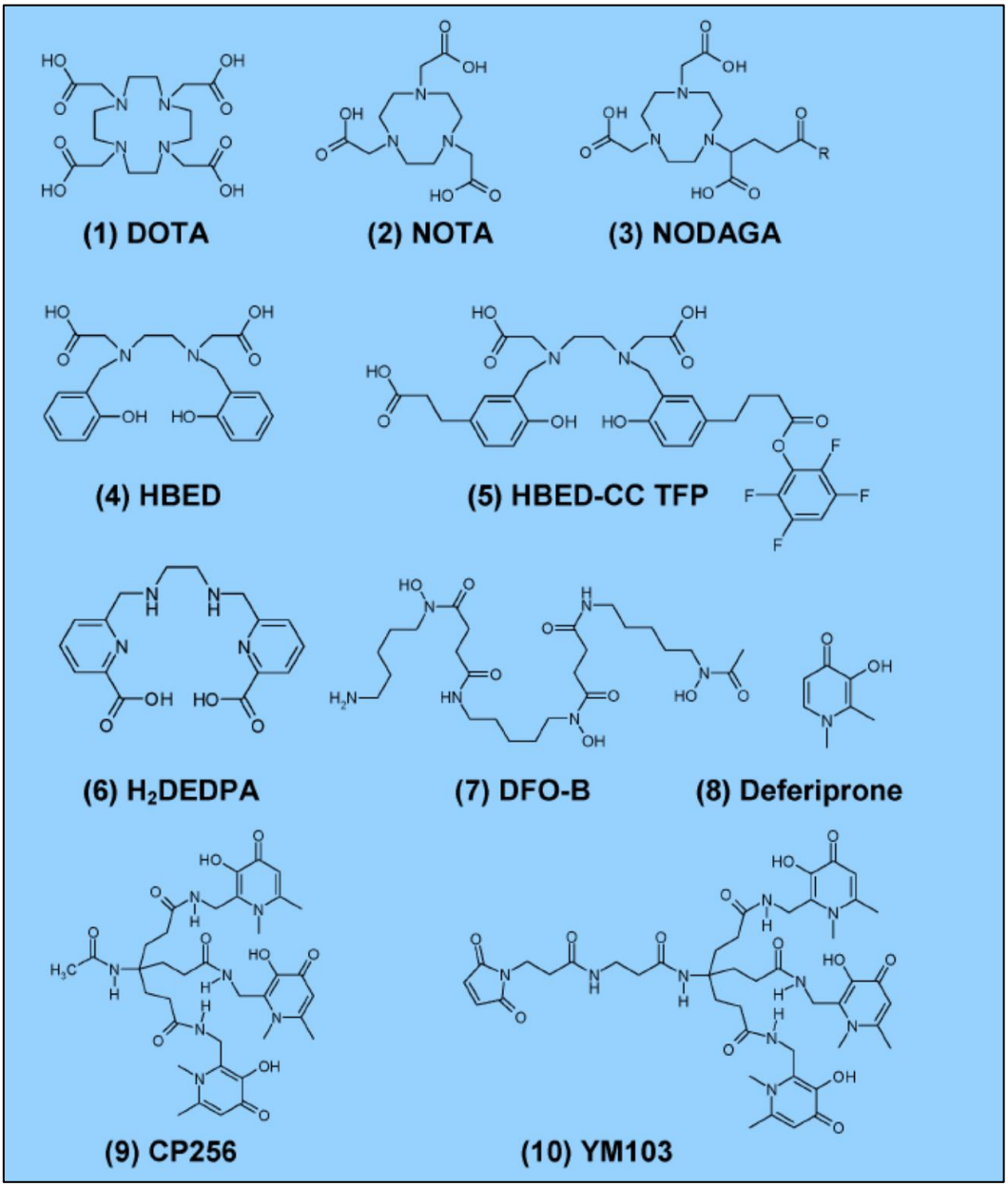


Figure 2.6: Examples of different ^{68}Ga-chelators: chemical structures and common abbreviations.[144]

2.5 PRECLINICAL STUDIES TO VALIDATE NOVEL RADIOPHARMACEUTICALS

2.5.1 CELL STUDIES

Pharmacodynamics is the study of the effects of the administered drug on the biological system.[145] Pharmacokinetics is the study of the effects of the biological system on a drug providing an understanding of the physiological effects of the drug. This discipline has become a very powerful tool in gaining knowledge and understanding of drug dosage in relation to achievable drug concentrations in plasma as well as the influence of disease states on the behaviour of a drug in the body.[146]

A receptor is commonly described as a macromolecule (mostly proteins) in the biological system to which endogenous ligands bind and the result of this binding is an alteration in the biochemical activity.[147] Therefore drugs that come from outside the body are referred to as exogenous ligands. In the development of receptor-based target-specific radiopharmaceuticals it is important to determine the interaction at the receptor. In the case of radiopharmaceuticals whereby the biologically active moiety is in some instances an analogue of an endogenous ligand, cell studies provide confirmation that the altered structure has retained the ability to bind selectively to cells with the target receptor.

The effect of a ligand-receptor interaction can be further classified as agonistic or antagonistic. An agonist-type ligand has the ability to mimic the endogenous ligand in its binding and therefore activates the receptor. A partial agonist-type ligand stimulates the receptor to a limited extent and in so doing prevents endogenous ligands from stimulating the receptor any further. An inverse agonist-type ligand is a full agonist except that it has the opposite pharmacological response to that of a normal agonist.[147]

An antagonist-type ligand binds to the receptor and does not elicit a response whilst simultaneously preventing the agonist from binding at that receptor. Competitive antagonists bind reversibly to the receptor and compete with the agonist. Non-competitive antagonists will bind irreversibly to the receptor and also affect the ability of the agonist to bind.

2.5.2 BIODISTRIBUTION AND PHARMACOKINETIC STUDIES

Biodistribution studies are essential for establishing the efficacy and usefulness of radiopharmaceuticals. These studies include image guided distribution, plasma clearance, and excretion (urinary, faecal) after administration. The radiopharmaceutical is injected into an animal model, allowed to distribute and the animals are then imaged at different time intervals. Regions of interest (ROI) are drawn over major organs such as the heart, lungs, liver, kidneys, spleen, bladder, abdomen and a background area to determine the radioactivity concentration in each organ over time. These values together with the information gained from the blood and urine samples, are used to construct time-activity curves to determine the biodistribution of the radiopharmaceutical. Semiquantitative analysis can be performed by calculating the ROI's Standard Uptake Values (SUV) from which the target-to-non-target-ratio between pathological and reference tissue can be determined. The resulting data is used to determine the efficacy of the radiopharmaceutical for imaging the organ of interest.

Pharmacokinetics is the study of the effects of the biological system on a drug. The information obtained provides an understanding of the physiological effects of the drug. This includes the study of the mechanisms of:[148]

- absorption and distribution of the drug;
- the rate at which the drug takes effect;
- the duration of the aforementioned effect;
- chemical changes of the substance caused by, for example, enzymes and;
- effects and routes of excretion of metabolites of the drug.

The pharmacokinetics of optimal imaging agents is that the radiopharmaceutical should have a fast blood clearance but at the same time remain long enough in the body to allow for sufficient accumulation in the targeted tissues. This implies that a radiopharmaceutical with ideal pharmacokinetics will possess substantial target uptake with a diagnostically useful signal-to-noise ratio in a short period of time.[111]

In plasma clearance studies the rate of localisation of a radiopharmaceutical is determined by measuring the activity in the blood pool. This can be achieved by taking

blood samples at predefined intervals following administration of the radiopharmaceutical. This information is then used to determine the half-time (time required for the radiopharmaceutical to reduce its plasma activity to one half) for plasma clearance of the radiopharmaceutical. The urinary and faecal excretions of a radiopharmaceutical are measured in a similar manner. Urine and faecal samples are taken at definite time intervals after injection and their radioactivity measured.

2.5.3 ORGAN LEVEL RADIATION DOSIMETRY

Dosimetry is the process of relating the amount of radioactivity that has been administered to the resultant dose of radiation that has been absorbed in the organs or whole body.[149] The process is more complicated for radionuclides that are internally distributed by way of radiopharmaceuticals as opposed to an external beam of radiation. Faster excretion of radiopharmaceutical implies a minimised and therefore favourable radiation dose. Animal imaging studies can also provide valuable additional information such as organ or tissue toxicity of the radiopharmaceutical as well as endotherapeutic damages to pathologies such as cancer.

2.6 THE IMAGING OF NK1 RECEPTORS AND CHRONIC PAIN

The identification of SP and the NK1 receptor in a variety of both neural and non-neural cells indicates that this neuropeptide and receptor have an important role in health and disease. More studies are necessary to gain a better understanding of their physiological roles. This study aimed to utilise nuclear imaging in order to elucidate the NK1R availability in conditions characterised by chronic pain.

2.6.1 IMAGING CHRONIC PAIN

Even though the nervous system is not solely responsible for chronic pain, the imaging of the brain has contributed greatly to identifying many of the neural mechanisms involved in chronic pain. Various neuroimaging modalities have been used to study several chronic pain disorders such as chronic low back pain,[150] chronic migraine[151] and osteoarthritis[152] amongst others. Neuroimaging studies have identified several cortical regions in humans that are involved in the perception of

pain. In terms of chronic pain, studies have revealed that there is altered structure, function and neurochemistry in the frontal-limbic-brainstem regions.[153]

For example, various imaging techniques have been used to assess pain processing in fibromyalgia, the results of which have indicated that pain is in fact real in fibromyalgia.[102] This includes functional MRI,[154] proton spectroscopy,[155,156,157] voxel based morphometry,[158] diffusion tensor imaging,[159] arterial spin labelling[160] and SPECT and PET imaging. For the purpose of this study SPECT and PET imaging will be further discussed.

SPECT studies that have used a tracer called [^{99m}Tc]Tc-Ethyl Cysteinate Dimer in a clinical trial has indicated that fibromyalgia patients exhibit brain perfusion abnormalities when compared to pain-free subjects. The degree of abnormalities was shown to correlate with the clinical severity of their diseases.[161] ^{99m}Tc-HMPAO brain perfusion SPECT imaging has also been used to determine if medical treatment with amitriptyline has an impact on cerebral blood flow in fibromyalgia patients. The study indicated that cerebral blood flow improved parallel to clinical response to treatment.[162]

Initial PET studies indicated no difference between healthy humans and fibromyalgia patients with respect to the uptake of [^{18}F]FDG (fluorodeoxyglucose) for all brain structures assessed.[163] Presynaptic dopaminergic function was investigated in six fibromyalgia patients using 6-[^{18}F]F-L-DOPA as a radiotracer.[164] Reduced uptake of the radiotracer was observed in several regions of the brain in the fibromyalgia patients indicating a disruption of presynaptic dopamine activity in which dopamine plays a role in natural analgesia. Another pilot study using [^{18}F]FDG-PET showed an association between increased metabolic activity in limbic structures and the improvement in a comprehensive treatment program.[165] In 2007 carfentanil, a selective μ-opioid receptor, was radiolabelled with carbon-11 (^{11}C) and used in PET studies revealing that fibromyalgia patients displayed receptor occupancy within several regions of the brain known to play a role in pain modulation.[166] Other authors used the binding of [^{11}C]-raclopride to determine the endogenous release of dopamine to painful stimulation during injection of painful hypertonic saline and non-painful normal saline.[167] The level of pain during the hypertonic saline injection was greater in the fibromyalgia patients compared to healthy humans. In addition, healthy

patients released dopamine in the basal ganglia, the levels of which correlated to the amount of perceived pain whereas in fibromyalgia patients no correlation was present.

Recent studies aimed at gaining a better understanding of the Sigma Receptor 1 (S1R) have led to the development of a highly selective S1R PET agent, [^{18}F]F-6-(3-fluoropropyl)-3-(2-azepan-1-yl)ethyl)benzo[*d*]thiazol-2(3H)-one ([^{18}F]FFTC-146).[168] S1R is a protein that is widely distributed in the central nervous system and peripheral tissues and has been linked to various neurologic conditions.[169] It has also been implicated in pain modulation both centrally and peripherally. First-in-human studies have been carried out on ten healthy volunteers using PET/MRI. No adverse reactions were reported. Considerable accumulation of the tracer was observed in S1R dense organs like the pancreas and spleen, moderate uptake in the brain and myocardium whilst there was only low uptake in bone and muscle. Clinical trials investigating different S1R-related diseases is in progress.

The results of neuroimaging studies of chronic pain states have repeatedly indicated extensive alterations in brain structure and function.[170] Furthermore these studies have shown that it is not a single pain centre in the brain but rather various regions that interact with each other and with other regions of the central nervous system such as the spinal cord. Developments involving S1R indicates a renewed interest in being able to visualise pain modulation to improve understanding of the underlying pathologies involved. This indicates that there is a need for further development of imaging techniques as diagnostic tools for the assessment of chronic pain in clinic.

2.6.2 IMAGING STUDIES OF NK1 RECEPTORS

Most imaging studies involving the NK1R have been focused on evaluating the effect of novel NK1 antagonists in the human brain as part of the drive to develop antagonist-based drugs that that will have therapeutic application in a range of disorders including pain and inflammation. Several studies have been aimed at developing and identifying ligands for use as potential radiotracers for *in vivo* imaging of the NK1R.[171,172] (2*S*,3*S*)-*N*-(1-(2- [1-^{11}C]ethoxy-5-(3-(trifluoromethyl)-4*H*-1,2,4-triazol-4-yl)phenyl)ethyl)-2-phenylpiperidin-3-amine demonstrated moderate specific binding in rhesus monkeys and was intended for further studies.[173] ^{11}C-CP-99,994

was proposed as a potential tracer for studying the central and peripheral SP receptors.[174] Though ligands have been suggested, none of them have progressed past pre-clinical studies to be implemented in the clinic. In 2000, two NK1 receptor antagonists (GR203040 and GR205171) were radiolabelled and used in PET studies in rhesus monkeys.[175] Both compounds were taken up rapidly in the brain, [^{11}C]GR205171 more than double the uptake of [^{11}C]GR203040. A pre-treatment of a cold dose of GR205171 was administered in order to carry out the Patlak[176] plot. There was a linear increase in the radioactivity ratio with time and no indication of a plateau. This indicates a slow dissociation from the receptor which would be useful for visualising the NK1R distribution. The use of this compound was however limited since it did not reach equilibrium within the maximum scanning time that is possible for a ^{11}C-radiolabelled ligand.

In 2002, another NK1 antagonist [^{18}F]SPA-RQ was evaluated in preclinical (guinea pig brain and rhesus monkey)[177] and clinical studies.[178] The compound showed an identically high affinity for the NK1R as that observed for endogenous SP. No specific binding was observed in the cerebellum which is an area of the brain that has been proven to contain no NK1Rs. This defined an internal reference region for determination of background, non-specific accumulation of the tracer. In the rhesus monkey the tracer was able to penetrate the cerebellum but washed out with time. Pre-treatment with cold antagonist decreased binding in the caudate and cortex and no effect in the cerebellum. The results of the clinical studies confirmed the high selectivity of the tracer for the NK1R and therefore its usefulness in being able to quantify NK1R occupancy by varying doses of the cold antagonist. The [^{18}F]SPA-RQ tracer only reached equilibrium after 6 h thereby limiting its clinical use. Due to fast plasma kinetics, the amount of parent compound present in the blood, 90 min post injection, was too low to be reliably measured.

In 2009, Wolfensberger *et* al[179] evaluated [^{11}C]R116301 as an *in vivo* tracer of NK1R in humans. The biodistribution of the tracer corresponded to the known distribution of NK1R. When the cold aprepitant (an NK1 antagonist) was administered there was a substantial reduction in the tracer signal to background levels. This served to confirm that the tracer was binding selectively to the NK1R making [^{11}C]R116301 feasible for imaging NK1R. Even though [^{18}F]SPA-RQ possessed a higher specific signal than

[^{11}C]R116301, it has slow kinetics resulting in longer study durations. [^{11}C]R116301 studies can be performed within 90 min.

Even though NK1 antagonists have been proposed for blocking the effects of SP and NK1R this strategy has failed to show any analgesic effect in the clinical setting. In addition, animal studies have indicated that SP is definitely involved in nociceptive signalling, yet similar evidence has not been found in human studies.[180] Clearly more work is necessary to gain a better understanding of the SP/NK1R pathway. Therefore, the aim of this study was to develop an imaging agent that can be used to detect increased NK1R availability in disorders which are characterised by chronic pain. Herein, [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP is further evaluated as the imaging agent with SP being chosen as the ligand with natural affinity for the NK1R in biological systems. [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP-PET/CT has never before been utilised for the evaluation of NK1R in chronic pain disorders.

2.7 REFERENCES

1. Von Euler US, Gaddum JH. An unidentified depressor substance in certain tissue extracts. J Physiol [Internet]. 1931 [cited 2019 Sep 3];72(1):74-87. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1403098/ doi:10.1113/jphysiol.1931.sp002763

2. Gaddum JH, Schild H. Depressor substances in extracts of intestine. J Physiol. 1934; 83:1-14.

3. Donnerer J. The chemical languages of the nervous system: History of scientists and substances. Switzerland: Karger Medical and Scientific Publishers; 2006.

4. Harrison S, Geppetti P. Substance P. Int J Biochem Cell Bio [Internet]. 2001 [cited 2019 Sep 16];33(6):555-76. Available from: https://www.sciencedirect.com/science/article/abs/pii/S1357272501000310?via%3Dihub doi: 10.1016/s1357-2725(01)00031-0

5. Chang MM, Leeman SE. Isolation of a sialogogic peptide from bovine hypothalamic tissue and its characterization as Substance P. J Biol Chem [Internet]. 1970 Sep [cited 2019 Sep 3];245(18):4784-4790. Available from: http://www.jbc.org/cgi/pmidlookup?view=long&pmid=5456150

6. Chang MM, Leeman SE, Niall HD. Amino-acid sequence of SP. Nat New Biol [Internet]. 1971 Jul [cited 2019 Sep 3];232(29):86-7. Available from: https://www.nature.com/articles/newbio232086a0 doi: 10.1038/newbio232086a0

7. Tregear GW, Niall HD, Potts Jun JT, Leeman SE, Chang MM. Synthesis of Substance P. Nat New Biol [Internet]. 1971 Jul [cited 2019 Sep 3];232:87-9. Available from: https://www.nature.com/articles/newbio232087a0 doi:10.1038/newbio232087a0

8. Powell D, Leeman S, Tregear GW, Niall HD, Potts JT. Radioimmunoassay for Substance P. Nat New Biol [Internet]. 1973 [cited 2019 Nov 23];241(112):252-4. Available from: https://www.nature.com/articles/newbio241252a0 doi: 10.1038/newbio241252a0

9. Otsuka M, Takahashi T. Putative peptide neurotransmitters. Annu Rev Pharmacol Toxicol [Internet]. 1977 Apr [cited 2019 Sep 6];17:425-39. Available from: https://doi.org/10.1146/annurev.pa.17.040177.002233

10. Nicoll RA, Schenker C, Leeman SE. Substance P as a transmitter candidate. Ann Rev Neurosci [Internet]. 1980 [cited 2019 Sep 10];3:227-68. Available from: https://www.annualreviews.org/doi/pdf/10.1146/annurev.ne.03.030180.001303 doi: 10.1146/annurev.ne.03.030180.001303

11. Pernow B. Substance P. Pharmacol Rev [Internet]. 1983 Jun [cited 2019 Sep 11];35(2):84-141. Available from: http://pharmrev.aspetjournals.org/content/35/2/85.long

12. Otsuka M, Yoshioka K. Neurotransmitter functions of mammalian tachykinins. Physiol Rev [Internet]. 1993 [cited 2019 Sep 9];73(2):229-307. Available from: https://europepmc.org/article/med/7682720 doi: 10.1152/physrev.1993.73.2.229

13. Lembeck F, Donnerer J. History of a pioneering neuropeptide: Substance P. In: Holzer P. editor. Tachykinins. Handbook of Experimental Pharmacology, vol 164. Berlin: Springer; 2004.

14. O' Connor TM, O' Connell J, O' Brien DI, Goode T, Bredin CP, Shanahan F. The role of Substance P in inflammatory disease. J Cell Phys [Internet]. 2004 [cited 2019 Oct 1];201:167-80 Available from: https://onlinelibrary.wiley.com/doi/full/10.1002/jcp.20061 doi:10.1002/jcp.20061

15. Mantyh PW. Neurobiology of Substance P and the NK1 receptor. J Clin Psychiat [Internet]. 2002 [cited 2019 Sep 25];63(S11):6-10. Available from: https://www.psychiatrist.com/jcp/article/pages/2002/v63s11/v63s1102.aspx

16. Hokfelt T, Pernow B, Wahren J. Substance P: a pioneer amongst neuropeptides. J Int Med [Internet]. 2001 Jan [cited 2019 Sep 14];249:27-40. Available from: https://doi.org/10.1046/j.0954-6820.2000.00773.x

17. Steinhoff MS, von Mentzer B, Geppetti P, Pothoulakis C, Bunnett NW. Tachykinins and their receptors: contributions to physiological control and the mechanisms of disease. Physiol Rev [Internet]. 2014 [cited 2019 Sep 30];94(1): 265–301. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3929113/ doi: 10.1152/physrev.00031.2013

18. Masu Y, Nakayama K, Tamaki H, Harada Y, Kuno M, Nakanishi S. cDNA cloning of bovine substance-K receptor through oocyte expression system. Nature [Internet]. 1987 [cited 2019 Sep 19];329:836-38. Available from: https://www.nature.com/articles/329836a0 doi: 10.1038/329836a0

19. Takahashi K, Tanaka A, Hara M, Nakanishi S. The primary structure and gene organization of human SP and neuromedin K receptors. Eur J Biochem [Internet]. 1992 Mar [cited 2019 Sep 20];204:1025-33. Available from: https://doi.org/10.1111/j.1432-1033.1992.tb16724.x

20. Gerard NP, Garraway LA, Eddy RL Jr, Shows TB, Iijima H, Paquet JL et al. Human Substance P receptor (NK1): organisation of the gene, chromosome localization, and functional expression of cDNA clones. Biochemistry [Internet]. 1991 Nov [cited 2019 Oct 1];30(44):10640-6. Available from: https://pubs.acs.org/doi/abs/10.1021/bi00108a006 doi: 10.1021/bi00108a006

21. Hopkins B, Powell SJ, Danks P, Briggs I, Graham A. Isolation and characterization of the human lung NK1 receptor cDNA. Biochem Biophys Res Commun [Internet]. 1992 [cited 2019 Oct 1];182(3):1514. Available from: https://www.sciencedirect.com/science/article/pii/0006291X92919056?via%3Dihub doi: 10.1016/0006-291x(92)91905-6

22. Snider RM, Constantine JW, Lowe JA 3rd, Longo KP, Lebel WS, Woody HA, et al. A potent nonpeptide antagonist of the Substance P (NK1) receptor. Science [Internet]. 1991 Jan [cited 2019 Oct 1];251:435-7. Available from: https://science.sciencemag.org/content/251/4992/435.long doi: 10.1126/science.1703323

23. Gether U, Johansen TE, Snider RM, Lowe JA 3rd, Nakanishi S, Schwartz TW. Different binding epitope for Substance P and the non-peptide antagonist, CP-96 345 on the NK1 receptor. Nature [Internet]. 1993 [cited 2019 Oct 2];362:345-8. Available from: https://www.nature.com/articles/362345a0 doi: 10.1038/362345a0

24. Cascieri MA, Huang RR, Fong TM, Cheung AH, Sadowski S, Ber E, et al. Determination of the amino acid residues in Substance P conferring selectivity and specificity for the rat neurokinin receptors. Mol Pharmacol [Internet]. 1992 Jun [cited 2019 Oct 4];41:1096-9. Available from: http://molpharm.aspetjournals.org/content/41/6/1096.long

25. DeVane, CL. Substance P: A new era, a new role. Pharmacotherapy [Internet]. 2001 [cited 2019 Oct 3];21(9):1061-9. Available from: https://accpjournals.onlinelibrary.wiley.com/doi/abs/10.1592/phco.21.13.1061.34612?sid=nlm%3Apubmed doi: 10.1592/phco.21.13.1061.34612

26. Quartara L, Maggi CA. The tachykinin NK1 receptor. Part I: ligands and mechanisms of cellular activation. Neuropeptides [Internet]. 1997 [cited 2019 Oct 3];31(6):537-63. Available from: https://www.sciencedirect.com/science/article/abs/pii/S0143417997900019?via%3Dihub doi: 10.1016/s0143-4179(97)90001-9

27. Saria A. The tachykinin NK1 receptor in the brain: pharmacology and putative functions. Eur J Pharmacol [Internet]. 1999 [cited 2019 Sep 27];375(1-3):51-60. Available from: https://www.sciencedirect.com/science/article/pii/S0014299999002599?via%3Dihub doi: 10.1016/s0014-2999(99)00259-9

28. Quartara L, Maggi CA. The tachykinin NK1 receptor. Part II: distribution and pathophysiological roles. Neuropeptides [Internet]. 1998 [cited 2019 Sep 23];32:1-49. Available from: https://www.sciencedirect.com/science/article/abs/pii/S0143417998900154?via%3Dihub doi: 10.1016/s0143-4179(98)90015-4

29. Nakaya Y, Kaneko T, Shigemoto R, Nakanishi S, Mizuno N. Immunohistochemical localization of Substance P receptor in the central nervous system of the adult rat. J Comp Neurol [Internet]. 1994 [cited 2019 Oct 6];347(2):249-74. Available from: https://onlinelibrary.wiley.com/doi/abs/10.1002/cne.903470208?sid=nlm%3Apubmed doi: 10.1002/cne.903470208

30. Bost KL, Breeding SA, Pascual DW. Modulation of the mRNAs encoding Substance P and its receptor in rat macrophages by LPS. Reg Immunol. 1992 Mar-Apr;4(2):105-12.

31. Joos GF, Germonpré PR, Pauwels RA. Role of tachykinins in asthma. Allergy [Internet]. 2000 [cited 2019 Oct 4];55(4):321-37. Available from: https://onlinelibrary.wiley.com/doi/full/10.1034/j.1398-9995.2000.00112.x?sid=nlm%3Apubmed doi: 10.1034/j.1398-9995.2000.00112.x

32. Killingsworth CR, Shore SA, Alessandrini F, Dey RD, Paulaskis JD. Rat alveolar macrophages express preprotachykinin gene-I mRNA encoding tachykinins. Am J Physiol Lung Cell [Internet]. 1997 Nov [cited 2019 Oct 12];273:L1073-81. Available from: https://doi.org/10.1152/ajplung.1997.273.5.L1073

33. Black PH. Stress and inflammatory response: A review of neurogenic inflammation. Brain Behav Immun [Internet]. 2002 [cited 2019 Oct 11];16(6);622-53. Available from: https://www.sciencedirect.com/science/article/pii/S0889159102000211?via%3Dihub doi: 10.1016/s0889-1591(02)00021-1

34. Walsh DT, Weg VB, Williams TJ, Nourshargh S. Substance P induced inflammatory responses in guinea-pig skin: the effect of specific NK1 receptor antangonists and the role of endogenous mediators. Br J Pharmacol [Internet]. 1995 [cited 2019 Oct 13];114:1343-50. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1510297/ doi: 10.1111/j.1476-5381.1995.tb13354.x

35. Baluk P, Geppetti P, McDonald M, Nadel JA. NK1 receptors mediate leukocyte adhesion in neurogenic inflammation in the rat trachea. Am J Physiology Lung Cell [Internet]. 1995 [cited 2019 Oct 9];268:L263-9. Available from: https://physiology.org/doi/abs/10.1152/ajplung.1995.268.2.L263 doi: 10.1152/ajplung.1995.268.2.L263

36. Perretti M, Ahluwalia A, Flower RJ, Manzini S. Endogenous tachykinins play a role in interleukin-1 induced neutrophil accumulation: involvement of NK1 receptors. Immunology. 1993 Sep [cited 2019 Oct 3];80(1):73-7.

37. van der Kleij HP, Ma D, Redegeld FA, Kraneveld AD, Nijkamp FP, Bienenstock J. Functional expression of neurokinin 1 receptors on mast cells induced by IL-4 and stem cell factor. J Immunol [Internet]. 2003 Aug [cited 2019 Sep 29];171(4):2074-9 Available from: https://doi.org/10.4049/jimmunol.171.4.2074

38. McGillis JP, Mitsuhashi M, Payan DG. Immunomodulation by tachykinin neuropeptides. Ann NY Acad Sci [Internet]. 1990 [cited 2019 Sep 21];594:85-94. Available from: https://nyaspubs.onlinelibrary.wiley.com/doi/abs/10.1111/j.1749-6632.1990.tb40470.x doi: 10.1111/j.1749-6632.1990.tb40470.x

39. Lucey DR, Novak JM, Polonis VR, Liu Y, Gartner S. Characterization of Substance P binding to human monocytes/macrophages. Clin Diagn Lab Immunol. 1994 May;1(3):330-5.

40. Payan DG, Brewster DR, Goetzl EJ. Stereospecific receptors for Substance P on cultured human IM-9 lymphoblasts. J Immunol [Internet]. 1984 Dec [cited 2019 Sep 28];133(6):3260-5. Available from: https://www.jimmunol.org/content/133/6/3260

41. Payan DG, Brewster DR, Missirian-Bastian A, Goetzl EJ. Substance P recognition by a subset of human T lymphocytes. J Clin Invest [Internet]. 1984 [cited 2019 Sep 10];74(4):1532-9. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC425324/ doi: 10.1172/JCI111567

42. Payan DG, McGillis JP, Organist ML. Binding characteristics and affinity labelling of protein constituents of the human IM-9 lymphoblast receptor for SP. J Biol Chem. 1986 Oct;261:14321-9.

43. Roberts AI, Taunk J, Ebert EC. Human lymphocytes lack Substance P receptors. Cell Immunol [Internet]. 1992 [cited 2019 Oct 8];141:457-65. Available from: https://www.sciencedirect.com/science/article/abs/pii/000887499290163J?via%3Dihub doi: 10.1016/0008-8749(92)90163-j

44. Kavelaars A, Jeurissen F, Heijnen CJ. Substance P receptors and signal transduction in leukocytes. Immunomethods [Internet]. 1994 [cited 2019 Oct 7];5(1):41-8. Available from: https://www.sciencedirect.com/science/article/abs/pii/S1058668784710369 doi: 10.1006/immu.1994.1036

45. Kähler CM, Sitte BA, Reinisch N, Wiedermann CJ. Stimulation of the chemotactic migration of human fibroblasts by Substance P. Eur J Pharmacol [Internet]. 1993 [cited 2019 Oct 9];249(3):281-6. Available from: https://www.sciencedirect.com/science/article/pii/001429999390523K?via%3Dihub doi: 10.1016/0014-2999(93)90523-k

46. Walsh DA, Mapp PI, Wharton J, Rutherford RA, Kidd BL, Revell PA et al. Localisation and characterization of Substance P binding to human synovial tissue in rheumatoid arthritis. Ann Rheum Dis [Internet]. 1992 Mar [cited 2019 Oct 11];51(3):313-7. Available from: http://dx.doi.org/10.1136/ard.51.3.313

47. Matayoshi T, Goto T, Fukuhara E, Takano H, Kobayashi S, Takahashi T. Neuropeptide Substance P stimulates the formation of osteoclasts via synovial fibroblastic cells. Biochem Biophys Res Commun [Internet]. 2005 [cited 2019 Oct 8];327(3):756-64. Available from: https://www.sciencedirect.com/science/article/pii/S0006291X04028323?via%3Dihub doi: 10.1016/j.bbrc.2004.12.055

48. Andersson G, Danielson P, Alfredson H, Forsgren S. Presence of Substance P and the neurokinin-1 receptor in tenocytes of the human Achilles tendon. Reg Peptides [Internet]. 2008 Oct [cited 2019 Oct 5];150(1-3):81-7. Available from: https://www.sciencedirect.com/science/article/abs/pii/S0167011508000359?via%3Dihub doi: 10.1016/j.regpep.2008.02.005

49. Backman LJ, Eriksson DE, Danielson P. Substance P reduces TNF-α-induced apoptosis in human tenocytes through NK1 receptor stimulation. Br J Sports Med

[Internet]. 2014 [cited 2019 Oct 5];48(19):1414-20. Available from: https://bjsm.bmj.com/content/48/19/1414 doi: 10.1136/bjsports-2013-092438

50. Munoz M, Covenas R. Involvement of Substance P and the NK1 receptor in pancreatic cancer. World J Gastroenterol [Internet]. 2014 [cited 2019 Oct 8];20(9):2321-34. Available from: https://www.wjgnet.com/1007-9327/full/v20/i9/2321.htm doi: 10.3748/wjg.v20.i9.2321

51. Hedley L, Henry JL. Participation of Substance P in spinal physiological responses to peripheral aversive stimulation. Regul Peptides. 1993;46:138-43.

52. Thompson SWN, Dray A, Urban L. Injury-induced plasticity of spinal reflex activity: NK1 neurokinin receptor activation and enhanced A- and C-fiber mediated responses in the rat spinal cord in vitro. J Neurosci [Internet]. 1994 [cited 2019 Oct 11];14(6):3672-8. Available from: https://www.jneurosci.org/content/14/6/3672.long doi:10.1523/JNEUROSCI.14-06-03672.1994

53. Gardner CJ, Twissell DJ, Dale TJ, Gale JD, Jordan CC, Kilpatrick GJ, et al. The broad spectrum anit-emetic activity of the novel non-peptide tachykinin NK1 receptor antagonist GR203040. Br J Pharmacol [Internet]. 1995 Dec [cited 2019 Oct 9];116(8):785-9. Available from: https://doi.org/10.1111/j.1476-5381.1995.tb15118.x

54. Teixeira RM, Santos AR, Ribeiro SJ, Calixto JB, Rae GA, De Lima TC. Effects of central administration of tachykinin receptor agonists and antagonists on plus-maze behavior in mice. Eur J Pharmacol [Internet]. 1996 [cited 2019 Oct 9];311(1):7-14. Available from: https://www.sciencedirect.com/science/article/pii/0014299996003901 doi: 10.1016/0014-2999(96)00390-1

55. Kidd BL, Morris VH, Urban L. Pathophysiology of joint pain. Ann Rheum Dis [Internet]. 1996 [cited 2019 Oct 9];55(5):276-83. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1010160 doi: 10.1136/ard.55.5.276

56. Gillen C, Maul C. New pharmacological strategies for pain relief. Expert Rev Neurother [Internet]. 2002 [cited 2019 Oct 1];2(5):691-702. Available from: https://www.tandfonline.com/doi/abs/10.1586/14737175.2.5.691 doi: 10.1586/14737175.2.5.691

57. Jessell T. Substance P in nociceptive sensory neurons. In: Porter R, O'Connor M, editors. Ciba Foundation Symposium 91 - Substance P in the Nervous System. USA: John Wiley & Sons; 1982.

58. Helme RD, Fletcher J. Substance P in the trigeminal system at postmortem: Evidence for a role in pain pathways in man. Clin Exp Neurol. 1983;19:37-44.

59. Oku R, Satoh M, Fujii N, Otaka A, Yajima H, Takagi H. Calcitonin gene-related peptide promotes mechanical nociception by potentiating release of Substance P from the spinal dorsal horn in rats. Brain Res [Internet]. 1987 [cited 2019 Oct 12];403(2):350-4. Available from: https://www.sciencedirect.com/science/article/abs/pii/0006899387900746?via%3Dihub doi: 10.1016/0006-8993(87)90074-6

60. Merskey H, Bogduk N. Classification of chronic pain. Seattle: IASP Press; 1994.

61. Ren K, Dubner R. Interactions between the immune and nervous systems in pain. Nat Med [Internet]. 2010 [cited 2019 Oct 2];16(11):1267-76.Available from: https://www.nature.com/articles/nm.2234 doi: 10.1038/nm.2234

62. Mannion RJ, Woolf CJ. Pain mechanisms and management: A central perspective. Clin J Pain [Internet]. 2000 [cited 2019 Oct 2];16(3):S144-56. Available from: https://insights.ovid.com/article/00002508-200009001-00006 doi: 10.1097/00002508-200009001-00006

63. Woolf CJ, Doubell TP. The pathophysiology of chronic pain – increased sensitivity to low threshold Aβ-fibre inputs. Curr Opin Neurol [Internet]. 1994 [cited

2019 Oct 9];4:525-34. Available from: https://www.sciencedirect.com/science/article/abs/pii/0959438894900531?via%3Dihub doi: 10.1016/0959-4388(94)90053-1

64. Gold MS, Gebhart GF. Nociceptor sensitization in pain pathogenesis. Nat Med [Internet]. 2010 [cited Oct 9];16(11):1248-57. Available from: https://www.nature.com/articles/nm.2235 doi: 10.1038/nm.2235

65. Nichols ML, Allen BJ, Rogers SD, Ghilardi JR, Honore P, Luger NM, et al. Transmission of chronic nociception by spinal neurons expressing the Substance P receptor. Science [Internet]. 1999 [cited 2019 Oct 7];286(5444):1558-61. Available from: https://science.sciencemag.org/content/286/5444/1558.long doi: 10.1126/science.286.5444.1558

66. Johnson MI. The physiology of the sensory dimensions of clinical pain. Physiotherapy. 1997 Oct;83(10):526-36.

67. Brooks PM. The burden of musculoskeletal disease – a global perspective. Clin Rheumatol [Internet]. 2006 [cited 2019 Oct 9];25(6):778-81. Available from: https://link.springer.com/article/10.1007%2Fs10067-006-0240-3 doi: 10.1007/s10067-006-0240-3

68. World Health Organization. The burden of musculoskeletal conditions at the start of the new millennium. WHO Technical Report Series 919. 2003.

69. Winkelstein B. Mechanisms of central sensitization, neuroimmunology and injury biomechanics in persistent pain: implications for musculoskeletal disorders. J Electromyogr Kinesiol [Internet]. 2004 [cited 2019 Oct 3];14:87-93. Available from: https://www.sciencedirect.com/science/article/abs/pii/S1050641103001433?via%3Dihub doi: 10.1016/j.jelekin.2003.09.017

70. Scott A, Bahr R. Neuropeptides in tendinopathy. Front Biosci [Internet]. 2009 [cited 2019 Oct 10];14:2203-11. Available from: https://europepmc.org/article/med/19273194 doi: 10.2741/3372

71. Andersson G, Backman LJ, Scott A, Lorentzon R, Forsgren S, Danielson P. Substance P accelerates hypercellularity and angiogenesis in tendon tissue and enhances paratendinitis in response to Achilles tendon overuse in a tendinopathy model. Br J Sports Med [Internet]. 2011 [cited 2019 Oct 11],45:1017–22. Avilable from: https://bjsm.bmj.com/content/early/2011/05/02/bjsm.2010.082750.info?versioned=true doi: 10.1136/bjsm.2010.082750

72. Lian Ø, Scott A, Engebretsen L, Bahr R, Duronio V, Khan K. Excessive apoptosis in patellar tendinopathy in athletes. Am J Sports Med [Internet]. 2007 [cited 2019 Oct 1];35:605-11. Available from: https://journals.sagepub.com/doi/abs/10.1177/0363546506295702?rfr_dat=cr_pub%3Dpubmed&url_ver=Z39.88-2003&rfr_id=ori%3Arid%3Acrossref.org&journalCode=ajsb doi: 10.1177/0363546506295702

73. Lotz M, Carson DA, Vaughan JH. Substance P activation of rheumatoid synoviocytes: neural pathway in pathogenesis of arthritis. Science [Internet]. 1987 [cited 2019 Dec 1];235:893-5. Available from: https://science.sciencemag.org/content/235/4791/893.long doi: 10.1126/science.2433770

74. Holzer P. Local effector functions of capsaicin-sensitive sensory nerve endings: involvement of tachykinins, calcitonin gene related peptide and other neuropeptides. Neuroscience [Internet]. 1988 [cited 2019 Oct 19],24(3):739-68. Available from: https://www.sciencedirect.com/science/article/abs/pii/0306452288900644?via%3Dihub doi: 10.1016/0306-4522(88)90064-4

75. Devillier P, Weill B, Renoux M, Menkès C, Pradelles P. Elevated levels of tachykinin-like immunoreactivity in joint fluids from patients with rheumatic inflammatory diseases. N Engl J Med [Internet]. 1986 [cited 2019 Oct 21];314:1323. Available from: https://www.nejm.org/doi/full/10.1056/NEJM198605153142018?url_ver=Z39.88-2003&rfr_id=ori%3Arid%3Acrossref.org&rfr_dat=cr_pub%3Dpubmed doi: 10.1056/NEJM198605153142018

76. Levine JD, Clark R, Devor M, Helms C, Moskowitz MA, Basbaum AI. Intraneuronal Substance P contributes the severity of experimental arthritis. Science [Internet]. 1984 [cited 2019 Oct 11];226(4674):547-9. Available from: https://science.sciencemag.org/content/226/4674/547.long doi: 10.1126/science.6208609

77. Sakai K, Matsuno H, Tsuji H, Tohyama M. Substance P receptor (NK1) gene expression in synovial tissue in rheumatoid arthritis and osteoarthritis. Scand J Rheumatol [Internet]. 1998 [cited 2019 Oct 25];27(2):135-41. Available from: https://www.tandfonline.com/doi/abs/10.1080/030097498441010 doi: 10.1080/030097498441010

78. Sluka KA, Westlund KN. Spinal cord amino acid release and content in an arthritis model: the effects of pretreatment with non-NMDA, NMDA and NK1 receptor antagonist. Brain Res [Internet]. 1993 [cited 2019 Oct 17];627(1):89-103. Available from: https://www.sciencedirect.com/science/article/abs/pii/0006899393907529 doi: 10.1016/0006-8993(93)90752-9

79. Binder W, Scott C, Walker JS. Involvement of Substance P in the anti-inflammatory effects of the peripherally selective kappa-opioid asimadoline and NK1 antagonist GR201171. Eur J Neurosci [Internet]. 1999 [cited 2019 Oct 27];11(6):2065-72. Available from: https://onlinelibrary.wiley.com/doi/abs/10.1046/j.1460-9568.1999.00625.x doi: 10.1046/j.1460-9568.1999.00625.x

80. Gowers WR. A lecture on lumbago: its lessons and analogues: delivered at the national hospital for the paralysed and epileptic. Br Med J [Internet]. 1904 Jan 16 [cited 2019 Oct 30];1(2246):117-21. Available form: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2352601/ doi: 10.1136/bmj.1.2246.117

81. Smythe HA, Moldofsky H. Two contributions to the understanding of the "fibrositis" syndrome. Bull Rheum Dis. 1977 Jan; 28(1):928-31.

82. Wolfe F, Smythe HA, Yunus MB, Bennett RM, Bombardier C, Goldenberg DL, et al. The American College of Rheumatology 1990 criteria for the classification of fibromyalgia. Report of the Multicenter Criteria Committee. Arthritis Rheumatol [Internet]. 1990 Feb [cited 2019 Oct 27];33(2):160-72. Available from: https://doi.org/10.1002/art.1780330203

83. Okifuji A, Turk DC, Sinclair JD, Starz TW, Marcus DA. A standardized manual tender point survey, I: Development and determination of a threshold point for the identification of positive tender points in fibromyalgia syndrome. J Rheumatol. 1997 Feb;24(2):377-83.

84. Wolfe F, Clauw DJ, Fitzcharles MA, Goldenberg DL, Katz RS, Mease P et al. The American College of Rheumatology preliminary diagnostic criteria for fibromyalgia and measurement of symptom severity. Arthritis Care Res (Hoboken) [Internet]. 2010 May [cited 2019 Oct 26];62(5):600-10. Available from: https://doi.org/10.1002/acr.20140

85. Arnold LM, Clauw DJ, McCarberg BH. Fibro collaborative. Improving the recognition and diagnosis of fibromyalgia. Mayo Clin Proc [Internet]. 2011 May [cited 2019 Oct 29];86(5):457-64. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3084648/ doi: 10.4065/mcp.2010.0738

86. Sim J, Madden S. Illness experience in fibromyalgia syndrome: a metasynthesis of qualitative studies. Soc Sci Med [Internet]. 2008 [cited 2019 Oct 29];67(1):57-67. Available from: https://www.sciencedirect.com/science/article/abs/pii/S0277953608001263?via%3Dihub doi: 10.1016/j.socscimed.2008.03.003

87. Schneider MJ, Brady DM, Perle SM. Commentary: differential diagnosis of fibromyalgia syndrome: proposal of a model and algorithm for patients presenting with the primary symptom of chronic widespread pain. J Manipulative Physiol Ther [Internet]. 2006 Jul [cited 2019 Oct 27];29(6):493-501. Available from: https://doi.org/10.1016/j.jmpt.2006.06.010

88. Aaron LA, Burke MM, Buchwald D. Overlapping conditions among patients with chronic fatigue syndrome, fibromyalgia and temporomandibular disorder. Arch Intern Med [Internet]. 2000 [cited 2019 Oct 29];160(2):221-7. Available from: https://jamanetwork.com/journals/jamainternalmedicine/fullarticle/485211 doi: 10.1001/archinte.160.2.221

89. Aaron LA, Buchwald D. A review of the evidence for overlap among unexplained clinical conditions. Ann Intern Med [Internet]. 2001 [cited 2019 Oct 28];134:868-81. Available from: https://annals.org/aim/article-abstract/714513/review-evidence-overlap-among-unexplained-clinical-conditions?doi=10.7326%2f0003-4819-134-9_Part_2-200105011-00011 doi: 10.7326/0003-4819-134-9_part_2-200105011-00011

90. Arnold LM, Crofford LJ, Mease PJ, Burgess SM, Palmer SC, Abetz L, et al. Patient perspectives on the impact of fibromyalgia. Patient Educ Couns [Internet]. 2008 [cited 2019 Oct 20];73(1):114-20. Available from: https://www.sciencedirect.com/science/article/abs/pii/S0738399108002796?via%3Dihub doi: 10.1016/j.pec.2008.06.005

91. Arnold LM, Hess EV, Hudson JI, Welge JA, Berno SE, Keck PE Jr. A randomized placebo-controlled, double-blind, flexible-dose study of fluoxetine in the

treatment of women with fibromyalgia. Am J Med [Internet]. 2002 Feb [cited 2019 Oct 29];112(3):191-7. Available from: doi: https://doi.org/10.1016/S0002-9343(01)01089-0

92. Bennett R. The fibromyalgia impact questionnaire (FIQ): a review of its development, current version, operating characteristics and use. Clin Exp Rheumatol [Internet]. 2005 [cited 2019 Sep 12];23(S39):S154-62. Available from: https://www.clinexprheumatol.org/article.asp?a=2701

93. Clauw DJ, Arnold LM, McCarberg BH. FibroCollaborative. The science of fibromyalgia. Mayo Clin Proc [Internet]. 2011 [cited 2019 Oct 26];86(9):907-11. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3258006/ doi: 10.4065/mcp.2011.0206

94. Russell IJ, Orr MD, Littman B, Vipraio GA, Alboukrek D, Michalek JE, et al. Elevated cerebrospinal fluid levels of Substance P in patients with the fibromyalgia syndrome. Arthritis Rheumatol [Internet]. 1994 Nov [cited 2019 Oct 28];37(11):1593-1601. Available from: https://onlinelibrary.wiley.com/doi/abs/10.1002/art.1780371106?sid=nlm%3Apubmed doi: 10.1002/art.1780371106

95. Russell IJ, Vaeroy H, Javors M, Nyberg F. Cerebrospinal fluid biogenic amine metabolites in fibromyalgia/fibrositis syndrome and rheumatoid arthritis. Arthritis Rheumatol [Internet]. 1992 [cited 2019 Oct 17];35(5):550-6. Available from: https://onlinelibrary.wiley.com/doi/abs/10.1002/art.1780350509?sid=nlm%3Apubmed doi: 10.1002/art.1780350509

96. Petersel DL, Dror V, Cheung R. Central amplification and fibromyalgia: Disorder of pain processing. J Neurosci Res [Internet]. 2011 [cited 2019 Sep 30];89(1):29-34. Available from: https://onlinelibrary.wiley.com/doi/abs/10.1002/jnr.22512 doi: 10.1002/jnr.22512

97. Schwarz MJ, Späth M, Müller-Bardorff H, Pongratz DE, Bondy B, Ackenheil M. Relationship of Substance P, 5-hydroxyindole acetic acid and tryptophan in serum of fibromyalgia patients. Neurosci Lett [Internet]. 1999 [cited 2019 Oct 19];259(3):196-8. Available from: https://www.sciencedirect.com/science/article/abs/pii/S0304394098009379?via%3Dihub doi: 10.1016/s0304-3940(98)00937-9

98. Bradley LA, Alberts KR, Alarcon G. Abnormal brain regional cerebral blood flow (rCBF) and cerebrospinal fluid (CSF) levels of Substance P (SP) in patients and non-patients with fibromyalgia (FM). Arthritis Rheumatol. 1996 Jan;39 (Suppl):S212.

99. Stratz T, Fiebich B, Haus U, Müller W. Influence of tropisetron on the serum Substance P levels in fibromyalgia patients. Scand J Rheumatol [Internet]. 2004 [cited 2019 Oct 26];119:41-3. Available from; https://www.tandfonline.com/doi/abs/10.1080/03009740410007023 doi: 10.1080/03009740410007023

100. Reynolds WJ, Chiu B, Inman RD. Plasma Substance P levels in fibrositis. J Rheumatol. 1988 Dec;15(12):1802-3.

101. Hill R. NK1(Substance P) receptor antagonists – why are they not analgesic in humans. Trends Pharmacol Sci [Internet]. 2000 [cited 2019 Dec 5];21(7):244-6. Available from: https://www.cell.com/trends/pharmacological-sciences/fulltext/S0165-6147(00)01502-9?_returnURL=https%3A%2F%2Flinkinghub.elsevier.com%2Fretrieve%2Fpii%2FS0165614700015029%3Fshowall%3Dtrue doi: 10.1016/s0165-6147(00)01502-9

102. Ablin JN, Buskila D, Clauw DJ. Biomarkers in fibromyalgia. Curr Pain Headache Rep [Internet]. 2009 [cited 2019 Oct 13];13(5):343-9. Available from: https://link.springer.com/article/10.1007%2Fs11916-009-0056-3 doi: 10.1007/s11916-009-0056-3

103. Julien N, Goffaux P, Arsenault P, Marchand S. Widespread pain in fibromyalgia is related to a deficit of endogenous pain inhibition. Pain [Internet]. 2005 [cited 2019 Oct 16];114(1-2):295-302. Available from: https://insights.ovid.com/article/00006396-200503000-00033 doi: 10.1016/j.pain.2004.12.032

104. Lautenbacher S, Rollman GB. Possible deficiencies of pain modulation in fibromyalgia. Clin J Pain [Internet]. 1997 [cited 2019 Oct 7];13(3):189-96. Available from: https://insights.ovid.com/crossref?an=00002508-199709000-00003 doi: 10.1097/00002508-199709000-00003

105. Lyon P, Cohen M, Quintner J. An evolutionary stress-response hypothesis for chronic widespread pain (fibromyalgia syndrome). Pain Med [Internet]. 2011 Aug [cited 2019 Oct 20];12(8):1167-78. Available from: https://doi.org/10.1111/j.1526-4637.2011.01168.x

106. Kim S-H, Kim DH, Oh D-H, Clauw DJ. Characteristic electron microscope findings in the skin of patients with fibromyalgia – a preliminary study. Clin Rheumatol [Internet]. 2008 [cited 2019 Oct 13];27(3):407-11. Available from: http://dx.doi.org/10.1007/s10067-007-0739-2

107. Hägermark O, Hökfelt T, Pernow B. Flare and itch induced by Substance P in human skin. J Investig Dermatol [Internet]. 1978 Oct [cited 2019 Nov 1];71(4):233-5. Available from: https://doi.org/10.1111/1523-1747.ep12515092

108. Theoharides TC, Zhang B, Kempuraj D, Tagen M, Vasiadi V, Angelidou A. IL-33 augments Substance P-induced VEGF secretion from human mast cells and is increased in psoriatic skin. Proc Natl Acad Sci USA [Internet]. 2010 Mar [cited 2019 Sep 22];107(9):4448-53. Available from: https://doi.org/10.1073/pnas.1000803107

109. Saha GB. Fundamentals of nuclear pharmacy. 4th ed. New York (NY): Springer-Verlag; 1998.

110. Türker S, Özer AY. Diagnostic radiopharmaceutical agents. FABAD J Pharm Sci [Internet]. 2004 [cited 2018 Jun 22];29:145-54. Available from: http://www.fabad.org.tr/eski/fabad.org/pdf/volum29/issue3/fabad2004j.pharm.sci.,2,145-154,2004.pdf.pdf

111. Liu S, Edwards DS. 99m-Tc-labeled small peptides as diagnostic radiopharmaceuticals. Chem Rev [Internet]. 1999 [cited 2018 June 19];99(9):2235-68. Available from: https://pubs.acs.org/doi/10.1021/cr980436l doi: 10.1021/cr980436l

112. Scott AM. Positron emission tomography: Basic sciences. In: Bailey DL, Townsend DW, Valk PE, Maisey MN, editors. PET imaging in oncology. London: Springer-Verlag; 2005.

113. Dash A, Chakravarty R. Radionuclide generators: the prospect of availing PET radiotracers to meet current clinical needs and future research demands. Am J Nucl Med Mol Imaging. 2019 Feb;9(1):30-66.

114. Holden NE. Table of the isotopes. In: Lide DR, editor. CRC Handbook of Chemistry and Physics. 77th ed. Boca Raton: CRC Press; 1997.

115. Perkins AC, Frier M, editors. Nuclear medicine in pharmaceutical research. London: CRC Press; 1999.

116. Shirley VS, Browne E, Lederer CM. Table of isotopes. 7th ed. New York: Wiley; 1978.

117. Walker FW, Parrington JR, Feiner F. Nuclides and isotopes (Chart of the nuclides). 14th ed. California: General Electric Company Nuclear Energy Operations; 1980.

118. Anger HO, Gottschalk A. Localisation of brain tumors with the positron scintillation camera. J Nucl Med. 1963 Jul;4:326-30.

119. Asti M, De Pietri G, Fraternali A, Grassi E, Sghedoni R, Fioroni F, et al. Validation of (^{68})Ge/(^{68})Ga generator processing by chemical purification for routine clinical application of (^{68})Ga-DOTATOC. Nucl Med Biol [Internet]. 2008 [cited 2019 Nov 11];35(6):721-4. Available from: https://www.sciencedirect.com/science/article/abs/pii/S0969805108001030?via%3Dihub doi: 10.1016/j.nucmedbio.2008.04.006

120. Henze M, Schuhmacher J, Hipp P, Kowalski J, Becker DW, Doll J, et al. PET imaging of somatostatin receptors using [^{68}Ga]DOTA-D-Phe1-tyr3-octretide: first results in patients with meningiomas. J Nucl Med. 2001 Jul;42(7):1053-6.

121. Razbash AA, Sevastianov YuG, Krasnov NN, Leonov AI, Pavlekin VE. Proceedings of the 5th International Conference on Isotopes, 5ICI; 2005 Apr 25-29; Medimond, Bologna: 2005. p.147-151.

122. Rosch F. Past, present and future of ^{68}Ge/^{68}Ga generators. Appl Radiat Isot. 2013 Jun; 76:24-30.

123. Banerjee SR, Pomper MG. Clinical applications of gallium-68. Appl Radiat Isot. 2013 Jun; 76:2-13.

124. Smith DL, Breeman WAP, Sims-Mourtada J. The untapped potential of gallium-68 PET: the next wave of ^{68}Ga agents. Appl Radiat Isot. 2013 Jun; 76:14-23.

125. Sanchez-Crespo A. Comparison of gallium-68 and fluorine-18 imaging characteristics in positron emission tomography. Appl Radiat Isot. 2013 Jun; 76:55-62.

126. Reubi JC, Schär JC, Waser B, Wenger S, Heppeler A, Schmitt JS, et al. Affinity profiles for human somatostatin receptor subtypes SST1-SST5 of somatostatin radiotracers selected for scintigraphic and radiotherapeutic use. Eur J Nucl Med [Internet]. 2000 [cited 2019 Nov 1];27(3):273-82. Available from:

https://link.springer.com/article/10.1007%2Fs002590050034 doi: 10.1007/s002590050034

127. Chorev M, Rubini E, Hart Y, Gilon C, Wormser U, Selinger Z. Metabolically stable analogues of Substance P: Persistent action of partially modified retro-inverso analogues of Substance P on rat parotid and hypothalamic slices. Eur J Pharmacol [Internet]. 1986 [cited 2019 Nov 1];127(3):187-95. Available from: https://www.sciencedirect.com/science/article/pii/0014299986903638?via%3Dihub doi: 10.1016/0014-2999(86)90363-8

128. Bennett VJ, Simmons MA. Analysis of fluorescently labelled Substance P analogs: binding, imaging and receptor activation. BMC Chem Biol [Internet]. 2001 June [cited 2019 Aug 3];1(1):1. Available from: https://doi.org/10.1186/1472-6769-1-1

129. Li YM, Marnerakis M, Stimson ER, Maggio JE. Mapping peptide-binding domains of the substance P (NK-1) receptor from P388D1 cells with photolabile agonists. J Biol Chem [Internet]. 1995 [cited 2019 Aug 16];270(3):1213-20. Available from: http://www.jbc.org/content/270/3/1213 doi: 10.1074/jbc.270.3.1213

130. Sagan S, Quancard J, Lequin O, Karoyan P, Chassaing G, Lavielle S. Conformational analysis of the C-terminal Gly-Leu-Met-NH_2 tripeptide of Substance P bound to the NK-1 Receptor. Cell Chem Biol [Internet]. 2005 May [cited 2019 Aug 12];12(5):555-65. Available from: https://doi.org/10.1016/j.chembiol.2005.03.005

131. Merlo A, Macke H, Reubi J-C, Good S. Radiolabeled conjugates based on Substance P and the uses thereof. Universitätsspital Basel, Universität Bern; EP 1603598 B1, 2008.

132. Merlo A, Macke H, Reubi J-C, Good S. Radiopharmaceuticals for cancer diagnosis and treatment. Kantonsspital Basel, Universität Bern; US 2007/0053837 A1, 2007.

133. De Araújo EB, Pujatti PB, Mengatti J. Radiolabeling of Substance P with ^{177}Lu and in vivo evaluation of tumor cell uptake in nude mice: Preliminary results. Cell Mol Biol (Noisy-le-grand) [Internet]. 2010 May [cited 2018 May 12];56(2):12-7. Available from: https://www.ipen.br/biblioteca/2008/eventos/13820.pdf

134. Pawlak D. Routine preparation of ^{68}Ga-DOTA- Substance P doses for locoregional administration follow up of ^{213}Bi-DOTA- Substance P in the course of glioma therapy. Proceedings on the Annual Congress of the EANM; 2013 Oct 19-23; Lyon, France: TP020.

135. Cordier D, Forrer F, Bruchertseifer F, Morgenstern A, Apostolidis C, Good S, et al. Targeted alpha-radionuclide therapy of functionally critically located gliomas with ^{213}Bi-DOTA-[Thi^{8}, Met(O_2)^{11}]-substance P: a pilot trial. Eur J Nucl Med Mol Imaging [Internet]. 2010 [cited 2019 Nov 11];37(7):1335-44. Available from: https://link.springer.com/article/10.1007%2Fs00259-010-1385-5 doi: 10.1007/s00259-010-1385-5

136. Krolicki L, Bruchertseifer F, Kunikowska J, Koziara H, Królicki B, Jakuciński M, et al. Prolonged survival in secondary glioblastoma following local injection of targeted alpha therapy with ^{213}Bi-substance P analogue. Eur J Nucl Med Mol Imaging [Internet]. 2018 Jul [cited 2019 Nov 12];45(9):1636-44. Available from: https://doi.org/10.1007/s00259-018-4015-2

137. Srivastava SC. A bridge not too far: personalised medicine with the use of theragnostic radiopharmaceuticals. J Postgrad Med Edu Res [Internet]. 2013 Jan [cited 2019 Nov 1];47(1):31-46. Available from: https://pdfs.semanticscholar.org/47c1/6635862090535d987e4ba00170ac839af99c.pdf

138. Jeelani S, Jagat Reddy RC, Maheswaran T, Asokan GS, Dany A, Anand B. Theranostics: A treasured tailor for tomorrow. J Pharm Bioall Sci [Internet]. 2014 [cited 2019 Nov 17];6(Suppl 1):S6-8. Available from:

https://www.ncbi.nlm.nih.gov/pmc/articles/pmid/25210387/ doi: 10.4103/0975-7406.137249

139. Chan HS, de Blois E, Konijnenberg MW, Morgenstern A, Bruchertseifer F, Norenberg JP, et al. Optimizing labelling conditions of ^{213}Bi-DOTATATE for preclinical applications of peptide receptor targeted alpha therapy. EJNMMI Radiopharm Chem [Internet]. 2016 May [cited 2019 Nov 17];1(9):1-15. Available from: https://doi.org/10.1186/s41181-016-0014-4

140. McDevitt MR, Finn RD, Sgouros G, Ma D, Scheinberg DA. An Ac-225/Bi-213 generator system for therapeutic clinical applications: construction and operation. Applied Radiat Isot [Internet]. 1999 [cited 2019 June 15];50(5):895-904. Available from: https://www.sciencedirect.com/science/article/abs/pii/S0969804398001511?via%3Dihub doi: 10.1016/s0969-8043(98)00151-1

141. Deng H, Wang H, Li Z. Matching chelators to radiometals for Positron Emission Tomography imaging-guided targeted drug delivery. Curr Drug Targets [Internet]. 2015 [cited 2019 Aug 1];16(6):610-24. Available from: http://www.eurekaselect.com/132850/article doi: 10.2174/1389450116666150707100702

142. Spang P, Herrmann C, Roesch F. Bifunctional gallium-68 chelators: past, present, and future. Semin Nucl Med [Internet]. 2016 [cited 2019 Aug 1];46(5):373-94. Available from: https://www.sciencedirect.com/science/article/abs/pii/S0001299816300125?via%3Dihub doi: 10.1053/j.semnuclmed.2016.04.003

143. Khan MU, Khan S. El-Refaie S, Win Z, Rubello D, Al-Nahhas A. Clinical indications for gallium-68 positron emission tomography imaging. Eur J Surg Oncol [Internet]. 2009 Jun [cited 2019 Nov 12];35(6):561-7. Available from: https://doi.org/10.1016/j.ejso.2009.01.007

144. Berry DJ, Ma Y, Ballinger JR, Tavare R, Koers A, Sunassee K, et al. Efficient bifunctional gallium-68 chelators for positron emission tomography: tris(hydroxypyridinone) ligands. Chem Commun (Camb) [Internet]. 2011 [cited 2019 Oct 13];47(25):7068-70. Available from: https://pubs.rsc.org/en/content/articlelanding/2011/cc/c1cc12123e#!divAbstract doi: 10.1039/c1cc12123e

145. Lees P, Cunningham FM, Elliott J. Principles of pharmacodynamics and their applications in veterinary pharmacology. J Vet Pharmacol Ther [Internet]. 2004 [cited 2019 Aug 1];27(6):397-414. Available from: https://onlinelibrary.wiley.com/doi/abs/10.1111/j.1365-2885.2004.00620.x?sid=nlm%3Apubmed doi: 10.1111/j.1365-2885.2004.00620.x

146. Benet LZ, Mancinelli L. Pharmacokinetics and pharmacodynamics. In: Swarbrick J, Boylan JC, editors. Encyclopedia of pharmaceutical technology clinical. 1st ed. New York: Marcel Dekker Inc; 2006.

147. Salahudeen MS, Nishtala PS. An overview of pharmacodynamic modelling, ligand-binding approach and its application in clinical practice. Saudi Pharm J [Internet]. 2017 Feb [cited 2019 Nov 12];25(2):165-75. Available from: https://doi.org/10.1016/j.jsps.2016.07.002

148. Bryant B, Knights K. Salerno E. Pharmacology for health professionals. 2nd ed. Sydney: Mosby Elsevier; 2006.

149. Jurcic JG, Wong JYC, Knox SJ, Wahl DR, Rosenblat TL, Meredith RF. Targeted radionuclide therapy. In: Gunderson LL, Tepper JL**,** editors. Clinical Radiation Oncology**.** 4th ed. Philadelphia: Elsevier, Inc.; 2015.

150. Ung H, Brown JE, Johnson KA, Younger J, Hush J, Mackey S. Multivariate classification of structural MRI data detects chronic low back pain. Cereb Cortex [Internet]. 2012 Apr [cited 2019 Aug 12];24(4):1037-44. Available from: https://doi.org/10.1093/cercor/bhs378

151. Chiapparini L, Ferraro S, Grazzi L, Bussone G. Neuroimaging in chronic migraine. Neurol Sci [Internet]. 2010 [cited 2019 Aug 4];31:S19-22. Available from: https://link.springer.com/article/10.1007%2Fs10072-010-0266-9 doi: 10.1007/s10072-010-0266-9

152. Howard MA, Sanders D, Krause K, O'Muircheartaigh J, Fotopoulou A, Zelaya F, et al. Alterations in resting-state regional cerebral blood flow demonstrate ongoing pain in osteoarthritis: an arterial spin-labeled magnetic resonance imaging study. Arthritis Rheumatol [Internet]. 2012 [cited 2019 Aug 4];64(12):3936-46. Available from: https://doi.org/10.1093/cercor/bhs378

153. Lee MC, Tracey I. Imaging pain: a potent means for investigating pain mechanisms in patients. Br J Anaesth [Internet]. 2013 Jul [cited 2019 Aug 4];111(1):64-72. Available from: https://doi.org/10.1093/bja/aet174

154. Gracely RH, Petzke F, Wolf JM, Clauw DJ. Functional magnetic resonance imaging evidence of augmented pain processing in fibromyalgia. Arthritis Rheumatol [Internet]. 2002 May [cited 2019 Aug 12];46(5):1333-43. Available from: https://doi.org/10.1002/art.10225

155. Harris RE, Sundgren PC, Pang Y, Hsu M, Petrou M, Kim SH, et al. Dynamic levels of glutamate within the insula are associated with improvements in multiple pain dominions in fibromyalgia. Arthritis Rheumatol [Internet]. 2008 Mar [cited 2019 Aug 12];58(3):903-7. Available from: https://doi.org/10.1002/art.23223 10.1002/art.23223

156. Harris RE, Sundgren PC, Craig AD, Kirshenbaum E, Sen A, Napadow V, et al. Elevated insular glutamate in fibromyalgia is associated with experimental pain. Arthritis Rheumatol [Internet]. 2009 Sep [cited 2019 Aug 12];60(10):3146-52. Available from: https://doi.org/10.1002/art.24849

157. Fayed N, Garcia-Campayo J, Magallón R, Andrés-Bergareche H, Luciano JV, Andres E, et al. Localised ^{1}H-NMR spectroscopy in patients with fibromyalgia: a

controlled study of changes in cerebral glutamate/glutamine, inositol, choline, and N-acetylaspartate. Arthritis Res Ther [Internet]. 2010 Jul [cited 2019 Nov 11];12(4):R134. Available from: https://doi.org/10.1186/ar3072

158. Bellato E, Marini E, Castoldi F, Barbasetti, Mattei L, Bonasia DE, et al. Fibromyalgia syndrome: etiology, pathogenesis, diagnosis, and treatment. Pain Res Treat [Internet]. 2012 Sep [cited 2019 Nov 5];2012: 426130. Available from: http://dx.doi.org/10.1155/2012/426130

159. Sundgren PC, Petrou M, Harris RE, Fan X, Foerster B, Mehrotra N, et al. Diffusion weighted and diffusion tensor imaging in fibromyalgia patients: a prospective study of whole brain diffusivity, apparent diffusion coefficient, and fraction anisotropy in different regions of the brain and correlation with symptom severity. Acad Radiol [Internet]. 2007 [cited 2019 Sep 1];14(7):839-46. Available from: https://linkinghub.elsevier.com/retrieve/pii/S1076-6332(07)00180-8 doi: 10.1016/j.acra.2007.03.015

160. Nebel MB, Gracely RH. Neuroimaging of fibromyalgia. Rheum Dis Clin North Am [Internet]. 2009 May [cited 2019 Oct 23];35(2):313-27. Available from: 10.1016/j.rdc.2009.06.004

161. Guedj E, Cammilleri S, Niboyet J, Dupont P, Vidal E, Dropinski JP, et al. Clinical correlate of brain SPECT perfusion abnormalities in fibromyalgia. J Nucl Med. 2008 Nov;49(11):1798-803.

162. Adigüzel O, Kaptanoglu E, Turgut B, Nacitarhan V. The possible effect of clinical recovery on regional cerebral blood flow deficits in fibromyalgia: a prospective study with semiquantitative SPECT. South Med J [Internet]. 2004 Jul [cited 2019 Oct 29];97(7):651–5. Available from: https://insights.ovid.com/article/00007611-200407000-00008 doi: 10.1097/00007611-200407000-00008

163. Yunus MB, Young CS, Saeed SA, Mountz JM, Aldag JC. Positron emission tomography in patients with fibromyalgia syndrome and healthy controls. Arthrit Care Res [Internet]. 2004 Aug 15 [cited 2019 Nov 1];51(4):513-8. doi: 10.1002/art.20544

164. Wood PB, Patterson JC 2nd, Sunderland JJ, Tainter KH, Glabus MF, Lilien DL. Reduced presynaptic dopamine activity in fibromyalgia syndrome demonstrated with positron emission tomography: A pilot study. J Pain [Internet]. 2007 [cited 2019 Nov 12];8(1):51-8. Available from: https://www.jpain.org/article/S1526-5900(06)00843-1/fulltext doi: 10.1016/j.jpain.2006.05.014

165. Walitt B, Roebuck-Spencer T, Esposito G, Atkins F, Bleiberg J, Foster G, et al. The effects of multidisciplinary therapy on positron emission tomography of the brain in fibromyalgia: a pilot study. Rheumatol Int [Internet]. 2007 [cited 2019 Nov 1];27(11):1019-24. Available from: https://link.springer.com/article/10.1007%2Fs00296-007-0405-7 doi: 10.1007/s00296-007-0405-7

166. Harris RE, Clauw DJ, Scott DJ, McLean SA, Gracely RH, Zubieta JK. Decreased Central mu-opioid receptor availability in fibromyalgia. J Neurosci [Internet]. 2007 Sep [cited 2019 Nov 16];27(37):10000 –6. Available from: https://doi.org/10.1523/JNEUROSCI.2849-07.2007

167. Wood PB, Schweinhardt P, Jaeger E, Dagher A, Hakyemez H, Rabiner EA, et al. Fibromyalgia patients show an abnormal dopamine response to pain. Eur J Neurosci 2007 [Internet]. 2007 [cited 2019 Nov 13];25(12):3576-82. Available from: https://onlinelibrary.wiley.com/doi/abs/10.1111/j.1460-9568.2007.05623.x doi: 10.1111/j.1460-9568.2007.05623.x

168. Hjornevik T, Cipriano PW, Shen B, Park JH, Gulaka P, Holley D, et al. Biodistribution and radiation dosimetry of ^{18}F-FTC-146 in humans. J Nucl Med. 2017 Dec;58(12):2004-9.

169. Shen B, Park JH, Hjornevik T, Cipriano PW, Yoon D, Gulaka PK, et al. Radiosynthesis and first-in-human PET/MRI evaluation with clinical-grade [^{18}F]FTC-146. Mol Imaging Biol [Internet]. 2017 Oct [cited 2019 Nov 13];19(5):779-86. Available from: https://doi.org/10.1007/s11307-017-1064-z

170. Martucci KT, Ng P, Mackay S. Neuroimaging chronic pain: what have we learned and where are we going? Future Neurol [Internet]. 2014 Nov [cited 2018 Nov 1];9(6):615-26. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5289824/

171. Breeman WA, VanHagen MP, Visser-Wisselaar HA, van der Pluijm ME, Koper JW, Setyono-Han B, et al. In vitro and in vivo studies of Substance P receptor expression in rats with the new analog [indium-111-DTPA-Arg1] Substance P. J Nucl Med. 1996 Jan;37(1):108-17.

172. Del Rosario RB, Mangner TJ, Gildersleeve DL, Shreve PD, Weiland DM, Lowe JA 3rd, et al. Synthesis of a nonpeptide C-11 labelled Substance-P antagonist for PET studies. Nucl Med Biol [Internet]. 1993 May [cited 2019 Nov 13];20(4):545-7. Available from: https://doi.org/10.1016/0969-8051(93)90085-9

173. Syvänen S, Eriksson J, Genchel T, Lindhe O, Antoni G, Långström B. Synthesis of two potential NK1-receptor ligands using [1-^{11}C]ethyl iodide and [1-^{11}C]propyl iodide and initial PET-imaging. BMC Med Imaging [Internet]. 2007 [cited 2019 Nov 30];7:6. Available from: https://doi.org/10.1186/1471-2342-7-6

174. Livni E, Babich JW, Desai MC, Godek DM, Wilkinson RA, Rubin RH, et al. Synthesis of a C-11 labeled NK1 receptor-ligand for PET studies. Nucl Med Biol [Internet]. 1995 [cited 2019 Nov 6];22:31-6. Available from: https://www.sciencedirect.com/science/article/abs/pii/0969805194000804?via%3Dihub doi: 10.1016/0969-8051(94)00080-4

175. Bergström M, Fasth KJ, Kilpatrick G, Ward P, Cable KM, Wipperman MD, et al. Brain uptake and receptor binding of two [^{11}C] labelled selective high affinity NK1-

antagonists, GR203040 and GR205171 – PET studies in rhesus monkey. Neuropharm [Internet]. 2000 [cited 2019 Nov 2];39(4):664-70. Available from: https://www.sciencedirect.com/science/article/abs/pii/S0028390899001823?via%3Dihub doi: 10.1016/s0028-3908(99)00182-3

176. Patlak CS, Blasberg RG, Fenstermacher JD. Graphic evaluation of blood-to-brain transfer constants from multiple-time uptake data. J Cereb Blood Flow Metab [Internet]. 1983 [cited 2019 Nov 1];3(2):1-7. Available from: https://journals.sagepub.com/doi/10.1038/jcbfm.1983.1 doi: 10.1038/jcbfm.1983.1

177. Hargreaves R. Imaging Substance P receptors (NK1) in the living human brain using Positron Emission Tomography. J Clin Psychiatry [Internet]. 2002 [cited 2019 Nov 1];63:18-24. Available from: https://www.psychiatrist.com/jcp/article/pages/2002/v63s11/v63s1104.aspx

178. Hietala J, Nyman MJ, Eskola O, Laakso A, Grönroos T, Oikonen V, et al. Visualisation and quantification of neurokinin-1 (NK1) receptors in the human brain. Mol Imaging Biol [Internet]. 2005 [cited 2019 Nov 17];7(4):262-72. Available from: https://link.springer.com/article/10.1007%2Fs11307-005-7001-6 doi: 10.1007/s11307-005-7001-6

179. Wolfensberger SPA, van Berckel BNM, Airaksinen AJ, Maruyama K, Lubberink M, Boellaard R, et al. First evaluation of [^{11}C]R116301 as an in vivo tracer of NK1 receptors in man. Mol Imaging Biol [Internet]. 2009 Jul [cited 2019 Nov 12];11(4):241-5. Available from: https://doi.org/10.3389/fncel.2019.00271

180. Haberberger RV, Barry C, Dominguez N, Matusica D. Human dorsal root ganglia. Front Cell NeuroSci [Internet]. 2019 June [cited 2019 Nov 11];13:1-17. Available from: https://doi.org/10.3389/fncel.2019.00271

PAPER 1

MANUSCRIPT FOR SUBMISSION TO THE JOURNAL

JOURNAL OF LABELLED COMPOUNDS AND RADIOPHARMACEUTICALS

RADIOSYNTHESIS, OPTIMISATION AND FORMULATION OF GALLIUM-68-1,4,7,10-TETRAAZACYCLODODECANE-1,4,7,10-TETRAACETIC ACID-[Thi^{8}, Met(O$_2$)^{11}]-SUBSTANCE P - A POTENTIAL NEUROKININ-1 RECEPTOR IMAGING AGENT

Janine Suthiram[1,2], Thomas Ebenhan[2], Biljana Marjanovic-Painter[1], Mike M. Sathekge[2] and Jan Rijn Zeevaart[1,3]

[1] Radiochemistry, The South African Nuclear Energy Corporation (Necsa), Pelindaba, Pretoria, South Africa

[2] Department of Nuclear Medicine, University of Pretoria and Steve Biko Academic Hospital, Pretoria, South Africa

[3] Department of Science and Technology, Preclinical Drug Development Platform, North West University, Potchefstroom, South Africa

For correspondence: janrijn.zeevaart@necsa.co.za

Keywords ^{68}Ga, ^{68}Ge/^{68}Ga generator, PET/CT-imaging, peptide radiolabelling, DOTA, Substance P, neurokinin-1 receptor, tachykinins, chronic pain

ABSTRACT

Substance P (SP) is a neuropeptide that is widely distributed in the mammalian system acting as an agonist ligand with a high preference for the neurokinin-1 receptor (NK1R). The involvement of SP in the facilitation of a diverse range of pathological and physiological processes makes it a biomarker of interest. There is a growing interest in determining the NK1R expression in diseases and conditions that are SP/NK1R mediated to inform the development of therapeutic agents. SP has been associated with inflammation and chronic pain and therefore has potential to enable visualisation of NK1R in these disease states. This study reports on the optimisation

and the "safe-to-administer" formulation of gallium-68-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid-[Thi^{8}, Met(O_2)^{11}]-Substance P ([^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP) as a potential radiotracer for PET/CT imaging of the NK1R expression in chronic pain disorders. The final radiolabelling methodology was found to be robust and repeatable and could be utilised for routine radiosynthesis going forward. [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP was prepared with high specific activity and in sufficient yield to serve as an injectable tracer for future pre-clinical studies.

1 INTRODUCTION

The tachykinin Substance P (SP) is a small undecapeptide[1] (Arg - Pro - Lys - Pro - Gln - Gln - Phe - Phe - Gly - Leu - Met) that has been identified as a neurotransmitter and neuromodulator.[2] It has long been studied for its involvement in the perception of pain[3] because it transmits information about tissue damage from peripheral receptors to the central nervous system where it is converted to the sensation of pain. SP, the preferential endogenous ligand for the neurokinin-1 receptor (NK1R),[4,5] has a high affinity for the glycoprotein receptor that is expressed in both the central and peripheral nervous systems.[6] Binding of SP to the receptor results in internalisation of the peptide into the cell via a clathrin-dependent mechanism.[7] Due to acidic conditions, the endosome which is a combination of peptide and receptor dissociates. The released receptor is recycled to the cell membrane whilst the internalized SP couples with lysosomes and subsequently undergoes further application by the cell.[8] Binding takes place at the second and third domains of the receptor at the C-terminal side. It has been reported that a specific motif of five amino acids, namely Phe-Phe-Gly-Leu-Met, imparts high affinity for tachykinins towards the NK1R.[9]

The SP/NK1 pathway is one of the most studied neurotransmitter pathways in the central nervous system because SP has been implicated as playing a role in inflammation and chronic pain.[10] It has also been implicated in emesis, asthma, psoriasis, inflammatory bowel disease, and in the regulation of depression and anxiety. In oncology, NK1R has been identified on a variety of cancer cells including astrocytoma, neuroblastoma, melanoma, and pancreatic cancer cells. SP is known to contribute to cancer cell progression, proliferation, metastasis, and

angiogenesis.[11,12,13] The involvement of SP and NK1R in a number of biological processes highlights the importance of gaining a better understanding of their interactions.

During the 1990s initial studies in animal models sparked interest in the concept of NK1R blockade[14,15] to illicit a reducing effect in chronic pain.[16,17] However, despite these promising results in animals, similar analgesic results were not achieved in the systemic blockade of the NK1R in humans.[18,19] Since then, the synthesis of metabolically stable analogues of SP[20,21] has been the key focus, always with the aim of retaining the biological activity of the peptide. Several of these analogues of SP have been investigated in both pre-clinical and clinical studies for application in general medicine, also extending into nuclear medicine.[22]

In the field of molecular imaging, non-invasive imaging techniques such as positron emission tomography/computed tomography (PET/CT) may be a powerful tool for gathering medical information about diseases with a complex pathology. Molecular and biochemical pathologies can be detected at early disease stages, often earlier than when conventional methods can detect disease symptoms.[22] To date, there have been relatively few studies in nuclear medicine reporting on imaging of the NK1R and most of these investigations have been aimed at the central nervous system. Three dimensional visualisation of the presence of the NK1R expressed on non-neural cells such as immune cells (macrophages, mast cells and lymphocytes) and tissue cells (tenocytes, fibroblasts, endothelial cells and synovial cells) could aid to further understand the pathology of pain disorders.

The commercial availability of multiple versions of the germanium-68/gallium-68 (^{68}Ge/^{68}Ga) generator[23] has made the development of ^{68}Ga-based PET diagnostic imaging agents that much more attractive.[24] Readily available on-site access, and frequently elutable radioactivity per day equates to the higher demand for ^{68}Ga-tracers and the on-demand patient management within nuclear medicine departments. The advent of the generator has also eliminated the need for a local cyclotron which translates to flexibility and convenience.[25] The drawback of ^{68}Ga *versus* fluorine-18 (^{18}F) is that the energy of the ^{68}Ga emitted positron is higher than the energy emitted

from ^{18}F resulting in PET ^{68}Ga-based images of lower resolution.[26] The $^{68}Ga^{3+}$-ion is versatile in its ability to complex a wide variety of ligands resulting in radiopharmaceuticals that have rapid blood clearance, quick diffusion, and effective target localization.[27] Furthermore the 68 min half-life of the radioisotope matches the pharmacokinetics of many peptides and other small molecules and allows for improved radiation dosimetry and a longitudinal imaging approach, if required.[28]

In an effort to gain a better understanding of the SP/NK1R pathway, the overall aim of our study was to develop a ^{68}Ga labelled SP-based tracer to evaluate the NK1R expression in osteoarthritic conditions characterised by chronic pain symptoms. Herein, the optimisation of the radiosynthesis and the "safe-to-administer" formulation of the potential radiotracer gallium-68-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid-[Thi^8, Met$(O_2)^{11}$]-Substance P ([^{68}Ga]Ga-DOTA-[Thi^8,Met$(O_2)^{11}$]SP) is reported. [^{68}Ga]Ga-DOTA-[Thi^8,Met$(O_2)^{11}$]SP has been reported as having an application in the imaging of gliomas and is currently undergoing clinical trials.[29,30] The radiotracer has been administered to critically-ill patients in combination with [^{213}Bi]Bi-DOTA-[Thi^8,Met$(O_2)^{11}$]SP that acts as targeted alpha-radionuclide therapy to attempt the treatment of inoperable gliomas. DOTA-[Thi^8,Met$(O_2)^{11}$]SP has also been previously radiolabelled with lutetium-177 (^{177}Lu) and evaluated in nude mice to study its uptake in pancreatic tumour cell xenografts.[31] However, to the authors knowledge the approach of investigating the interactions of SP and NK1R in disease states associated with chronic pain using a ^{68}Ga-tracer based PET imaging approach has not been reported to date.

Findings on the development of a robust method for the radiolabelling of DOTA-[Thi^8,Met$(O_2)^{11}$]SP with Ga-68 is reported herein. The effect of varying radiolabelling parameters such as peptide molarity, pH, reaction temperature, incubation period and the benefit of purification was evaluated. A purification procedure of the radiolabelled product was applied and the optimised [^{68}Ga]Ga-DOTA-[Thi^8,Met$(O_2)^{11}$]SP radiolabelling protocol was tested for robustness and repeatability. In preparation for pre-clinical work, the "safe-for-administration" formulation was determined and the optimal radiolabelling method, purification and formulation of DOTA-[Thi^8,Met$(O_2)^{11}$]SP is presented.

2 MATERIAL AND METHODS

2.1 CHEMICALS AND MATERIAL

Pharmaceutical grade chemicals including 32% hydrochloric acid (HCl; suprapure grade), ethanol & sodium acetate were purchased from Merck & Sigma Aldrich (Johannesburg, South Africa). C18 Sep-Pak® light cartridges for solid-phase extraction (SPE) were obtained from Waters (Microsep, Johannesburg, South Africa). Glass-microfiber chromatography paper impregnated with silica-gel (ITLC-SG) was purchased from Chemetrix (Midrand, South Africa). High-performance liquid chromatography (HPLC)-grade water (resistivity of 18.2 MΩ.cm) was made in-house using a Simplicity-185 Millipore system (Cambridge, Massachusetts, USA).

2.2 SUMMARY OF PEPTIDES INVESTIGATED

DOTA-Substance P (DSP) was purchased from GL Biochem (Shanghai, China) (DSP-A) and ABx-Advanced Biochemical Compounds (Radeberg, Germany) (DSP-B). DOTA-[Thi^{8},Met(O$_2$)^{11}]SP was purchased from piChem (Raaba-Grambach, Austria). For HPLC studies, DOTA and SP were purchased from Sigma-Aldrich (St Louis, MO, USA). The amino acid sequences of the aforementioned peptides are provided in **Table 1**.

Table 1. The amino acid sequence of the different peptides and the chemical structure of the chelator utilised in the study.

Peptide/ Compound	**Sequence / Name**	**Purity**
DSP-A	DOTA-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH2	> 98%
DSP-B	DOTA-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH2*	> 95%
DOTA-[Thi^{8},Met(O$_2$)^{11}]SP	DOTA-Arg-Pro-Lys- Pro- Gln- Gln- Phe- Thi- Gly- Leu- Met(O2)-NH2	> 95%
SP	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH2#	> 95%
DOTA	1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid	> 97%

*) acetate salt; #) acetate salt hydrate

2.3 ^{68}GE/^{68}GA GENERATOR ELUTION

^{68}Ga-activity (89%; electron capture; Emax (β+) 1.9 MeV) was eluted from the tin-dioxide-based ^{68}Ge/^{68}Ga generator (1.85 GBq, iThemba Labs, South Africa) using a moderately acidic solution (10 ml, 0.6 M HCl) via eluate fractionation as follows: first 1 ml into waste vial, followed by 2 ml ^{68}Ga-eluate for radiolabelling into eluate vial, remaining 7 ml into waste vial. Between 75 and 90% of peak activity/elutable [^{68}Ga]$GaCl_3$ was obtained in the elution volume of 2 ml, the activity of which was quantified using a Capintec C25 Ionisation Chamber (New Jersey, USA).

2.4 CHROMATOGRAPHIC ANALYSIS OF [^{68}GA]GA-DSP-A/-B AND [^{68}GA]GA-DOTA-[THI^{8},MET(O_2)^{11}]SP

2.4.1 Determination of labelling efficiency and colloid formation using instant thin layer chromatography (ITLC)

The percentage labelling efficiency (%LE) was determined using ITLC-SG paper as stationary phase and 0.1 M citrate, pH 5 to distinguish free ^{68}Ga from the ^{68}Ga-product. For detection of colloidal-^{68}Ga a different mobile phase (1 M ammonium acetate/methanol 1:1 (v/v)) was utilized. An amount of ~3-6 µl of the crude ^{68}Ga-peptide mixture or the purified sample was spotted at the bottom of the ITLC-SG strip using an insulin needle (Hamilton, Milano, Italy) and thereafter developed using the mobile phase until the solvent front had progressed to near the top of the strip. The strip was removed from the mobile phase, rapidly dried and analysed on an ITLC scanner (VSC-201, Veenstra Ind., Oldenzaal, Netherlands) using a gamma radiation detector (Scionix 25B25/1.5-E2, Bunnik, Netherlands) to result in an activity chromatogram. Genie2000 software (Veenstra Ind., Oldenzaal, Netherlands) was used for peak identification and to calculate the percentage labelling efficiency by means of an “area under the curve analysis”.

2.4.2 Determination of labelling efficiency and radiochemical purity using high performance liquid chromatography (HPLC)

The percentage radiochemical purity (% RCP) was determined by combined radio/UV-HPLC analysis using an Agilent 1200-series instrument (Agilent Technologies Inc.,

Wilmington DE, USA), with aligned diode array detector and radioactive detector (Gina Star, Raytest, Straubenhardt, Germany).

The mobile phase consisted of a 0.1% trifluoroacetic acid (TFA) in water (Solvent A) mixture and a 0.1% TFA in acetonitrile (Solvent B) mixture. An isocratic elution profile of 75% Solvent A and 25% Solvent B was carried out at 40 °C (flow rate of 1 ml/min) using a Zorbax Stable Bond C18 (4.6 mm x 250 mm; 5 µm) column (Agilent Technologies Inc., Wilmington DE, USA).

2.5 PREPARATION OF FREEZE-DRIED ALIQUOTS OF DOTA-[THI^{8},MET(O$_2$)^{11}]SP

An aliquot of 1 mg of DOTA-[Thi^{8},Met(O$_2$)^{11}]SP was dissolved in 2 ml of 0.01 M HCl (suprapure). Aliquots (100 µl) of the peptide solution were transferred into preconditioned glass vials (sterile, pyrogen free, vacuum sealed borosilicate glass; NTP Radioisotopes, Pelindaba, South Africa). The vials were closed with their rubber stoppers and placed in the freezer overnight. The rubber stoppers of the frozen vials were opened slightly and placed in the bench-top freeze dryer (Alpha I-5, Martin Christ Gefriertrocknungsanlagen GmbH, Germany) overnight. The vials were removed the next day and sealed with a metal cap. They were stored in a freezer (-20 ºC) until used.

2.6 DEVELOPMENT OF RADIOLABELLING METHODS FOR DOTA CONJUGATED PEPTIDES

2.6.1 ^{68}Ga-Radiolabelling of DSP-A/B and DOTA-[Thi^{8},Met(O$_2$)^{11}]SP

Phase 1) in-house information available for ^{68}Ga-radiolabelling of other peptides was used in the first phase to perform an un-customized radiolabelling technique as follows: the ^{68}Ga-activity was buffered to pH 3.5-4 using a 2.5 M sodium acetate buffer solution. The acidity of all ^{68}Ga-mixtures was determined using a narrow range pH paper (pH Fix 0-6, Macherey-Nagel, Düren, Germany) by exposing the strip to ~5 µl of the test solution. The wet strip was compared to the colour scale provided on the container. The corresponding pH value was obtained in 0.5 increments. At this stage 100-200 µg of DSP-A, DSP-B or DOTA-[Thi^{8},Met(O$_2$)^{11}]SP was added to the eluate,

vortex mixed, incubated at 95 ˚C for 15 min and further analysed using ITLC and HPLC.

Phase 2) the dependency of the labelling efficiency upon changes to the acidity of the ^{68}Ga-mixture, peptide molarity and incubation time or temperature was carried out in sufficient repeats (n ≥ 3) to suggest the optimum conditions for a robust labelling solution.

2.6.2 ^{68}Ga-product Purification

The crude radiolabelled solution was purified adopting and further optimizing a previously published procedure using a pre-conditioned C18 reversed-phase cartridge (Sep-Pak online, light, 100 mg) or a C18 reversed-phase column (Sep-Pak, 500 mg, 3 cc).[32] Briefly, the crude ^{68}Ga-labeled peptide mixtures were loaded at a flow rate of 2.5 ml/min followed by 1 ml of saline which was used to rinse the reaction vial to ensure transfer of any residual ^{68}Ga-labeled peptide that may have remained in the vial. A volume of 6 ml saline was used to rinse cartridge/column followed by increasing concentrations of ethanolic saline solutions (1 ml each of 10, 20, 25, 50 %, v/v) as an eluent to recover the radiolabelled product from the C18-matrix. All solutions were measured for their radioactivity content using an ionisation chamber (Capintec Inc, Pittsburgh, PA, USA). ITLC analysis was used to determine free-$^{68}Ga^{3+}$ and ^{68}Ga-labeled peptide to calculate the percentage labelling efficiency (%LE). HPLC analysis was used to confirm the ^{68}Ga-labeled peptide identity and determine its percentage radiochemical purity (%RCP). In phase 2, the concentration and sequence of the ethanolic saline solutions were optimised.

2.6.3 Optimised Radiolabelling procedure

The step-by-step radiolabelling procedure is outlined in **Table 2** and relevant data recorded using a radiolabelling data capture sheet (**Appendix 2**). ^{68}Ga-activity was hereby obtained by eluate fractionation from a tin-dioxide-based ^{68}Ga/^{68}Ge generator (0.6 M HCl elute). Aliquots of 50 µg of freeze dried DOTA-[Thi^{8},Met(O_2)^{11}]SP was first reconstituted with 250 µl of a 2.5 M buffer solution of sodium acetate to a pH of ~8 followed by ^{68}Ga radiolabelling (pH range of 3.5-4 on addition of the ^{68}Ga eluate). The majority of the ^{68}Ga-purified radiolabelled peptide was obtained using only a 30% ethanol/saline (v/v) mixture.

Table 2: Step by step procedure – ^{68}Ga-radiolabelling of DOTA-[Thi^{8},Met(O$_2$)^{11}]SP.

Time (min)	**Procedure**
-10	Freeze dried kit vial was allowed to defrost, prepare syringes with required volumes of 1) HCl and 2) buffering agent 3) Ethanolic saline mixtures 4) saline
-5	Preparation of all other consumables and equipment
-1	The Freeze dried kit containing 50 µg DOTA-[Thi^{8},Met(O$_2$)^{11}]SP was reconstituted with 250 µl 2.5 M sodium acetate
0	Obtaining the ^{68}Ga-activity by eluate fractionation from a tin-dioxide-based ^{68}Ga/^{68}Ge generator (2 ml of 0.6 M HCl eluate)
01	^{68}Ga was transferred into the peptide containing vial
02	Measurement of pH to be 3.5-4
03	Vortex mixing of the reaction vial for 0.5 min
04	Heat block incubation of the reaction vial at 95 ˚C for 15 min
05	Preconditioning of the C18 light cartridge: Gently push 4 ml EtOH followed by 2 ml deionised water through the cartridge
06	Prepare for sterile dispensing of the purified product (sterile filter, product vial)
09	Vortex the reaction vial for 0.25 min
14	Vortex the reaction vial for 0.25 min
19	Allow the reaction vial to cool for 2 min and withdraw a small sample for chromatographic analysis. (25 µl sample for HPLC injection, 4 µl for ITLC spotting)
23	Load the contents of the reaction vial onto the preconditioned C18 cartridge; repeat loading step after rinsing residual radioactivity from the reaction vial with 1 ml saline
24	Rinse the cartridge with 6 ml saline – washout of free ^{68}Ga-activity into a new vial
25	Recover [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP from colloidal-^{68}Ga using 30% ethanolic saline mixture
26	Measure radioactivity in product vial, used C18 cartridge and reaction vial, load-and-rinse fractions and miscellaneous material (syringes, pH paper, needles)
27	Reconstitute [^{68}Ga]Ga- DOTA-[Thi^{8},Met(O$_2$)^{11}]SP with saline to yield < 5% EtOH
29	Sterilise product by 0.22 µm filtration using a sterile syringe filter and withdraw quality control sample
30	Prepare [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP safe to administer doses

2.7 THERMODYNAMIC STABILITY OF [^{68}GA]GA-DSP PEPTIDES

Purified radiolabelled solutions of [^{68}Ga]Ga-DSP-A/B and [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP were maintained at 37 °C for up to 120 min to determine the thermodynamic compound stability. Samples were taken at defined time points, cooled to ambient temperature to quench any possible further reaction and then analysed using HPLC. The resulting chromatograms were compared to determine if there was any degradation of the radiolabelled peptide or reoccurrence of free ^{68}Ga-species.

2.8 STATISTICAL ANALYSIS

In order to obtain statistically relevant data, a minimum of three data sets for each parameter varied is presented. Analytical data was reported as an average ± standard deviation (SD) or an average ± standard error of the mean (SEM). Calculations were done using MS Excel Software (Microsoft, Albuquerque, USA). The variance was calculated by using a regression analysis or a Student's t-test with differences at the 95% confidence interval ($p < 0.05$) considered to be statistically significant.

3 RESULTS AND DISCUSSION

3.1 GENERATOR ELUTION

An average generator elution yielded 312 MBq ± 119 MBq (n=15) (over a period of 6 months) ^{68}Ga-activity which was collected in 1.0 ml ready to be used in radiolabelling. This was achieved using an eluate fractionation allowing for an improved radioactivity concentration as compared to a routinely performed generator purge.[32]

3.2 CHROMATOGRAPHIC IDENTIFICATION OF ^{68}GA-DSP PEPTIDES

The radioactivity yield using the improved generator elution protocol was deemed sufficiently high to perform the required experiments. Previous experience influenced the choice of HPLC material required; however, further development was required to optimise the HPLC analysis (data not shown).

3.2.1 Radioanalysis of buffered ^{68}Ga-activity

Generator eluates were buffered using 2.5 M sodium acetate to identify the changes in retention time of the radioactivity as a function of eluate acidity (**Figure 1**).

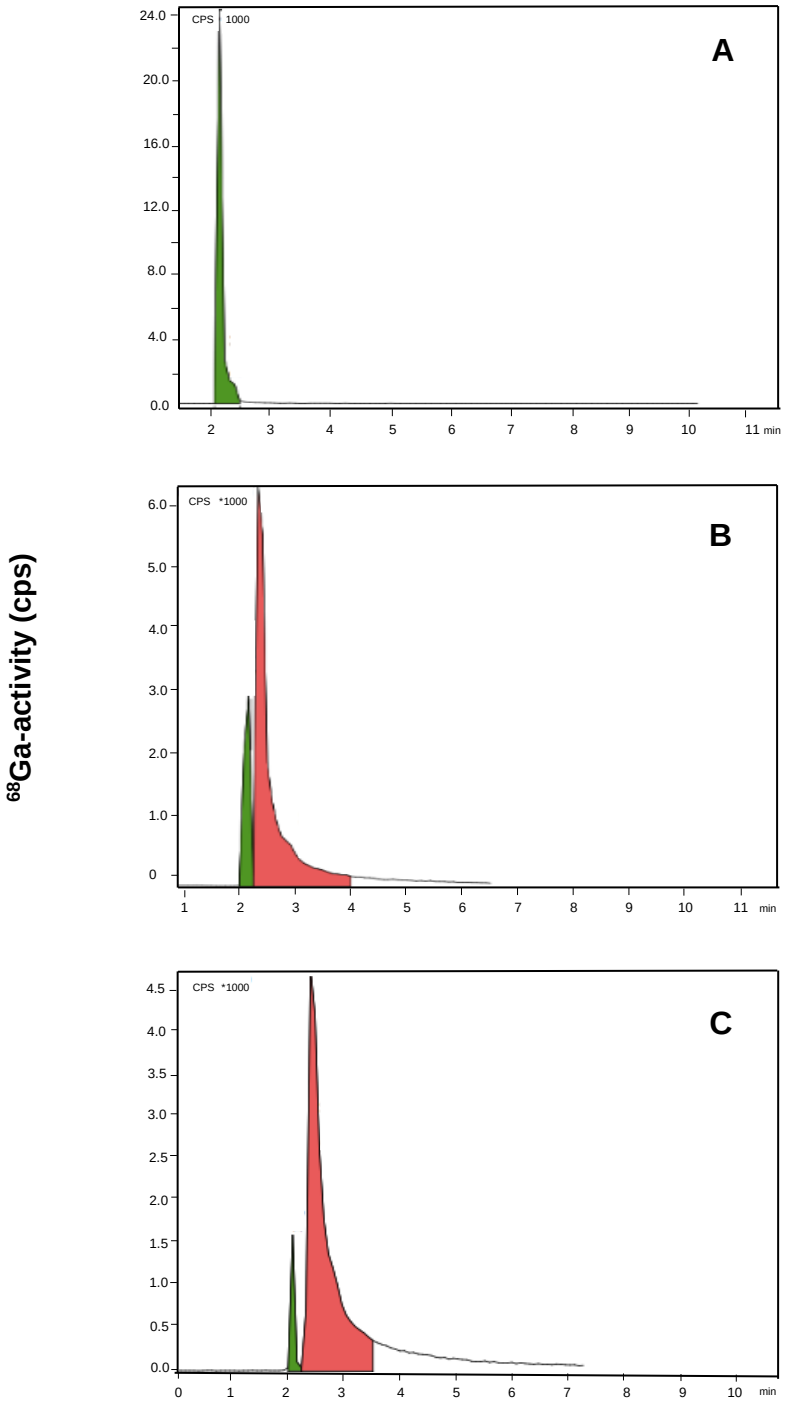


Figure 1: Radio-chromatograms of generator-derived ^{68}Ga-activity buffered with 2.5 M sodium acetate to obtain different pH: **A**) pH 3.7 **B**) pH 3.3 **C**) pH 2.3 (n=2). Isocratic HPLC

elution: 75% Solvent A (0.1% TFA in water) / 25% Solvent B (0. 1% TFA in acetonitrile) with a Zorbax Stable Bond C18 (4.6 mm x 250 mm; 5 µm) column (40 °C, 1 ml/min).

Buffered ^{68}Ga-activity showed a single peak of [^{68}Ga]$GaCl_3$ (**Figure 1A**; green; retention time = 2 min; 100%) at pH 2.3, whereas buffered ^{68}Ga-activities of pH 3.3 demonstrated a smaller radioactivity peak at the same retention time (RT) (**Figure 1B**; 2 min; green; ~22%) together with a major radioactivity peak appearing between 2.5-4 min (**Figure 1B**; red; ~78%) indicating the [^{68}Ga]$Ga^{3+}(CH_3COO^-)_3$ complex. At pH 3.7 (**Figure 1C**), the [^{68}Ga]$GaCl_3$ peak (green; ~15%) can be resolved from the major peak of the [^{68}Ga]$Ga^{3+}(CH_3COO^-)_3$ complex (red; ~85%).

3.2.2 UV HPLC Analysis

Results of the HPLC analysis are illustrated in
Figure 2 DSP-A, DSP-B and DOTA-[Thi^8,Met(O_2)^{11}]SP were analysed by UV-HPLC with the intention to identify the retention times of the DSP-peptides. UV signal at 214 nm showed peak RTs (min) of DSP-A, -B and DOTA-[Thi^8,Met(O_2)^{11}]SP of ~21-22 min and 7 min, respectively. SP and DOTA (controls) were retained at 23 and 11 min, respectively. Peaks recorded at ≤ 3 min were considered unspecific.

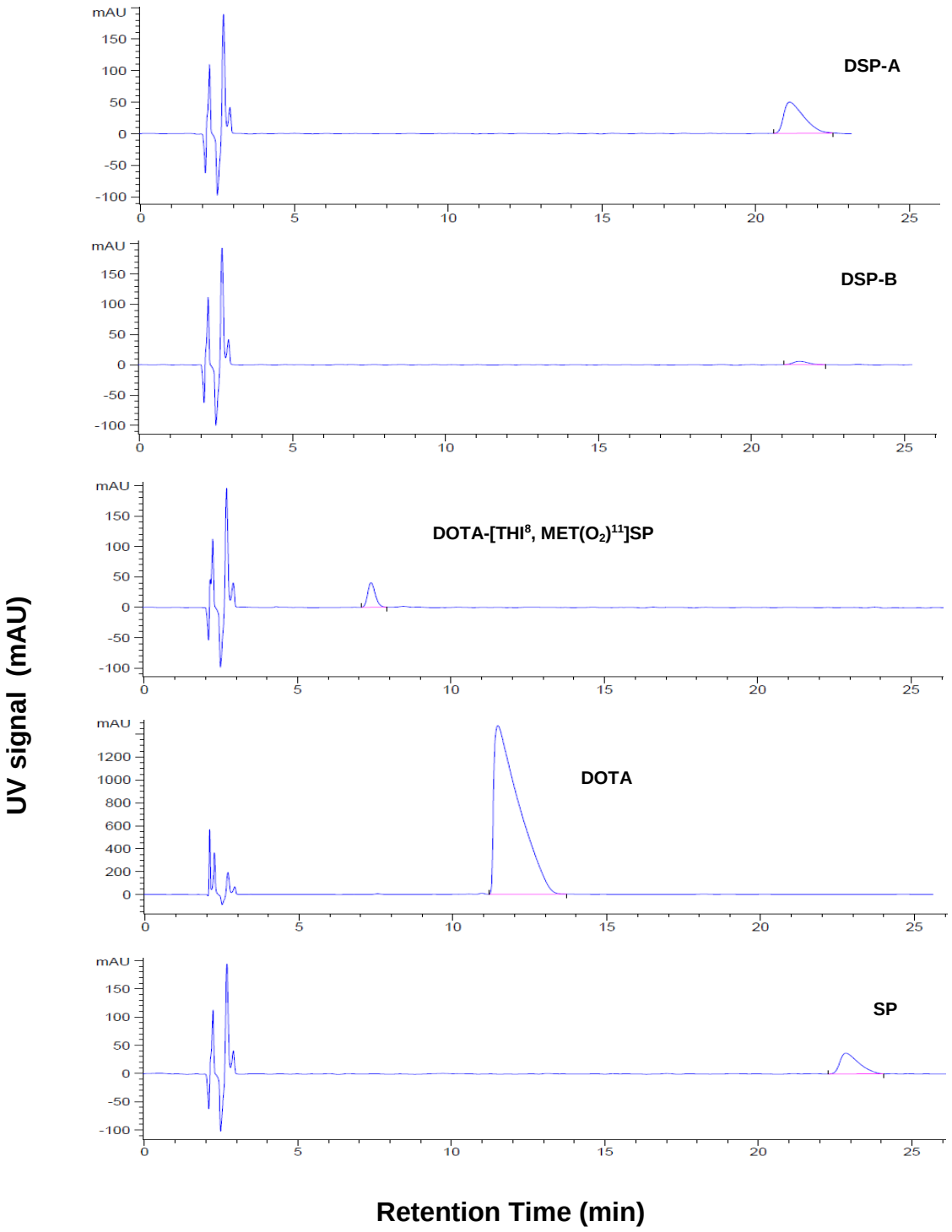


Figure 2: Representative HPLC analysis (UV signal at wavelengths of 214 nm and 254 nm recorded simultaneously) comparing the RTs of the DSP-peptides, SP and DOTA (controls). Isocratic HPLC elution with 75% Solvent A (0.1 % TFA in water) / 25% Solvent B (0.1% TFA in acetonitrile) on a Zorbax Stable Bond C18 (4.6 mm x 250 mm; 5 µm) column (40 °C, 1 ml/min).

3.2.3 Radioanalysis of [^{68}Ga]Ga-DSP peptides

The corresponding HPLC radio-chromatograms were analysed and peak radioactivity detected as summarized in **Table 3**: RT1) < 4 min, RT2) between 6 -12 min and R3) > 15 min. As crude [^{68}Ga]Ga-DSP-peptide preparations were analysed radioactivity was detected in all samples at a RT = ~2.15 min indicating uncomplexed ^{68}Ga-species similar to what has been already confirmed by HPLC analysis of buffered ^{68}Ga-eluate samples (**Figure 1**; control). [^{68}Ga]Ga-DSP-A analysis revealed the presence of two radiolabelled peaks with RTs of ~6 min (unknown) and ~21.5 min (co-registered with the UV signal for DSP-A). Although structurally identical (3% difference in chemical purity) analysis for [^{68}Ga]Ga-DSP-B identified peak activity eluted at ~22.5 min (co-registering with the DSP-B-derived UV signal) and a small amount of unknown radioactivity (<5 %) eluted at a RT of 19 min. Furthermore; unexpected radioactivity was detected (similar to [^{68}Ga]Ga-DSP-A) at a RT of ~ 6 min. None of the peaks found for [^{68}Ga]Ga-DSP-A/-B can be attributed to [^{68}Ga]Ga-DOTA (RT=11 min) or SP incubated with ^{68}Ga-activity (where no radiolabelling occurred). The calculation of the percentage peak analysis is summarized in **Table 3**.

Table 3: Radio-chromatographic analysis of [^{68}Ga]Ga-DSP peptides.*

HPLC Analysis**	**Normalised radioactivity per elution period (%)**		
	RT1 < 4 min	**RT2 = 6-12 min**	**RT3 <15 min**
[^{68}Ga]Ga-DSP-A	23 ± 2	18 ± 3	51 ± 17
[^{68}Ga]Ga-DSP-B	20 ± 3	15 ± 4	68 ± 5
[^{68}Ga]Ga- DOTA-[Thi^{8},Met(O_2)^{11}]SP	29 ± 2	70 ± 2	nd
DOTA	< 1	98 ± 2	nd
SP	~99	nd	nd

*) ^{68}Ga-labelling: 2 ml ^{68}Ga-activity, 90 °C, 15 min, 100 µg DSP-peptide, SP or DOTA, pH 3.5-4 (n=2),

**) Isocratic HPLC elution with 75% Solvent A (0.1% TFA in water) / 25% Solvent B (0.1% TFA in acetonitrile) using a Zorbax StableBond C18 (4.6 mm x 250 mm; 5 µm) column (40 °C, 1 ml/min); nd = no activity detected.

The RT of DOTA-[Thi^{8},Met(O_2)^{11}]SP is significantly different to DSP-A/-B and this would be due to the modifications in the amino acid residues at positions 8 and 11 which may alter the overall polarity of the peptide thus affecting its retention time.

[^{68}Ga]Ga-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP analysis showed a single radioactive product peak (RT = 7.5 min) co-registering with its UV signal.

Peptide stability and potential degradation is a probable limitation when applying a new radiolabelling procedure. The primary factor influencing the peptide stability is the amino acid composition and sequence. Hydrolysis, oxidization and de-amination are common non-enzymatic processes of peptide degradation. The unknown radioactivity detected (~6 min) for the [^{68}Ga]Ga-DSP-A/-B samples could be degradation of the peptide during the radiolabelling process, possibly because of a pH sensitivity or oxidization of Met^{11} to a sulfone and sulfoxide. Thus, this degradation could yield a mixture of intact [^{68}Ga]Ga-DSP-A/-B together with a [^{68}Ga]Ga-DOTA complexed to modified DSP depending on where the peptide underwent possible oxidation or fragmentation.

The initial radiolabelling approach used for DSP-A/-B and DOTA-[Thi^{8},Met(O$_2$)^{11}]SP favoured adjusting the ^{68}Ga-eluate to a pH of 3.5-4 before adding the peptide (pre-weighed powder or freeze-dried aliquots) which protected the peptide from an apparent degradation at low pH levels. It was observed that the %LE for these initial experiments were relatively low. Evaluation of the free ^{68}Ga^{3+}-peak in generator-derived eluates that had been pH adjusted indicated that varying the acidity (i.e. pH level) influenced the peak appearance in the HPLC chromatogram which in turn correlated with labelling efficiency. This trend can be explained by published evidence of different species of gallium complexes as a function of pH. In a strongly acidic millieu (pH<2) Ga^{3+} tends to precipitate and therefore becomes unavailable for radiolabelling. This is not the case for pH >3 at which pH a reactive [^{68}Ga]Ga{Cl$_4$}$^{-}$ intermediate complex susceptable for complexation with DOTA forms.[33] Metals such as gallium exhibit hydrolytic properties and give rise to polycationic structures at a given pH.[33] For any radiolabelling going forward, the ^{68}Ga-eluate was not pH buffered but was rather added directly to a pre-buffered, alkaline solution containing the DOTA-[Thi^{8},Met(O$_2$)^{11}]SP which consequently improved the %LE. Further attempts to purify [^{68}Ga]Ga-DSP-A from its detected radiolabeled by-products using a C18-based matrix (SPE cartridges/columns) were unsuccessful. For instance variations in the ethanol/saline mixtures (for C18 SPE cartridge or column elution procedures) normally

deemed sufficient to recover pure product, had no impact on the separation of the two radio peaks in different ethanolic fractions. All fractions eluted from the C18 SPE cartridge or -column showed similar RT pattern after HPLC analysis. The average %LE for [^{68}Ga]Ga-DSP-A was 51±17 (crude) which was still lower than the required minimum of 95% after purification (77±10; n=5; 50% ethanolic saline used for [^{68}Ga]Ga-DSP-A recovery from the cartridge). Similar results were achieved in a test run using DSP-B instead of DSP-A; yielding 66% and 85%LE for the crude and purified ^{68}Ga-product, respectively.

Results obtained from the preliminary evaluation of the DOTA-peptides indicated that although radiolabelling could be achieved, the lack of stability and the presence of possible by-products or impurities in [^{68}Ga]Ga-DSP-A/-B eliminates this version of DOTA-conjugated SP as a suitable precursor for ^{68}Ga-radiolabelling. DOTA-[Thi^{8},Met(O_2)^{11}]SP was successfully radiolabelled and also passed the required chemical stability and radiochemical properties. Therefore Phase 2 involved further development and optimisation of the radiolabelling of [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP.

3.3 OPTIMISATION

In an effort to develop a robust radiolabelling method for [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP, investigations into the effect of varying conditions during the radiolabelling process, namely pH, time, temperature, peptide concentration and the benefit of product purification were evaluated. For better standardization, ready-to use vials containing freeze-dried DOTA-[Thi^{8},Met(O_2)^{11}]SP were produced in a large batch and ^{68}Ga-activities from the same generator were used in the optimisation process to ensure that observed changes in the %LE were as a result of the parameter being varied. At least three radiosyntheses were carried out per parameter investigation to ensure minimum statistically robust data. Both HPLC and ITLC analysis were performed to improve the reliability of the ITLC data. Colloid formation was evaluated with ITLC and there were no ^{68}Ga-colloids present in any of the radiolabelled preparations.

3.3.1 Summary of optimised radiolabelling data

Parameters were further optimized to determine the optimal conditions for ^{68}Ga-radiolabelling as displayed in **Figure 3**. The reported %LE refers to crude radiolabelling conditions (supplementary data in **Appendix 3**). Radiolabelling reactions (n = 14) were carried out comparing two pH ranges; < 3.5 and ≥ 3.5 - 4.2 through adjustment of the radiolabelling solution at t = 0. On average, a marginal improvement in the %LE was observed using radiolabelling solutions buffered at pH 3.5 and above. The highest %LE was achieved at 3.5, amounting to > 90%. For gallium complexation, the pH should be low enough to prevent formation of [^{68}Ga]Ga-oxide or [^{68}Ga]Ga-hydroxide-species which would adversely affect the radiolabelling reaction. At the same time, the pH should allow for deprotonation of the donor pendant acid groups and this occurs at higher pH values.[34] In general, DOTA-conjugated peptides are able to incorporate radionuclides, such as ^{68}Ga, optimally at pH 3.5 - 4.[35] For radionuclides that are supplied or obtained in acidic solutions such as hydrochloric acid (HCl), the best reaction kinetics are achieved when a minimal volume of sodium acetate buffer is added to control the pH during the radiolabelling reaction.[36] Studies of Rossouw & Breeman[32] has proven that even though literature indicates that sodium ions compete with ^{68}Ga^{3+} for complex formation with DOTA, the use of 2.5 M sodium acetate in small[37] or large reaction volumes has no adverse impact on the radiolabelling reaction. At a pH above 4 there is a tendency for colloidal-^{68}Ga formation, therefore ^{68}Ga-peptide complexes that require a pH of 4 to form must be able to effectively compete against colloid formation.[38]

Optimal labelling efficiencies were achieved at 95 ºC (p < 0.05) in comparison to ambient temperature and 60 ºC (**Figure 3, B**). The higher labelling efficiencies achieved at 95 ºC were expected as there is literature evidence that DOTA requires heat to facilitate complexation of radiopharmaceutical concentrations of [^{68}Ga]Ga^{3+} to give labelling yields greater than 80%.[39,40,41] Supporting the latter argument, no radiolabelling occurred at ambient temperature. If the reaction solution is allowed to stand for more than 120 min, ~3% of [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP product was detected further emphasising the requirement for the heating step.

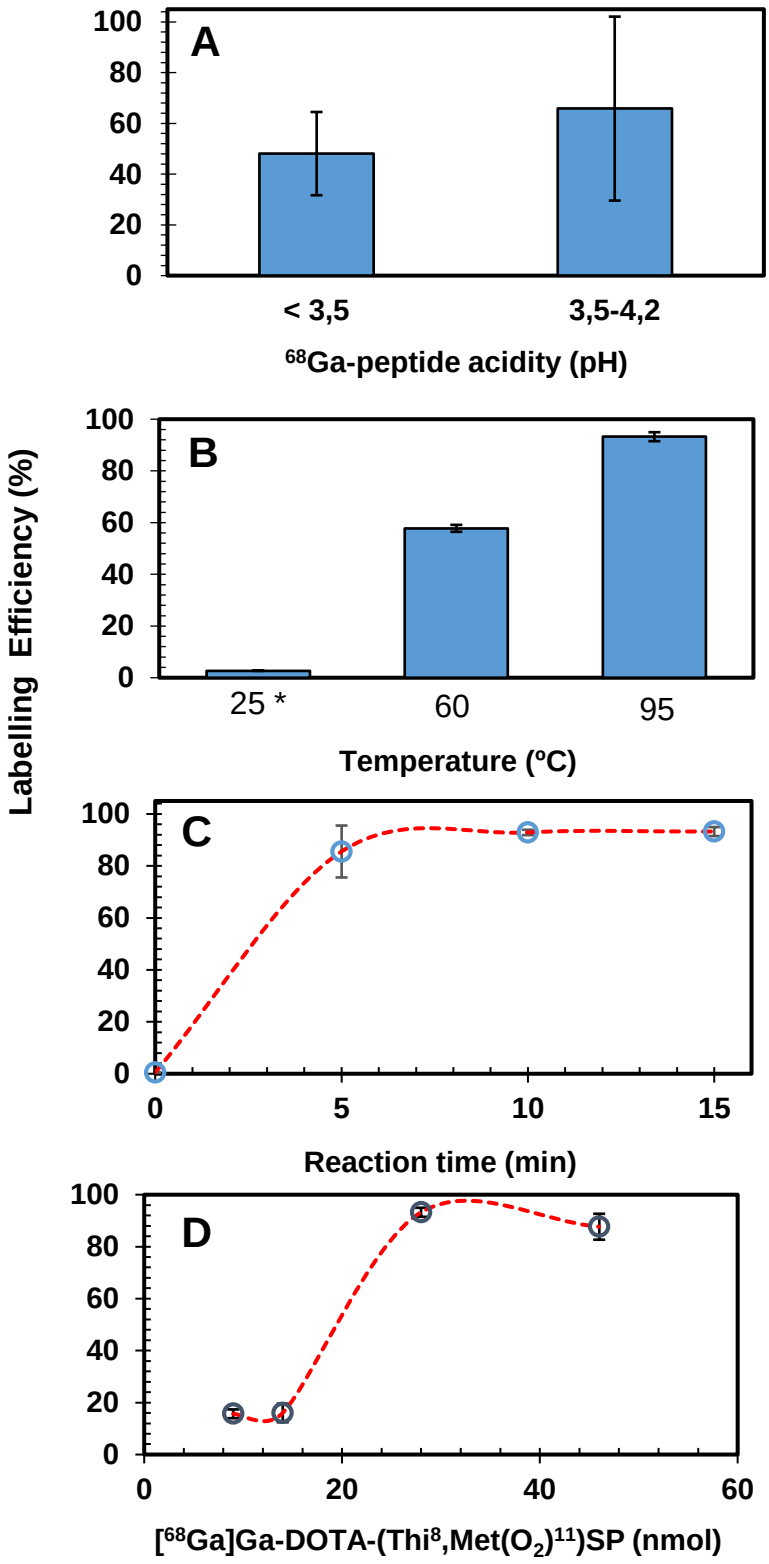


Figure 3: The effect of varying the radiolabelling parameters during radiosynthesis * of [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP measured as percentage labelling efficiency. (A) ^{68}Ga-peptide solution acidity prior to heating (pH value) (B) Reaction temperature (°C) (C) Reaction time (min) (95 °C) and (D) Precursor molarity of DOTA-[Thi^{8},Met(O$_2$)^{11}]SP (nmol).

(*) if not varied otherwise relevant to A-D, the other labelling parameters were as follows: 28 nmol DOTA-[Thi^{8},Met(O$_2$)^{11}]SP, 15 min incubation time at 95 °C, pH 3.5-4).

The requirement to apply heat to DOTA complexes during radiolabelling with metals compared to chelators such as di-ethylene-tri-amine-pentaacetic acid (DTPA) and 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA) that are able to complex radiometals at ambient temperature, is a shortcoming. Chelators such as NOTA and its derivative 1,4,7-triazacyclononane-1-glutaric acid-4,7-acetic acid (NODAGA) (for proteins) (**Figure 4**) are able to complex [^{68}Ga]Ga^{3+} in a shorter time at ambient temperature and still demonstrate excellent stability in plasma.[42] In general, peptides are mostly stable to heating; therefore, in most instances DOTA is still the preferred chelator as it results in complexes that are kinetically and thermodynamically stable.[36] In this study, we have demonstrated that DOTA-[Thi^{8},Met(O$_2$)^{11}]SP is able to withstand being heated to 95 ºC as evident in the single radioactive peak of [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP which correlates to the RT of the DOTA-[Thi^{8},Met(O$_2$)^{11}]SP UV signal.

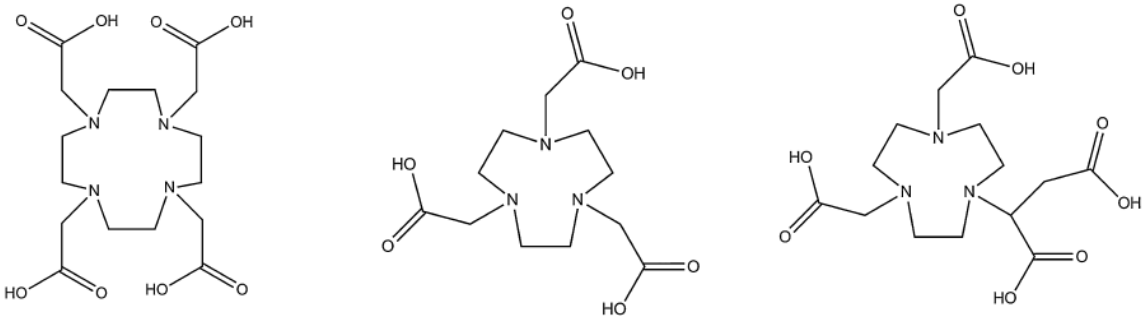


Figure 4: Structure of azamacrocyclic chelators for ^{68}Ga radiolabelling: 1,4,7,10 - tetrazacyclononane-1,4,7-tetraacetic acid **(**DOTA), 1,4,7-triazacyclononane-1,4,7-triacetic acid **(**NOTA) and 1,4,7-triazacyclononane-1-glutaric acid-4,7-acetic acid (NODAGA).

A minimum of 5 minutes incubation time is required to achieve > 90%LE, with the highest (more robust) %LE achieved at 15 min without being compromised by significant ^{68}Ga-colloid formation (**Figure 3C**). The type of buffering agent and amount required (herein sodium acetate) to adjust the pH is widely used and did not appear to play a key role in the improvement of the radiolabelling (**Figure 3A**). More importantly,

lower peptide masses of 9-14 nmol resulted in a low %LE. A significant increase (p <0.001) is observed in the %LE (up to > 90%) if peptide mass was increased to 28 nmol (50 µg) (**Figure 3D**). Further doubling the peptide content did not result in further increasing the %LE. Since SP may result in pharmacological effects, such as transient hot flushing due to vasodilatation of the blood vessels of the skin, its application as a radiopharmaceutical should be considering quantities as small as possible.[43,44] Therefore a lower peptide molarity is preferred.

3.4 PURIFICATION

Radiosynthesis, purification, quality control and formulation can take up to 40 minutes to complete which is short enough to ensure that there is enough ^{68}Ga-activity for injection, however a lower starting ^{68}Ga-activity could benefit from the shorter 5 minute reaction time. The correlation coefficient for the time dependent %LE data is 1.0 indicating that there is a positive direct correlation between the variables. Ethanol/saline solutions (10, 20, 30, 40%) were applied to purify crude [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP using a pre-conditioned C18-based SPE cartridge. The fractions were analysed using ITLC to verify the radioactive purity of the product. The radioactive yield was calculated from the decay corrected product activity taking into account the free ^{68}Ga in the load and rinse solutions as well as the colloids trapped on the C18 cartridge and adhering to the reaction vial. The activity in the different ethanol/saline solutions were considered to collectively contain the product (**Table 4**). The 30% ethanol/saline solution was found to be the most effective at stripping the product from the C18 cartridge. The 40% fraction also contained considerable activity. The results obtained from these purification experiments were not based on full scale radiolabelling and were only intended to identify the optimal ethanol concentration that would result in desorption of the ^{68}Ga-peptide from the C18 cartridge. The use of 30% ethanol/saline mixture ensures that the ethanol content in the final eluate is low making formulation of a “safe-to-administer” product that contains less than 7% (w/v) ethanol (without the need to evaporate the ethanol) more achievable.

Table 4: Summary of %LE obtained during the purification process for different sequences of varying ethanol/saline mixtures.

Step-wise C18 SPE Elution (Ethanol/Saline concentration)	**Radiochemical Yield (%)**		
	[^{68}Ga]Ga-DOTA-[Thi^{8}, Met(O$_2$)^{11}]-SP*	**Free-^{68}Ga$^{¥}$**	**colloidal-^{68}Ga$^{\#}$**
10, 10, 30% (n=1)	47.2	3.3	37.6
20, 30% (n=1)	58.3	4.4	31.5
10, 20, 30, 40% (n=9)	72 ± 11	5.8 ± 4.4	15 ± 11

*) Total [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP activity recovered within all of the ethanol/saline fractions.

$^{¥}$) Radioactivity that washes through the column following loading of the crude solution and rinsing.

$^{\#}$) Radioactivity that was found retained on the C18 SPE cartridge/column and any residual activity in the reaction vial.

3.5 THERMODYNAMIC STABILITY

Purified [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP was incubated at ambient temperature and sampled every 30 minutes for analysis on HPLC, up to 2.5 hours. The [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP was found to be stable over that period demonstrating an overall integrity of 98%, with no shift in the retention time of the product peak or the formation of any additional peaks.

3.6 OPTIMISED RADIOLABELLING AND FORMULATION OF [^{68}GA]Ga-DOTA-[THI^{8},MET(O$_2$)^{11}]SP

The optimised radiolabelling parameters were implemented and tested for repeatability and robustness. This included the utilisation of freeze dried DOTA-[Thi^{8},Met(O$_2$)^{11}]SP to ensure accuracy in the molarity of the peptide per aliquot and to increase the shelf life of the peptide. A summary of the results of these radiosyntheses are reported in **Table 5**.

The optimised parameters resulted in improved labelling efficiency with < 6% colloids and < 3% free uncomplexed ^{68}Ga. High radiochemical purity > 99% could be achieved following SPE purification.

Table 5: Summary of four consecutive preparations of [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP.

Parameter Measurement	**[^{68}Ga]Ga-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP**
DOTA-[Thi^{8},Met(O$_2$)^{11}]SP (nmol)	28
Sodium acetate (M); Volume (µl)	2.5; 250
Starting activity (MBq)$^{+}$; Volume (µl)	616 ± 148; 990
Incubation time (min)	15
Temperature (ºC)	95
Buffered Eluate Acidity (pH value)	3.5 - 4
Radiolabelling Efficiency (%)*	79 ± 0.6
Colloidal-^{68}Ga (%)*	5.9 ± 1.5
Uncomplexed-^{68}Ga (%)*	2.7 ± 0.4
C18 SPE recovery (%)*	90.7 ± 0.4
Radiochemical Purity (%)¥	99.8 ± 0.4
Instant Labelling (>95 %LE)**	100 (4/4)
Radiosynthesis Yield (MBq) #	519 ± 127
Specific Activity (MBq/nmol)	13.5 ± 3.9
Average activity (MBq) of end product	379 ± 108
Radiosynthesis time (min)	31 ± 3

$^{+}$) All data is based on a half-scale starting ^{68}Ga-activity which refers to use of only ~1 ml of the generator eluate.
$^{\#}$) Crude radiolabelled product, before purification, quantified from ITLC analysis (not decay corrected).
*) Decay-corrected values.
**) ITLC-based quantification.

Additional half-scale radiosyntheses which did not undergo purification due to instant labelling of 99.0 ±0.7 % (n=4) and a radiosynthesis yield of 501 ± 140 MBq (sufficient for production of 3-4 doses for larger animals) are also reported here. ^{68}Ga-colloid formation was negligible (≤ 1.2%) as determined by ITLC analysis. The specific activity of 18 ± 4 MBq/nmol was significantly higher (p < 0.05) compared to that achieved performing a C18-based SPE purification on [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP (**Table 4**).

3.7 FINAL FORMULATION OF [^{68}GA]GA-DOTA-[THI^{8},MET(O$_2$)^{11}]SP

The final formulation was carried out by passing the 1 ml 30% ethanol/saline fraction of [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP through a pre-wet sterile filter (Millex-GV, 0.22

µm membrane sterile filters) (use 1 ml saline to rinse product vial) into a sterile vial of pre-buffered saline solution (5.5 ml of 0.9% sodium chloride, 2 ml 2.5 M sodium acetate). The resultant “safe-to-administer” product in saline had a final pH of 6.5 and < 5% ethanol which is suitable for intravenous injection.

It should be noted that even though the protocol yields a final product with a suitable specific activity, the generator shelf-life is an important factor that has to be considered because it yields different degrees of eluate quality and quantity. Good generator maintenance and adherence to Good Radiopharmacy Practice (GRPP) will have a positive impact on the characteristics of the final product.

4 CONCLUSION

Different DSP peptides were selected for their potential use as ^{68}Ga-labelled NK1R PET imaging agents. Even though radiolabelling could be achieved, only [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP was identified by a single radio peak product with no impurities or by-products (i.e. only uncomplexed ^{68}Ga-species). A robust radiolabelling method was developed and optimised for the preparation of “safe-to-administer” [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP. The final radiolabelling procedure was evaluated towards routine preparation practice and repeatability was successfully demonstrated. The optimal incubation time and temperature, peptide molarity, optimal pH, and purification conditions were in line with or improved when compared with other ^{68}Ga-peptide preparations suitable for pre-clinical or clinical application.[32,45] Excellent labelling efficiencies were achieved to the point that some preparations did not require further purification (i.e. instant radiolabelling). C18-based SPE purification yielded > 99% radiochemical purity and [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP remained stable and intact up to 150 minutes at ambient temperature thus, ensuring ease of any administration. The optimised, “safe-to-administer” preparation and formulation of [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP yielded injectable doses with suitable specific activity that can be effectively applied to preclinical studies to evaluate the NK1R expression.

5 ACKNOWLEDGEMENTS

This work was supported by the Nuclear Technologies in Medicine and the Biosciences Initiative (NTeMBI), a national technology platform developed and managed by the South African Nuclear Energy Corporation (Necsa) and funded by the Department of Science and Technology (DST).

6 CONFLICT OF INTEREST

The authors report no conflict of interest.

7 REFERENCES

1. Chang MM, Leeman SE, Niall HD. Amino-acid sequence of SP. Nat New Biol [Internet]. 1971 [cited 2019 Sep 3];232(29):86-7. Available from: https://www.nature.com/articles/newbio232086a0 doi: 10.1038/newbio232086a0

2. Garcia-Recio S, Gascon P. Biological and pharmacological aspects of the NK1-receptor. Biomed Res Int [Internet]. 2015 Apr [cited 2019 Nov 11]; 495704. Available from: http://dx.doi.org/10.1155/2015/495704

3. DeVane, CL. Substance P: A new era, a new role. Pharmacotherapy [Internet]. 2001 [cited 2019 Oct 3];21(9):1061-9. Available from: https://accpjournals.onlinelibrary.wiley.com/doi/abs/10.1592/phco.21.13.1061.34612?sid=nlm%3Apubmed doi: 10.1592/phco.21.13.1061.34612

4. Otsuka M, Yoshioka K. Neurotransmitter functions of mammalian tachykinins. Physiol Rev [Internet]. 1993 [cited 2019 Sep 9];73(2):229-307. Available from: https://europepmc.org/article/med/7682720 doi: 10.1152/physrev.1993.73.2.229

5. Maggi CA, Patacchini R, Rovero P, Giachetti A. Tachykinin receptors and tachykinin receptor antagonists. J Auton Pharmacol [Internet]. 1993 Feb [cited 2019

Nov 30];13:23–93. Available from: https://doi.org/10.1111/j.1474-8673.1993.tb00396.x

6. Quartara L, Maggi CA. The tachykinin NK1 receptor. Part I: ligands and mechanisms of cellular activation. Neuropeptides [Internet]. 1997 [cited 2019 Oct 3];31(6):537-63. Available from: https://www.sciencedirect.com/science/article/abs/pii/S0143417997900019?via%3Dihub doi: 10.1016/s0143-4179(97)90001-9

7. Majkowska-Pilip A, Koźmiński P, Wawrzynowska A, Budlewski T, Kostkiewicz B, Gniazdowska E. Application of Neurokinin-1 Receptor in Targeted Strategies for Glioma Treatment. Part I: Synthesis and Evaluation of Substance P Fragments Labeled with 99mTc and 177Lu as Potential Receptor Radiopharmaceuticals. Molecules [Internet]. 2018 Oct [cited 2019 Nov 12];23(10):2542. Available from: https://doi.org/10.3390/molecules23102542

8. Muñoz M., Martinez-Armesto J., Coveñas R. NK-1 receptor antagonists as antitumor drugs: a survey of the literature from 2000 to 2011. Expert Opin Ther Pat [Internet]. 2012 [cited 2019 Nov 17];22(7):735–46. Available from: https://www.tandfonline.com/doi/abs/10.1517/13543776.2012.697153?journalCode=ietp20 doi: 10.1517/13543776.2012.697153

9. Saria A. The tachykinin NK1 receptor in the brain: pharmacology and putative functions. Eur J Pharmacol [Internet]. 1999 [cited 2019 Sep 27];375(1-3):51-60. Available from: https://www.sciencedirect.com/science/article/pii/S0014299999002599?via%3Dihub doi: 10.1016/s0014-2999(99)00259-9

10. Zieglgänsberger W. Substance P and pain chronicity. Cell Tissue Res [Internet]. 2019 [cited 2019 Oct 28]; 375(1):227–41. Available from: https://link.springer.com/article/10.1007/s00441-018-2922-y doi: 10.1007/s00441-018-2922-y

11. Harrison S, Geppetti P. Substance P. Int J Biochem Cell Bio [Internet]. 2001 [cited 2019 Sep 16];33(6):555-76. Available from: https://www.sciencedirect.com/science/article/abs/pii/S1357272501000310?via%3Dihub doi: 10.1016/s1357-2725(01)00031-0

12. Goto T, Nakao K, Gunjigake KK, Kido MA, Kobayashi S, Tanaka T. Substance P stimulates late-stage rat osteoblastic bone formation through neurokinin-1 receptors. Neuropeptides [Internet]. 2007 [cited 2019 Nov 4];41:25–31. Available from: https://www.sciencedirect.com/science/article/abs/pii/S0143417906001491?via%3Dihub doi: 10.1016/j.npep.2006.11.002

13. Nowicki M, Ostalska-Nowicka D, Konwerska A, Miskowiak B. The predicting role of substance P in the neoplastic transformation of the hypoplastic bone marrow. J Clin Pathol [Internet]. 2006 [cited 2019 Nov 5];59:935–41. Available from: https://jcp.bmj.com/content/59/9/935 doi: 10.1016/j.npep.2006.11.002

14. Murray CW, Cowan A, Larson AA. Neurokinin and NMDA antagonists (but not a kainic acid antagonist) are antinociceptive in the mouse formalin model. Pain [Internet]. 1991 [cited 2019 Nov 6];44(2):179-85. Available from: https://insights.ovid.com/article/00006396-199102000-00013 doi:10.1016/0304-3959(91)90135-K

15. Seguin L, Le Marouille-Girardon S, Millan MJ. Antinociceptive profiles of non-peptidergic neurokinin1 and neurokinin2 receptor antagonists: a comparison to other classes of antinociceptive agent. Pain [Internet].1995 [cited 2019 Nov 18];161:325-43. Available from: https://insights.ovid.com/article/00006396-199505000-00020 doi:10.1016/0304-3959(94)00194-J

16. Sakurada T, Katsumata K, Yogo H, Tan-No K, Sakurada S, Ohba M, et al. The neurokinin-1 receptor antagonist, sendide, exhibits antinociceptive activity in the formalin test. Pain [Internet]. 1995 Feb [cited 2019 Nov 8];60:175-80. Available from: https://doi.org/10.1016/0304-3959(94)00107-P

17. Traub RJ. The spinal contribution of substance P to the generation and maintenance of inflammatory hyperalgesia in the rat. Pain [Internet]. 1996 Sep [cited 2019 Nov 5];67:151-61. Available from: https://doi.org/10.1016/0304-3959(96)03076-X

18. Hill R. NK1(Substance P) receptor antagonists – why are they not analgesic in humans. Trends Pharmacol Sci [Internet]. 2000 [cited 2019 Nov 22];21(7):244-6. Available from: https://www.cell.com/trends/pharmacological-sciences/fulltext/S0165-6147(00)01502-9?_returnURL=https%3A%2F%2Flinkinghub.elsevier.com%2Fretrieve%2Fpii%2FS0165614700015029%3Fshowall%3Dtrue doi: 10.1016/s0165-6147(00)01502-9

19. Boyce S, Hill R. Discrepant results from precinical and clinical studies on the potential of substance P-receptor antagonist compounds as analgesics. In: Mea D, editor. Proceedings of the 9th World Congress on Pain: Seattle. IASP Press; 2000; pp. 313-324.

20. Chorev M, Rubini E, Hart Y, Gilon C, Wormser U, Selinger Z. Metabolically stable analogues of Substance P: Persistent action of partially modified retro-inverso analogues of Substance P on rat parotid and hypothalamic slices. Eur J Pharmacol [Internet]. 1986 [cited 2019 Nov 1];127(3):187-95. Available from: https://www.sciencedirect.com/science/article/pii/0014299986903638?via%3Dihub doi: 10.1016/0014-2999(86)90363-8

21. Sandberg BEB, Lee C-M, Hanley MR, Iversen LL. Synthesis and Biological Properties of Enzyme-Resistant Analogues of Substance P. Eur J Biochem [Internet]. 1981 Feb [cited 2019 Nov 12];114(2):329-37. Available from: https://doi.org/10.1111/j.1432-1033.1981.tb05152.x

22. Majkowska-Pilip A, Halik PK, Gniazdowska E. The significance of NK1 receptor ligands and their application in targeted radionuclide tumour therapy. Pharmaceutics [Internet]. 2019 Sep [cited 2019 Dec 2];11(443):1-28. Available from: https://doi.org/10.3390/pharmaceutics11090443

23. Dash A, Chakravarty R. Radionuclide generators: the prospect of availing PET radiotracers to meet current clinical needs and future research demands. Am J Nucl Med Mol Imaging [Internet]. 2019 Feb [cited 2019 Nov 11];9(1):30-66. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6420712/

24. Velikyan I. Prospective of 68Ga-Radiopharmaceutical Development. Theranostics [Internet]. 2014 [cited 2019 Nov 1];4(1):47-80. Available from: http://www.thno.org/v04p0047.htm

25. Breeman WAP, Verbruggen AM. The 68Ge/68Ga generator has high potential, but when can we use 68Ga-labelled tracers in clinical routine? Eur J Nucl Med Mol Imaging [Internet]. 2007 Mar [cited 2019 Nov 11];34:978-81. Available from: https://doi.org/10.1007/s00259-007-0387-4.

26. Sanchez-Crespo A, Andreo P, Larsson, SA. Positron flight in human tissues and its influence on PET image spatial resolution. Eur J Nucl Med Mol Imaging [Internet]. 2004 [cited 2019 Nov 23];31:44–51. Available from: https://link.springer.com/article/10.1007%2Fs00259-003-1330-y doi: 10.1007/s00259-003-1330-y

27. Smith DL, Breeman WAP, Sims-Mourtada J. The untapped potential of gallium-68 PET: the next wave of 68Ga agents. Appl Radiat Isot [Internet]. 2013 Jun [cited 2019 Nov 19];76:14-23. Available from: https://doi.org/10.1016/j.apradiso.2012.10.014

28. Banerjee SR, Pomper MG. Clinical applications of gallium-68. Appl Radiat Isot [Internet]. 2013 Jun [cited 2019 Nov 21];76:2-13. Available from: https://doi.org/10.1016/j.apradiso.2013.01.039

29. Merlo A, Macke H, Reubi J-C, Good S. Radiolabeled conjugates based on Substance P and the uses thereof. Universitätsspital Basel, Universität Bern; EP 1603598 B1, 2008.

30. Merlo A, Macke H, Reubi J-C, Good S. Radiopharmaceuticals for cancer diagnosis and treatment. Kantonsspital Basel, Universität Bern; US 2007/0053837 A1, 2007.

31. De Araújo EB, Pujatti PB, Mengatti J. Radiolabeling of Substance P with 177Lu and in vivo evaluation of tumor cell uptake in nude mice: Preliminary results. Cell Mol Biol (Noisy-le-grand) [Internet]. 2010 May [cited 2018 May 12];56(2):12-7. Available from: https://www.ipen.br/biblioteca/2008/eventos/13820.pdf

32. Rossouw DD, Breeman WA. Scaled-up radiolabelling of DOTATATE with 68Ga eluted from a SnO2-based 68Ga/68Ge generator. Appl Radiat Isot [Internet]. 2012 [cited 2019 Nov 23];70(1):171-5. Available from: https://www.sciencedirect.com/science/article/abs/pii/S0969804311003988?via%3Dihub doi: 10.1016/j.apradiso.2011.07.016

33. Hacht B. Gallium(III) Ion Hydrolysis under Physiological Conditions. Bulletin of the Korean Chemical Society [Internet]. 2008 Feb [cited 2019 Nov 23];29(2):372-6. Available from: https://doi.org/10.5012/BKCS.2008.29.2.372

34. Pellegrini PA, Howell NR, Shepherd RK, Lengkeek NA, Oehlke E, Katsifis AG, et al. Synthesis and Radiolabelling of DOTA-Linked Glutamine oncolodyues with 67,68Ga as Markers for Increased Glutamine Metabolism in Tumour Cells. Molecules [Internet]. 2013 Jun [cited 2019 Nov 6];18:7160-78. Available from: https://doi.org/10.3390/molecules18067160

35. Breeman WAP. Practical Aspects of labeling DTPA- and DOTA Peptides with 90Y, 111In, 177Lu, and 68Ga for Peptide-Receptor Scintigraphy and Peptide-Receptor Radionuclide Therapy in Preclinical and Clinical Applications Continuing Education for Nuclear Pharmacists And Nuclear Medicine Professionals. 2012 Nov 16;16(5). Available from: https://pharmacyce.unm.edu/program_information/freelessonfiles/Vol16Lesson5.pdf

36. Sosabowski JK, Mather SJ. Conjugation of DOTA-like chelating agents to peptides and radiolabeling with trivalent metallic isotopes. Nat Protoc [Internet]. 2006 [cited 2019 Dec 1];1(2):972-6. Available from: https://www.nature.com/articles/nprot.2006.175 doi: 10.1038/nprot.2006.175

37. De Blois E, Chan HS, Naidoo C, Prince D, Krenning EP, Breeman WAP. Characteristics of SnO2-based 68Ga/68Ge generator and aspects of radiolabelling DOTA-peptides. Appl Radiat Isot [Internet]. 2011 Feb [cited 2019 Nov 17];69:308-15. Available from: https://doi.org/10.1016/j.apradiso.2010.11.015

38. Tsionou MI, Knapp CE, Foley CA, Munteanu CR, Cakebread A, Imberti C, et al. Comparison of macrocyclic and acyclic chelators for gallium-68 radiolabelling RSC Adv [Internet]. 2017 [cited 2019 Nov 16];7:49586-99. Available from: https://pubs.rsc.org/en/content/articlelanding/2017/RA/C7RA09076E#!divAbstract doi: 10.1039/c7ra09076e

39. Zhernosekov KP, Filosofov DV, Baum P, Aschoff P, Bihl H, Razbash A, et al. Processing of generator-produced 68Ga for medical application. J Nucl Med [Internet]. 2007 [cited 2019 Nov 11];48(10):1741-8. Available from: http://jnm.snmjournals.org/content/48/10/1741 doi: 10.2967/jnumed.107.040378

40. Eppard E, Wuttke M, Nicodemus PL, Rosch F. Ethanol-Based Post-processing of Generator-Derived ^{68}Ga Toward Kit-Type Preparation of ^{68}Ga-Radiopharmaceuticals. J Nucl Med [Internet]. 2014 [cited 2019 Nov 23];55(6):1023-8. Available from: https://doi.org/10.2967/jnumed.113.133041 doi: 10.2967/jnumed.113.133041

41. Velikyan I, Beyer GJ, Langstroem B. Microwave-supported preparation of (68)Ga bioconjugates with high specific radioactivity. Bioconjugate Chem [Internet]. 2004 [cited 2019 Nov 30];15(3):554-60. Available from: https://pubs.acs.org/doi/10.1021/bc030078f doi: https://doi.org/10.1021/bc030078f

42. Velikyan I, Maecke H, Langstrom B. Convenient Preparation of 68Ga-Based PET-Radiopharmaceuticals at Room Temperature. Bioconjugate Chem [Internet]. 2008 Jan [cited 2019 Nov 7];19(2):569-73. Available from: https://doi.org/10.1021/bc700341x

43. Schaffalitzky, De Muckadell OB, Aggestrup S, Stentoft P. Flushing and plasma substance P concentration during infusion of synthetic substance P in normal man. Scand J Gastroenterol [Internet]. 1986 May [cited 2019 Nov 7];21(4):498-502. Available from: https://doi.org/10.3109/00365528609015169

44. van Hagen RM, Breeman WAP, Reubi JC, Postema RTE, van den Anker-Lugtenburg PJ, Kwekkeboom DJ et al. Visualization of the thymus by substance P receptor scintigraphy in man. Eur J Nuc Med [Internet]. 1996 Nov [cited 2019 Nov 13];23(11):1508-13. Available from: https://doi.org/10.1007/BF01254476

45. Ebenhan T, Chadwick N, Sathekge MM, Govender P, Govender T, Kruger HG, Marjanovic-Painter B, Zeevaart JR. Peptide synthesis, characterization and 68Ga-radiolabeling of NOTA-conjugated ubiquicidin fragments for prospective infection imaging with PET/CT. Nucl Med Biol [Internet]. 2014 Feb [cited 2019 Nov 23];41:390-400. Available from: https://doi.org/10.1016/j.nucmedbio.2014.02.001

PAPER 2

MANUSCRIPT FOR SUBMISSION TO THE JOURNAL

APPLIED RADIATION AND ISOTOPES

GALLIUM-68/BISMUTH-213 RADIOLABELLING OF PEPTIDES AS PROSPECTIVE THERANOSTIC AGENTS

Janine Suthiram[1,2] , Thomas Ebenhan[1], Danka Erasmus[1], Judith Wagener[2], Biljana Marjanovic-Painter[2], Jan Rijn Zeevaart[2,3], Sebastian Marx[4], Marian Meckel[4], Mike M Sathekge[1]

[1] *Nuclear Medicine, University of Pretoria and Steve Biko Academic Hospital, Gezina, Pretoria, Gauteng, South Africa*

[2] *Radiochemistry, The South African Nuclear Energy Corporation (Necsa), Pelindaba, Pretoria, South Africa*

[3] *Department of Science and Technology, Preclinical Drug Development Platform, North West University, Potchefstroom, North West Province, South Africa*

[4] *ITG Isotope Technologies Garching GmbH, Lichtenbergstrasse 1, 85748 Garching, Germany*

For correspondence: Mike.Sathekge@up.ac.za

Keywords ^{213}Bi, ^{68}Ga, ^{225}Ac/^{213}Bi generator, theranostic, PET/CT-imaging, peptide radiolabelling, DOTA-TATE, PSMA, DOTA-[Thi^{8},Met(O_2)^{11}]SP

TECHNICAL NOTE

ABSTRACT

The success of personalised nuclear medicine relies on the development of an interdependent, collaborative targeted therapeutic agent and a companion diagnostic test known as a theranostic pair. In this study, we evaluated [^{213}Bi]Bi-DOTA-functionalized peptides for targeted alpha radionuclide therapy together with their

companion [^{68}Ga]Ga-labelled peptides that are prioritized for diagnostic PET/CT imaging. Initial empirical values gained from radiolabelling DOTA-[D-Phe^{1},Tyr^{3}]-octreotate (DOTA-TATE), PSMA-11/PSMA-617 and DOTA-[Thi^{8},Met(O_2)^{11}]SP with ^{68}Ga and ^{213}Bi are reported.

1 INTRODUCTION

In recent years in the medical fraternity, there has been an emphasis on the benefits of personalised medicine. Drug design is moving away from a "one-size-fits-all" approach to a more unique method that customises therapy based on the interpersonal variation[1] observed in patients' responses to a particular drug. The concept of theranostics involves integrating imaging and therapy in a single system[2] and aims to translate conventional medicine to patient-centred care[1] ultimately leading to an improved patient outcome. In the early 2000s the theranostics principle started to be pursued in more branches of medicine. In nuclear medicine however, theranostics is by no means a new concept. Though classified retrospectively, the earliest prominent example of a nuclear theranostic medicine was the use of radioactive iodine to diagnose[3] and treat thyroid disorders[4,5] which was initiated over 80 years ago.[6]

Radiotheranostics refers to the incorporation of radionuclides in the paired imaging and therapeutic agents.[7] The successful radiolabelling of somatostatin analogues has enabled the development of peptide receptor radionuclide therapy (PRRT) for neuroendocrine tumours and this modality has greatly contributed to the expansion of the concept of theranostics to other oncological applications.[8] 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) is an aminocarboxylate macrocycle that conjugates to the peptide allowing for radiolabelling of the ligand with a variety of radiotherapeutic isotopes such as yttrium-90, lutetium-177 (^{177}Lu), actinium-225 (^{225}Ac) and bismuth-213 (^{213}Bi). The theranostic pair of [^{68}Ga]Ga-PSMA-617 and [^{177}Lu]Lu-PSMA-617 are currently in clinical use for the diagnosis and therapy of prostate cancer. [^{225}Ac]Ac-PSMA-617[9] and [^{213}Bi]Bi-PSMA-617[10] are in the early stages of human evaluation.

Bismuth-213 (t½ = 45.7 min) is a mixed alpha/beta emitting radionuclide that decays by emission of a gamma photon (440.5 keV) suitable for single photon emission

computerised tomography (SPECT) imaging.[11] The benefit of using alpha-emitter containing radiopharmaceuticals is that they deposit high energy, which effectively kills cancer cells, in a short range in tissue (~80 µm) thus preventing surrounding non-target tissue from receiving an unnecessarily high radiation dose.[12] Furthermore, ^{213}Bi decays to a stable isotope resulting in reduced radiotoxicity to the patient.[13] The cost and limited availability of ^{225}Ac to produce ^{213}Bi together with its short half-life are considered drawbacks to the use of this alpha-emitter.[14] The introduction of a clinically approved ^{225}Ac/^{213}Bi-generator[15] which is able to produce eluates of ^{213}Bi in the order of 25 to100 mCi, has allowed for the labelling of antibodies and peptides, some of which have been used successfully in preclinical and clinical studies.[16] Recent advances have seen the development of a high activity, reliable generator system[17] that can be loaded with up to 4 GBq of ^{225}Ac and has already been used to prepare therapeutic doses of up to 2.3 GBq of ^{213}Bi.[18]

Tachykinins are one of the most studied classes of neuropeptides due to their involvement in important physiological processes and diseases. This includes chronic pain, inflammation,[19] cancer,[20] and infection.[21] Substance P (SP) has a high affinity for the neurokinin-1 receptor (NK1R) and has the potential to visualize the involvement of NK1R in conditions characterised by chronic osteoarthritic pain symptoms. We previously reported on the radiosynthesis, optimisation and safe for administration formulation of ^{68}Ga-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid-[Thi^{8},Met(O_2)^{11}]-Substance P ([^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP) as a potential PET/CT tracer for that purpose. [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP and [^{213}Bi]Bi-DOTA-[Thi^{8},Met(O_2)^{11}]SP has, in the last few years, been reported in literature for their use in the imaging and therapy of glioblastoma multiforme with reliable success. The reported radiosynthesis of [^{213}Bi]Bi-DOTA-[Thi^{8},Met(O_2)^{11}]SP is achieved using a microwave synthesiser where the peptide, generator eluate and 2 M TRIS buffer are heated at 95 °C for 5 min.[22,23] Herein we report on the radiosynthesis of [^{213}Bi]Bi-DOTA-[Thi^{8},Met(O_2)^{11}]SP using a simpler, robust radiolabelling method similar to that employed for [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP. The labelling efficiency achieved is compared to that obtained for DOTA-TATE and ligands targeting the prostate-specific membrane antigen (PSMA) that have been introduced recently as ^{177}Lu-labeled theranostics for neuroendocrine tumours and prostate cancer, respectively.[24,25] Even though the theranostic pair [^{68}Ga]Ga-/[^{213}Bi]Bi-DOTA-

[Thi^{8},Met(O_2)^{11}]SP may not have an application in chronic pain disorders, the safe to administer formulation may be of benefit to researchers in the oncology space.

2 MATERIAL AND METHODS

2.1 CHEMICALS AND MATERIAL

Suprapure-grade 30% hydrochloric acid (HCl) and pharmaceutical-grade ethanol, sodium acetate and sodium iodide were purchased from Merck & Sigma Aldrich (Johannesburg, South Africa). C18 Sep-Pak® (light) cartridges for solid-phase extraction (SPE) were obtained from Microsep (Johannesburg, South Africa). Silica-gel coated aluminium-backed thin-layer chromatography (TLC) sheets were purchased from Sigma Aldrich (Johannesburg, South Africa) and glass microfiber chromatography paper impregnated with silica gel (ITLC-SG) from Chemetrix (Johannesburg, South Africa). High-performance liquid chromatography (HPLC)-grade water (resistivity of 18.2 MΩ.cm) was made in-house using a Simplicity-185 Millipore system (Cambridge, Massachusetts, USA). DOTA-TATE was purchased from Auspep (Victoria, Australia), PSMA-11 and PSMA-617 were purchased from ABX-Advanced Biochemical Compounds (Radeberg, Germany). DOTA-[Thi^{8},Met(O_2)^{11}]SP was purchased from piCHEM (Raaba-Grambach, Austria).

2.2 GENERATOR ELUTION

^{68}Ga (89%; EC β+ max. 1.9 MeV) was eluted from a tin-dioxide-based ^{68}Ge/^{68}Ga generator (1.85 GBq, iThemba Labs, South Africa) as previously described using moderately acidic conditions (10 ml, 0.6 M HCl) via eluate fractionation as follows: first 1 ml into waste vial, followed by 2 ml ^{68}Ga-eluate for radiolabelling into eluate vial, remaining 7 ml into waste vial. Between 75 and 90% of elutable [^{68}Ga]$GaCl_3$ activity was obtained in the 2 ml which was quantified using a Capintec C25 Ionisation Chamber (New Jersey, USA)

The ^{225}Ac/^{213}Bi generators (3.7 GBq) were supplied by the Institute for Isotope Technologies Munich (ITG, Garching, Germany). The column was first flushed with 1.5 ml of 0.01 M HCl. ^{213}Bi was eluted using 1.5 ml of a mixture of 0.1 M NaI and 0.1 M HCl. Following elution, the generator column was rinsed and stored with 2 ml of 0.01 M HCl.

2.3 RADIOLABELLING PROCEDURES

^{68}Ga-labelling of DOTA-TATE was performed as previously reported[26] using 30-50 µg peptide whereas ^{213}Bi-labelling was carried out with 100 µg. PSMA-11 was radiolabelled using an in-house kit-based production method[27] whereas 100 µg of PSMA-617 was radiolabelled with ^{213}Bi following a method previously used for a single patient case report.[10] DOTA-[Thi^{8},Met(O_2)^{11}]SP was labelled with ^{68}Ga and ^{213}Bi using 50 µg.

Briefly, [^{68}Ga]Ga- and [^{213}Bi]Bi-bioconjugates were buffered with sodium acetate to pH 3.5-4 and pH 5 respectively, incubated at 93-95 °C for 15 min followed by purification. Final solutions were sterilized by filtration and directly dispensed into syringes diluting the product to ~10 ml. Radiochemical purity and yields were assessed by ITLC-SG or TLC.

2.4 CHROMATOGRAPHIC ANALYSIS

The percentage ^{68}Ga-labelling efficiency (%LE) was determined using ITLC-SG as stationary phase and 0.1 M citrate, pH 5, as the mobile phase to determine free ^{68}Ga-species *versus* ^{68}Ga-peptide. The crude or pure ^{68}Ga-peptide (~3-6 µl) was spotted at the bottom of the ITLC-SG strip and developed using the mobile phase till the solvent front has progressed to the near-top of the strip (within 4-5 min). The strip was then removed from the developing chamber, dried and analysed on a TLC scanner (Veenstra Ind., Oldenzaal, Netherlands) using a gamma radiation detector (Bunnik, Netherlands). The resulting chromatograms were quantified using Genie2000 software (Veenstra Ind., Oldenzaal, Netherlands); peak identification and calculation of the percentage labelling efficiency were achieved by way of “area under the curve” analysis. The same mobile phase was used for analysis of the ^{213}Bi-peptides; however, silica-gel plates with aluminium backing were utilized (running at 0.5 cm/min) to improve the sensitivity of the TLC.

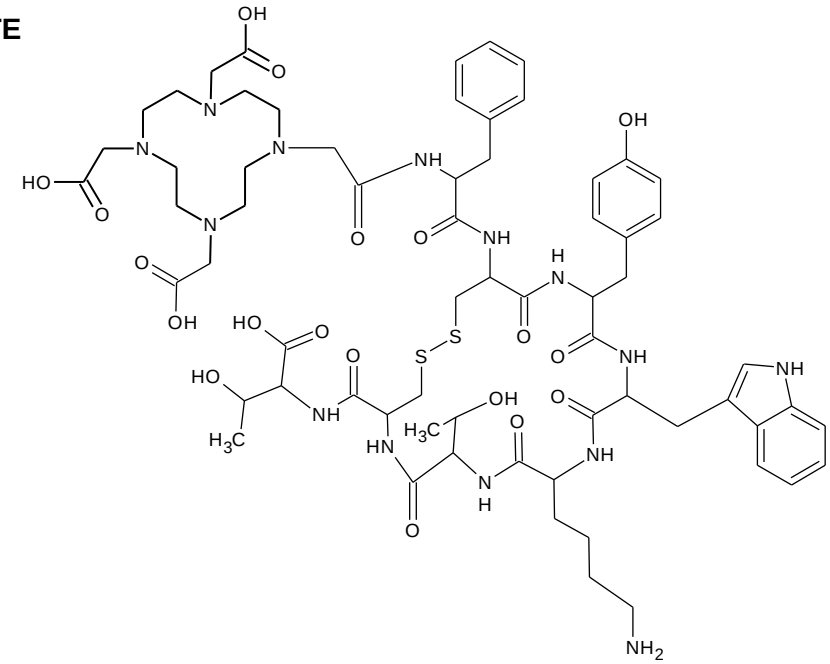
3 RESULTS AND DISCUSSION

The development of therapeutic agents in nuclear medicine has progressed from β-emitters to higher energy short range α-emitters such as ^{213}Bi. Auger emitters that decay with the emission of Auger electrons deposit very high energy over very short

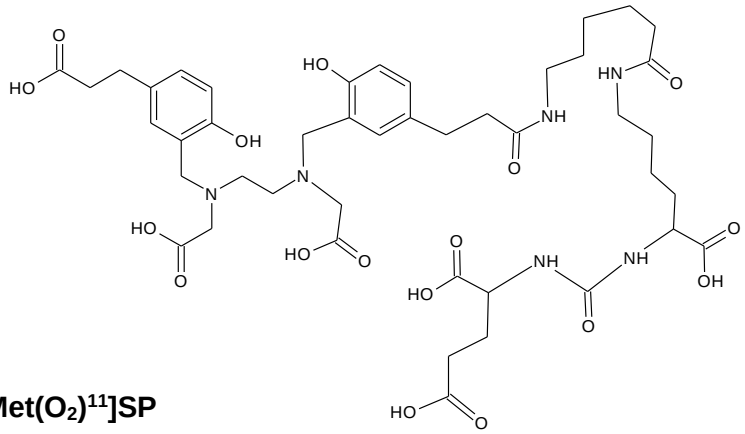
distances and these radionuclides have also gained interest. Previously reported methods for the radiosynthesis of [^{213}Bi]Bi- DOTA-[Thi^{8},Met(O_2)^{11}]SP involved the use of a microwave synthesiser.[22,23] We report a simpler method to prepare [^{213}Bi]Bi-DOTA-[Thi^{8},Met(O_2)^{11}]SP that is similar to the methodology used to prepare ^{68}Ga radiolabelled peptides. Radiolabelling of DOTA-TATE and PSMA-11/-617 peptides (**Figure 1**) with ^{68}Ga and ^{213}Bi, were for separate studies. Therefore, the data that was obtained is included here for comparative purposes only.

^{68}Ge/^{68}Ga generators (1.85 GBq, iThemba Labs, South Africa) that were utilised for this study routinely yielded 312 MBq ± 119 MBq (n = 15) of ^{68}Ga activity in 1.0 ml (half-scale) of eluate obtained through fractionated elution. The ^{225}Ac/^{213}Bi generator yielded 40-67 MBq/ml ^{213}Bi in a total volume of 1.5 ml of non-toxic eluent. Generator details are summarised in **Table 1**.

DOTA-TATE



PSMA



DOTA-[Thi^{8},Met(O$_2$)^{11}]SP

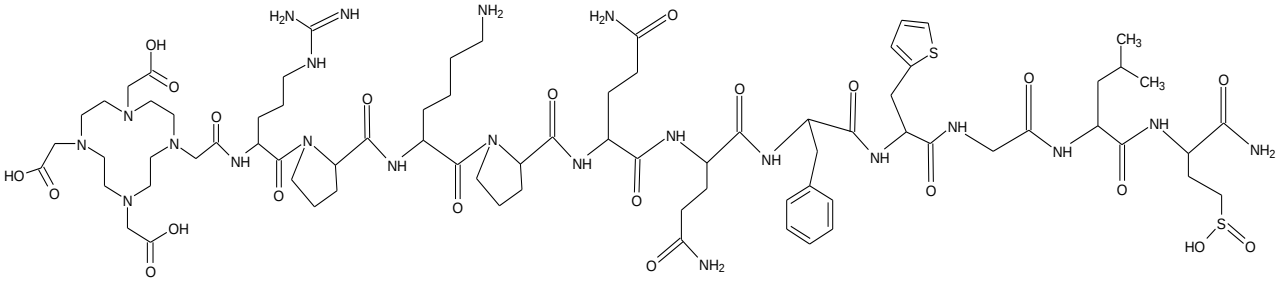


Figure 1: Structures of the different peptides used in the study. DOTA-TATE for neuroendocrine tumours, PSMA for prostate cancer and DOTA-[Thi^{8},Met(O$_2$)^{11}]SP for chronic pain disorders and oncology.

Table 1: Overview of the ^{68}Ga/^{68}Ge and ^{225}Ac/^{213}Bi generator systems.

Generators	**^{68}Ga/^{68}Ge**	**^{225}Ac/^{213}Bi**
Manufacturer	iThemba LABS	ITG
Mother radionuclide	^{68}Ge	^{225}Ac
Mother half-life (days)	271	10
Matrix of resin	Tin dioxide	AG-MP-50
Eluent	0.6 M HCl	0.1 M NaI/0.1 M HCl
Daughter radionuclide	^{68}Ga	^{213}Bi
Daughter half-life (min)	67	46
Emitting (keV)	1900; β^+; EC	8400; β; high LET

Footnotes:

LET = linear energy transfer; EC = Electron Capture

A summary of the results achieved for the different peptides with both ^{68}Ga and ^{213}Bi is reported in **Table 2**. Less than 0.1 mg of each of the peptides (DOTA-TATE, PSMA-11/PSMA-617, DOTA-[Thi^8,Met(O_2)^{11}]SP) were used in all preparations with sodium acetate as the buffer. With the exception of PSMA-11, all radiolabelling reactions required heating to 95 °C. [^{68}Ga]Ga-DOTATATE-PET/CT product activity was 133-259 MBq (n = 15) and supported [^{213}Bi]Bi-DOTATATE treatment (259-370 MBq). [^{68}Ga]Ga-PSMA-11-PET/CT (120-240 MBq, n > 20) was performed to facilitate [^{213}Bi]Bi-DOTA-PSMA-617 treatment (222-407 MBq). All patient administrations were successful but are not discussed in detail as they are not part of the scope of this study.

The optimised radiosynthesis of [^{68}Ga]Ga-DOTA-[Thi^8,Met(O_2)^{11}]SP (using 0.05 mg, RCP ≥ 99.8%, 194-461 MBq, n = 4) was achieved with a labelling efficiency of 99.9 ± 0.2% and translated well to [^{213}Bi]Bi- DOTA-[Thi^8,Met(O_2)^{11}]SP with a RCP of 65.7%, 91.2% and 97.5% (using 0.05 mg, 222-259 MBq, n = 3) after 5, 10 and 15 min incubation, respectively. This initial data for ^{213}Bi labelling indicates that with a small mass of peptide and 15 min of heating at 95 °C, an RCP of 97.5 % can be achieved. These results are comparable to that achieved for both DOTA-TATE and PSMA. Furthermore the radiosynthetic method that we propose for the radiolabelling of [^{68}Ga]Ga-/[^{213}Bi]Bi-DOTA-[Thi^8,Met(O_2)^{11}]SP is in keeping with the routine preparation of DOTA-TATE and PSMA, both of which are being used with significant success in pre-clinical and clinical studies.

Table 2: Summary of radiolabelling data for ^{68}Ga-peptides and ^{213}Bi-peptides.

Radionuclide	**^{68}Ga**	**^{213}Bi***	**^{68}Ga**	**^{213}Bi***	**^{68}Ga**	**^{213}Bi**
Peptides	**DOTA-TATE**	**DOTA-TATE**	**PSMA-11**	**PSMA-617**	**DOTA-[Thi^{8},Met(O$_2$)^{11}]SP**	**DOTA-[Thi^{8},Met(O$_2$)^{11}]SP**
Mass of peptide (mg)	0.05	0.1	0.005	0.1	0.05	0.05
Reaction temp (°C)	95	95	RT	95	95	95
pH^{+}	3-3.5	4	4-4.5	4	3.5	3.5
Dose (MBq)#	133-259	259-370	120-240	222-407	-	-
Radiosyntheses (n)	15	5	>20	>20	4	3
RCP (%)	>95	>95	>95	>95	≥99.8	65.7 91.2 97.5
Incubation time (min)	15	15	15	15	15	5 10 15

$^{+}$) Na-acetate was used as buffering agent in all ^{68}Ga-preparations, final solutions sterilized by filtration and directly dispensed into syringes diluting the product to ~10 ml.

*) Data has been included for comparison purposes only. These radiosyntheses were carried out by Sathekge MM, et al. (unpublished data).

* All quality control was carried out using ITLC-SG analysis.

All product activity and RCP data were decay corrected.

Existing radiosynthesis methods for [^{213}Bi]Bi-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP using a microwave synthesiser report an RCP of 98.0 ± 1.4 % within 5 min for 10-30 µg of the peptide.[22] Later data released from the same group of researchers reported an improved RCP of higher than 99 % with no indication of peptide mass used.[23] The radiolabelling of the co-administered [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP was performed using a peptide mass of 150 µg compared to this study preparation which used 50 µg. This is an important consideration given that Substance P is a vasodilator[28] and can have pharmacological effects on patients. Clinical studies of [^{213}Bi]Bi-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP for the treatment of gliomas have recorded some

side effects including mild flush and transient worsening of paresis and aphasia.[23] Even though the reaction time is reduced which is a great benefit for the short half-life of ^{213}Bi, the method reported herein uses a conventional heating plate and would be well suited for nuclear medicine departments at hospitals that do not have access to additional equipment such as a microwave synthesiser. The similarity in the method used for the radiolabelling of DOTA-[Thi^{8},Met(O$_2$)^{11}]SP with both ^{68}Ga and ^{213}Bi also implies an easy transition between preparations.

4 CONCLUSION

The radiolabelling method for [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP was successfully translated to [^{213}Bi]Bi-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP. Quantitative labelling of [^{213}Bi]Bi-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP was achieved within 15 min. High radiochemical purity (> 95%) can be achieved with a simple, reliable method that uses conventional heating with a low peptide mass. The data indicates that a robust preparation and safe to administer formulation is achievable. The theranostic pair [^{68}Ga]Ga-/[^{213}Bi]Bi-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP is not likely to have an application in chronic pain disorders, however the safe-to-administer formulation may be of benefit to researchers for use as a theranostic pair in oncological applications.

5 ACKNOWLEDGEMENTS

The authors would like to express their gratitude to The South African Nuclear Energy Corporation SOC Ltd (Necsa) and the Steve Biko Academic Hospital for access to the iThemba LABS and ITG generator and for the use of the DOTA-TATE and PSMA data.

6 CONFLICT OF INTEREST

The authors report no conflict of interest.

7 REFERENCES

1. Jeelani S, Jagat Reddy RC, Maheswaran T, Asokan GS, Dany A, Anand B. Theranostics: A treasured tailor for tomorrow. J Pharm Bioall Sci [Internet]. 2014 [cited 2019 Nov 17];6(Suppl 1):S6-8. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/pmid/25210387/ doi: 10.4103/0975-7406.137249

2. Airaksinen AJ. Radiolabeled porous silicon for bioimaging applications. In: Santos H, editor. Porous silicon for biomedical applications. United Kingdom: Woodhead Publishing; 2014. p. 253-269.

3. Hertz S, Roberts A, Evans RD. Radioactive iodine as an indicator in the study of thyroid physiology. Proc Soc Exp Biol Med [Internet]. 1938 [cited 2019 Nov 17]; 38:510–3. Available from: https://journals.sagepub.com/doi/abs/10.3181/00379727-38-9915P doi: 10.3181/00379727-38-9915P

4. Hertz S, Roberts A. Application of radioactive iodine in Graves disease. J Clin Invest [Internet]. 1942 [cited 2019 Nov 1];21:624-33. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC435114/ doi: 10.1172/JCI101276

5. Seidlin SM, Marinelli LD, Oshry E. Radioactive iodine therapy: effect on functioning metastases of adenocarcinoma of the thyroid. JAMA [Internet]. 1946 [cited 2019 Jun 21];132:838-47. Available from: https://jamanetwork.com/journals/jama/article-abstract/290151 doi: 10.1001/jama.1946.02870490016004

6. Verburg FA, Heinzel A, Hänscheid H, Mottaghy FM, Luster M, Giovanella L. Nothing new under the nuclear sun: towards 80 years of theranostics in nuclear medicine. Eur J Nucl Med Mol Imaging [Internet]. 2014 [cited 2019 Oct 13];41(2)199-201. Available from: https://link.springer.com/article/10.1007/s00259-013-2609-2 doi: 10.1007/s00259-013-2609-2

7. Jadvar H, Chen X, Cai W, Mahmood U. Radiotheranostics in cancer diagnosis and management. Radiology [Internet]. 2018 [cited 2019 Oct 16];286(2):388-400. Available from: https://pubs.rsna.org/doi/10.1148/radiol.2017170346 doi: 10.1148/radiol.2017170346

8. Levine R, Krenning EP. Clinical history of the theranostic radionuclide approach to neuroendocrine tumours and other types of cancer: Historical review based on an interview of Eric P. Krenning by Rachel Levine. J Nucl Med [Internet]. 2017 [cited 2019 Aug 1];58(Suppl 2):3S-9S. Available from: http://jnm.snmjournals.org/content/58/Supplement_2/3S.long doi: 10.2967/jnumed.116.186502

9. Kratochwil C, Bruchertseifer F, Rathke H, Bronzel M, Apostolidis C, Weichert W, et al. Targeted a-therapy of metastatic castration-resistant prostate cancer with ^{225}Ac-PSMA-617: dosimetry estimate and empiric dose finding. J Nucl Med [Internet]. 2017 [cited 2019 Jul 13];58(10):1624–31. Available from: http://jnm.snmjournals.org/content/58/10/1624.long doi: 10.2967/jnumed.117.191395

10. Sathekge M, Knoesen O, Meckel M, Modiselle M, Vorster M, Marx S. ^{213}Bi-PSMA-617 targeted alpha-radionuclide therapy in metastatic castration-resistant prostate cancer. Eur J Nucl Med Mol Imaging [Internet]. 2017 [cited 2019 Aug 14];44(6):1099–100.Available from: https://link.springer.com/article/10.1007/s00259-017-3657-9 doi: 10.1007/s00259-017-3657-9

11. Srivastava SC. A bridge not too far: personalised medicine with the use of theragnostic radiopharmaceuticals. J Postgrad Med Edu Res [Internet]. 2013 Jan [cited 2019 Nov 1];47(1):31-46. Available from: https://pdfs.semanticscholar.org/47c1/6635862090535d987e4ba00170ac839af99c.pdf

12. Chan HS, de Blois E, Konijnenberg MW, Morgenstern A, Bruchertseifer F, Norenberg JP, et al. Optimizing labelling conditions of ^{213}Bi-DOTATATE for preclinical applications of peptide receptor targeted alpha therapy. EJNMMI

Radiopharm Chem [Internet]. 2016 [cited 2019 Nov 17];1(9):1-15 Available from: https://link.springer.com/article/10.1186/s41181-016-0014-4 doi: 10.1186/s41181-016-0014-4

13. Keogan DM, Griffith DM. Current and potential applications of bismuth-based drugs. Molecules [Internet]. 2014 Sep [cited 2019 Aug 23];19(9):15258-97. Available from: https://doi.org/10.3390/molecules190915258

14. Dekempeneer Y, Keyaerts M, Krasniqi A, Puttemans J, Muyldermans S, Lahoutte T, et al. Targeted alpha therapy using short-live alpha-particles and the promise of nanobodies as targeting vehicle. Expert Opin Biol Ther [Internet]. 2016 Aug [cited 2019 Aug 23];16(8):1035-47. Available from: https://doi.org/10.1080/14712598.2016.1185412

15. McDevitt MR, Finn RD, Sgouros G, Ma D, Scheinberg DA. An Ac-225/Bi-213 generator system for therapeutic clinical applications: construction and operation. Appl Radiat Isot [Internet]. 1999 [cited 2019 June 15];50(5):895-904. Available from: https://www.sciencedirect.com/science/article/abs/pii/S0969804398001511?via%3Dihub doi: 10.1016/s0969-8043(98)00151-1

16. Norenberg JP, Krenning BJ, Konings I, Kusewitt DF, Nayak TK, Anderson TL. Bi-213-DOTA(0),Tyr(3) octreotide peptide receptor radionuclide therapy of pancreatic tumors in a preclinical animal model. Clin Cancer Res [Internet]. 2006 [cited 2019 Nov 20];12(3):897. Available from: https://clincancerres.aacrjournals.org/content/12/3/897 doi: 10.1158/1078-0432.CCR-05-1264

17. Bruchertseifer F, Apostolidis C, Mirzadeh S, Boll R, Murphy K, Morgenstern A. Development of a high-activity 225Ac/213Bi radionuclide generator for synthesis of clinical doses of ^{213}Bi-labelled biomolecules. In: Proceedings of the 8th International Symposium on Target Alpha Therapy; 2013 Jun 4-6; Oak Ridge, USA.

18. Morgenstern A, Apostolidis C, Kratochwii C, Sathekge M, Krolicki L, Bruchertseifer F. An overview of targeted alpha therapy with ^{225}actinium and ^{213}bismuth. Curr Radiopharm [Internet]. 2018 [cited 2019 Nov 22];11(3):200-8. Available from: http://www.eurekaselect.com/161764/article doi: 10.2174/1874471011666180502104524

19. Canning BJ. Potential role of tachykinins in inflammatory diseases. J Allergy Clin Immunol [Internet]. 1997 May [cited 2019 Nov 11];99(5):579-82. Available from: https://doi.org/10.1016/S0091-6749(97)70015-0

20. Palma C. Tachykinins and their receptors in human malignancies. Curr Drug Targets [Internet]. 2006 [cited 2019 Nov 20];7(8):1043-52. Available from: http://www.eurekaselect.com/56449/article doi: 10.2174/138945006778019282

21. Steinhoff MS, von Mentzer B, Geppetti P, Pothoulakis C, Bunnett NW. Tachykinins and their receptors: contributions to physiological control and the mechanisms of disease. Physiol Rev [Internet]. 2014 Jan [cited 2019 Sep 30];94(1): 265–301. Available from: https://doi.org/10.1152/physrev.00031.2013

22. Cordier D, Forrer F, Bruchertseifer F, Morgenstern A, Apostolidis C, Good S, et al. Targeted alpha-radionuclide therapy of functionally critically located gliomas with ^{213}Bi-DOTA-[Thi^{8}, Met(O_2)^{11}]-substance P: a pilot trial. Eur J Nucl Med Mol Imaging [Internet]. 2010 [cited 2019 Nov 11];37(7):1335-44. Available from: https://link.springer.com/article/10.1007%2Fs00259-010-1385-5 doi: 10.1007/s00259-010-1385-5

23. Krolicki L, Bruchertseifer F, Kunikowska J, Koziara H, Królicki B, Jakuciński M, et al. Prolonged survival in secondary glioblastoma following local injection of targeted alpha therapy with ^{213}Bi-substance P analogue. Eur J Nucl Med Mol Imaging [Internet]. 2018 Jul [cited 2019 Nov 12];45(9):1636-44. Available from: https://doi.org/10.1007/s00259-018-4015-2

24. Baum RP, Kulkarni HR. Theranostics: From molecular imaging using Ga-68 labeled tracers and PET/CT to personalized radionuclide therapy - The bad Berka experience. Theranostics [Internet]. 2012 [cited 2019 Nov 20];2(5):437-47. Available from: http://www.thno.org/v02p0437.htm doi: 10.7150/thno.3645

25. Weineisen M, Schottelius M, Simecek J, Baum RP, Yildiz A, Beykan S et al. ^{68}Ga- and ^{177}Lu-Labeled PSMA I&T: Optimization of a PSMA-targeted theranostic concept and first proof-of-concept human studies. J Nucl Med [Internet]. 2015 [cited 2019 Nov 23];56(8):1169-76. Available from: http://jnm.snmjournals.org/content/56/8/1169 doi: 10.2967/jnumed.115.158550

26. Rossouw DD, Breeman WA. Scaled-up radiolabelling of DOTATATE with ^{68}Ga eluted from a SnO_2-based ^{68}Ga/^{68}Ge generator. Appl Radiat Isot [Internet]. 2012 [cited 2019 Nov 23];70(1):171-5. Available from: https://www.sciencedirect.com/science/article/abs/pii/S0969804311003988?via%3Dihub doi: 10.1016/j.apradiso.2011.07.016

27. Ebenhan T, Vorster M, Marjanovic-Painter B, Wagener J, Suthiram J, Modiselle M, et al. Development of a single vial kit solution for radiolabeling of ^{68}Ga-DKFZ-PSMA-11 and its performance in prostate cancer patients. Molecules [Internet]. 2015 Aug [cited 2019 Nov 30];20(8):14860-78. Available from: https://doi.org/10.3390/molecules200814860

28. Walsh DA, Mapp PI, Wharton J, Rutherford RA, Kidd BL, Revell PA, et al. Localization and characterization of Substance P binding to human synovial tissue in rheumatoid arthritis. Ann Rheum Dis [Internet]. 1992 Mar [cited 2019 Nov 18];51(3):313-7. Available from: http://dx.doi.org/10.1136/ard.51.3.313

PAPER 3

MANUSCRIPT FOR SUBMISSION TO THE JOURNAL

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS

IN VITRO EVALUATION OF THE TACHYKININ RECEPTOR ACTIVITY AND SELECTIVITY OF DOTA-[THI^{8}, MET(O$_2$)^{11}]-SUBSTANCE P

Janine Suthiram[1,2] , Thomas Ebenhan[2,3], Jan Rijn Zeevaart[1,4], Mike M Sathekge[5], Alexis Schwulst[6], Ross Anderson[6], Zulfiah Mohamed-Moosa[6], Claire Newton[6]

[1] *The South African Nuclear Energy Corporation (Necsa SOC Ltd.), Radiochemistry, Pelindaba, South Africa*

[2] *University of Pretoria, Department of Nuclear Medicine, Pretoria, South Africa*

[3] *Nuclear Medicine Research Infrastructure/NPC, Preclinical Imaging Facility, Pretoria, South Africa*

[4] *Department of Science and Technology, Preclinical Drug Development Platform, North West University, Potchefstroom, South Africa*

[5] *University of Pretoria, Nuclear Medicine & Steve Biko Academic Hospital, Pretoria, South Africa*

[6] *Centre for Neuroendocrinology, Department of Immunology (CN) and Department of Physiology (RA, AS and ZM), Faculty of Health Sciences, University of Pretoria.*

For correspondence: Janine.Suthiram@necsa.co.za

Keywords Tachykinin, neurokinin-1 receptor, ^{68}Ga, Substance P, PET/CT-imaging, peptide radiolabelling, specificity, dose response, EC$_{50}$

ABSTRACT

The success of radiopharmaceutical development hinges on the affinity and selectivity of the biological component for the intended target. Specific binding of a peptide ligand to a receptor makes for a high target to non-target ratio, which is desirable in drug design. For diagnostic radiopharmaceuticals, target engagement must be rapid with high affinity and selectivity to ensure imaging of the tracer depending on the modality

(SPECT, PET), whilst therapeutic radiopharmaceuticals benefit from longer residence times that improve therapeutic efficacy. An analogue of Substance P (SP), DOTA-[Thi^{8},Met(O_2)^{11}]SP (where DOTA = 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) was selected as the ligand of interest in an effort to develop a [^{68}Ga]Ga-labelled PET/CT radiotracer to assess the neurokinin-1 receptor (NK1R) availability in chronic pain disorders. Modifications to the amino acid residues in SP could interfere with the ability of the peptide to selectively bind to the intended NK1R target. Tachykinin receptor signalling studies using an inositol phosphate accumulation study, found DOTA-[Thi^{8},Met(O_2)^{11}]SP to have similar efficacy and potency as native SP but greater selectivity for NK1R. DOTA-[Thi^{8},Met(O_2)^{11}]SP was unable to elicit significant activation of any of the other tachykinin receptors at high concentrations and also did not have any antagonistic behaviour at these receptors. The inability to activate and stimulate the other tachykinins supports the result that DOTA-[Thi^{8},Met(O_2)^{11}]SP has a high potency and selectivity for NK1R.

1 INTRODUCTION

Substance P (SP), an undecapeptide first described by Gaddum and Shild,[1] is widely distributed in the central and peripheral nervous system of mammalian species.[2] Both neuronal and non-neuronal cells, including immune cells, produce SP.[3] The biological activity of SP is mediated through interactions with all three tachykinin receptors, neurokinin-1 receptor (NK1R), neurokinin-2 receptor (NK2R), and neurokinin-3 receptor (NK3R). However, a preferential interaction at a much higher affinity takes place at NK1R.[4] Under certain conditions, such as differential receptor availability and high peptide concentration, endogenous tachykinins are not selective and can therefore act on any of the receptors.[5] SP has also been shown to act as a low potency agonist at MRGPRX2, a mast-cell specific receptor, which is implicated in mast-cell-mediated allergic-type reactions.[6]

The tachykinin receptors (NK1R, NK2R, and NK3R) and MRGPRX2 are G protein-coupled receptors (GPCRs). GPCRs form the largest protein family of cell surface receptors and are accountable for response to a large variety of chemical (chemoattractant, calcium ions, hormones, and neurotransmitters) and sensory (light, odorants and taste molecule) stimuli.[7] As a result they are responsible for regulating

most signalling pathways. Like all GPCRs, the NK1R consists of seven transmembrane spanning α helical domains (TM domains), with an extracellular amino-terminus and intracellular carboxyl tail.[6,8] It consists of 407 amino acid residues amounting to a relative molar mass of 46 kDa.[9] The second and third TM domains of the receptor is the location of the endogenous agonist binding site and also provides the location of the binding site for current peptide antagonists. When SP binds to NK1R, coupling to downstream signalling partners is elicited.[4]

Despite the structural similarities between different GPCRs, individual receptors display unique combinations of signal transduction activities. This involves interaction with various G proteins and their subtypes, as well as G protein-independent signalling pathways. Ligand binding to GPCRs induces a conformational change in the TM bundle. Evaluation of crystal structures of activated/agonist-bound and inactive/inverse agonist-bound GPCRs have determined that the biggest conformational change observed is the outward movement of the cytoplasmic component of TM6.[10,11] This movement allows interactions with downstream hetero-trimeric guanine nucleotide-binding proteins (G proteins) which consist of Gα, Gβ, and Gγ subunits.[12] GPCR acts as a guanine nucleotide exchange factor (GEF) which catalyses the exchange of bound guanosine diphosphate (GDP) for guanosine triphosphate (GTP) on the Gα-subunit of the G protein.[13] Exchange in guanine nucleotides in turn engages conformational changes in the G protein subunits and results in dissociation between the Gα and dimeric Gβγ subunits.[14] The dissociated Gα and Gβγ subunits are then able to continue signal transduction by interacting with other available intracellular proteins. This takes place until the intrinsic GTP-ase activity of the Gα subunit catalyses the hydrolysis of the bound GTP to GDP, allowing re-association of the G protein subunits.

G proteins are categorised into four major families, based on the downstream signalling partners of the Gα subunit ($G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11,}$ and $G\alpha_{12/13}$). Most GPCRs have the ability to activate more than one Gα-subtype; however they generally show preference for one specific subtype. MRGPRX2 and all of the tachykinin receptors couple preferentially to the $G\alpha_{q/11}$ family of G proteins. This $G\alpha_{q/11}$ family activates the enzyme phospholipase C (PLC), which cleaves plasma membrane lipids (phosphatidylinositol (4,5)-bisphosphate (PIP2)) into diacylglycerol (DAG) and inositol (1,4,5)-trisphosphate (IP_3). IP_3 diffuses through the cytosol to activate IP_3-gated Ca^{2+}

channels in the membranes of the endoplasmic reticulum, causing the release of stored Ca^{2+} into the cytosol (**Figure 1**).[15] The increase in cytosolic Ca^{2+} can induce physiological responses such as calcium-mediated exocytosis as well as promote the translocation of the enzyme phosphokinase C (PKC) to the plasma membrane where it can be activated by DAG. PKC phosphorylates target proteins in the cell to induce physiological responses and alter gene transcription. IP_3 is recycled back to PIP2 through a number of enzymatic steps that convert it to inositol bisphosphate (IP_2), inositol monophosphate (IP), myo-inositol and then PIP2 (**Figure 1**). Loading cells grown in low-inositol/inositol-free media with radiolabelled myo-inositol (a precursor of PIP2), ensures that the inositol phosphates generated upon receptor stimulation are radiolabelled and can be measured after separation and collection on an ion exchange column. Performing the assay in the presence of lithium chloride (an inhibitor of inositol monophosphatase [IMPase] which catalyses the conversion of IP to myo-inositol) leads to accumulation of the soluble radiolabelled inositol phosphates such that the amount of radioactivity measured is proportional to receptor signalling activity. This is the basis of the assay that will be used to assess tachykinin receptor activity when stimulated with peptide ligands neurokinin A (NKA), neurokinin B (NKB), SP and DOTA-[Thi^8,$Met(O_2)^{11}$]SP.

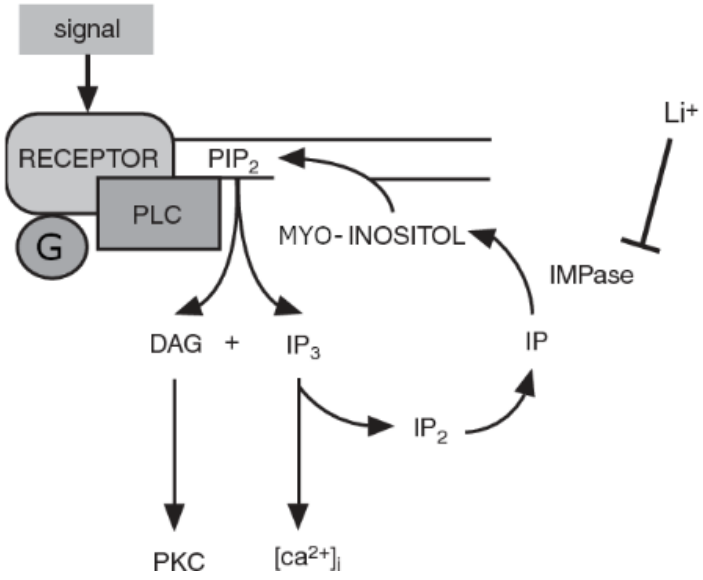


Figure 1: The phosphatidylinositol signalling pathway that is exploited in the inositol phosphate accumulation assay for the purpose of evaluating tachykinin receptor activity.[16]

Tachykinins are one of the most studied classes of neuropeptides due to their involvement in numerous important physiological processes and diseases such as inflammation,[17] cancer,[18] infection,[4] and chronic pain. We previously reported on the radiosynthesis, optimisation and safe for administration formulation of ^{68}Ga-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid-[Thi^{8},Met(O_2)^{11}]-Substance P ([^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP) as a potential PET/CT tracer for imaging NK1R availability in chronic pain disorders. DOTA-[Thi^{8},Met(O_2)^{11}]SP has been proposed as a more metabolically stable analogue of native SP. [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP and [^{213}Bi]Bi-DOTA-[Thi^{8},Met(O_2)^{11}]SP have, in the last few years, been reported in literature for their use in the imaging and therapy of glioblastoma multiforme with great success.[19,20] The therapeutic effectiveness of [^{213}Bi]Bi-DOTA-[Thi^{8},Met(O_2)^{11}]SP was reduced due to the short half-life of ^{213}Bi. [^{225}Ac]Ac-DOTA-[Thi^{8},Met(O_2)^{11}]SP was proposed as an attractive alternative[21] due to the longer half-life of ^{225}Ac and has been assessed *in vitro* in human glioblastoma cell lines. The results indicated a high affinity of the radiobioconjugate for NK1R on isolated glioblastoma stem cells and cell lines derived from a human glioblastoma tumour.[22,23] However, to our knowledge no investigation into the receptor specificity of this modified SP analogue has been undertaken. Modification of native tachykinins can significantly alter their receptor selectivity profiles[24,25,26,27,28] and thus can have an adverse impact on the target to non-target ratio. Therefore, determining receptor selectivity/target specificity is an important prerequisite to any *in vivo* investigation. In our study, the potency and selectivity of DOTA-[Thi^{8},Met(O_2)^{11}]SP for NK1R was evaluated in HEK 293-T cells (a 'blank' background into which different tachykinin receptors could be introduced) to verify that the amino acid modifications on DOTA-[Thi^{8},Met(O_2)^{11}]SP have not inadvertently altered the receptor specificity of this SP analogue.

2 MATERIAL AND METHODS

2.1 CHEMICALS AND MATERIAL

pcDNA mammalian expression vectors encoding human NK3R, NK2R, NK1R, and MRGPRX2 were obtained from the Bloomsburg University cDNA Resource Center (www.cdna.org). The empty vector pcDNA3.1 was obtained from Invitrogen (Thermo

Fisher Scientific, Waltham, MA, USA). The peptide-based ligands NKA and NKB were purchased from CPC Scientific (Sunnydale, CA, USA), SP from Sigma Aldrich (St Louis, MO, USA) and DOTA-[Thi^{8}, Met(O_2)^{11}]-SP from piCHEM (Raaba-Grambach, Austria). [^{3}H]-myo-inositol was purchased from Perkin Elmer (Waltham, MA, USA).

2.2 CELL PREPARATION

Modified human embryonic kidney cells (HEK 293-T) (which provide a 'blank' background for measurement of GPCR signalling and stably express large T-antigen for optimal expression of exogenous proteins) were utilised. Cells were cultured in a humidified atmosphere at 37 ^{o}C with 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with Glutamax (Thermo Fisher Scientific, Waltham, MA, USA). The media was supplemented with 10% foetal calf serum (FCS) (Biochrome, Berlin, Germany) and cells were sub-cultured every 2-3 days to maintain cell density of less than 90%.

2.3 INOSITOL PHOSPHATE ACCUMULATION ASSAY

Cells were seeded at a density of 1x10^{5} cells/well in 24 well tissue culture plates coated with Matrigel, Growth Factor Reduced Matrix (BD Biosciences, San Jose, CA, USA) at a 1:30 dilution (to aid cell attachment). Post-seeding (24 h), cells were transfected with receptor cDNA (0.5 µg/well) using X-treme GENE HP transfection reagent (Sigma Aldrich, St Louis, MO, USA) at a 1:2 ratio. The media was aspirated 24 h after transfection and replaced with reduced inositol Media 99 (Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 1% FCS and 0.5 µCi/well [^{3}H]myo-inositol. Cells were then incubated overnight at 37 °C before aspiration of media and addition of Buffer I (DMEM supplemented with 20 mM HEPES, 10 mM LiCl and 0.1 % Bovine Serum Albumin). Plates were then incubated for 30 min at 37 °C before aspiration and stimulation with appropriate ligands (refer to Section 2.3.1-2.3.3 for details) prepared in Buffer. Following stimulation, cells were lysed by addition of 10 mM formic acid and incubated at 4 °C for 60 min. Cell lysates were transferred to tubes containing 100-200 mesh 1X8 Dowex beads (Sigma, St Louis, MO, USA) and were incubated for 5 min before washing twice with water and twice with wash buffer (60 mM ammonium formate / 5 mM sodium tetraborate). Samples were then eluted from the beads in 1 ml elution buffer (1 M ammonium formate/0.1 M formic acid) and

2.5 ml Optiphase Hisafe3-scintillation fluid (Perkin Elmer, Waltham, MA, USA) was added. The radioactivity (decays per minute; dpm) of the eluate was measured by liquid scintillation counting using a Packard Tri-Carb 4810TR liquid scintillation analyser with QuantaSmart software (Perkin Elmer, Waltham, MA, USA).

2.3.1 Activation of tachykinin receptors by DOTA-[Thi^{8},Met(O$_2$)^{11}]SP

Cells transfected with human NK1R, NK2R, NK3R, or MRGPRX2 were stimulated for 1 h at 37 ^{o}C in the presence of vehicle (0.1% dimethyl sulphoxide [DMSO]), 1 µM endogenous ligand (NK1R and MRGPRX2, SP; NK2R, NKA and NK3R, NKB) or 1 µM DOTA-[Thi^{8}, Met(O$_2$)^{11}]-SP (test compound) prior to measurement of [^{3}H]-inositol phosphate (IP) accumulation.

2.3.2 Dose response analysis

Cells expressing human NK1, NK2, NK3, or MRGPRX2 receptors were stimulated for 1 h at 37 ^{o}C in the presence of vehicle (0.1% DMSO) or a range of concentrations of DOTA-[Thi^{8},Met(O$_2$)^{11}]SP (test compound) or endogenous ligand (NK1R and MRGPRX2, SP; NK2R, NKA and NK3R, NKB) prior to measurement of [^{3}H]- IP accumulation.

2.3.3 Antagonism of tachykinin receptors by DOTA-[Thi^{8},Met(O$_2$)^{11}]SP

Cells expressing human NK2, NK2, or MRGPRX2 receptors were stimulated for 30 min at 37 ^{o}C in the presence of vehicle (0.1% DMSO), 1 µM DOTA-[Thi^{8},Met(O$_2$)^{11}]SP (test compound) or 1 µM talnetant (a validated peptide antagonist of the NK3R) prior to stimulation for 1 h at 37 ^{o}C with endogenous ligands at close to their EC$_{50}$ concentration (MRGPRX2, 10 µM SP; NK2R, 10 nM NKA and NK3R, 30 nM NKB) prior to measurement of [^{3}H]-IP accumulation.

2.4 DATA ANALYSIS

Data was analysed using GraphPad Prism Version 5 (La Jolla, CA, USA). Where a range of doses were applied (dose-response analyses), three-parameter sigmoidal dose response curves were generated and were used to calculate values for potency (EC$_{50}$) and maximal response (E$_{max}$) for each ligand. Data are mean ± SEM from at least three independent experiments unless otherwise stated. Statistical comparisons

were made using one-way ANOVA or Student's t-test, as appropriate, with $p < 0.05$ considered significant.

3 RESULTS AND DISCUSSION

To determine agonist activity at each of the receptors, HEK 293-T cells expressing human NK1R, NK2R, NK3R, or MEGPRX2 were stimulated for 1 h in the presence of vehicle, endogenous ligand (SP for NK1R and MRGPRX2; NKA for NK2R, and NKB for NK3R) or DOTA-[Thi^8,Met(O$_2$)^{11}]SP (test compound). Cells transfected with empty vector served as a negative control to show that any responses in receptor transfected cells are selective for that receptor. DOTA-[Thi^8,Met(O$_2$)^{11}]SP induced high levels of stimulation of the NK1R, similar to SP (**Figure 2**).

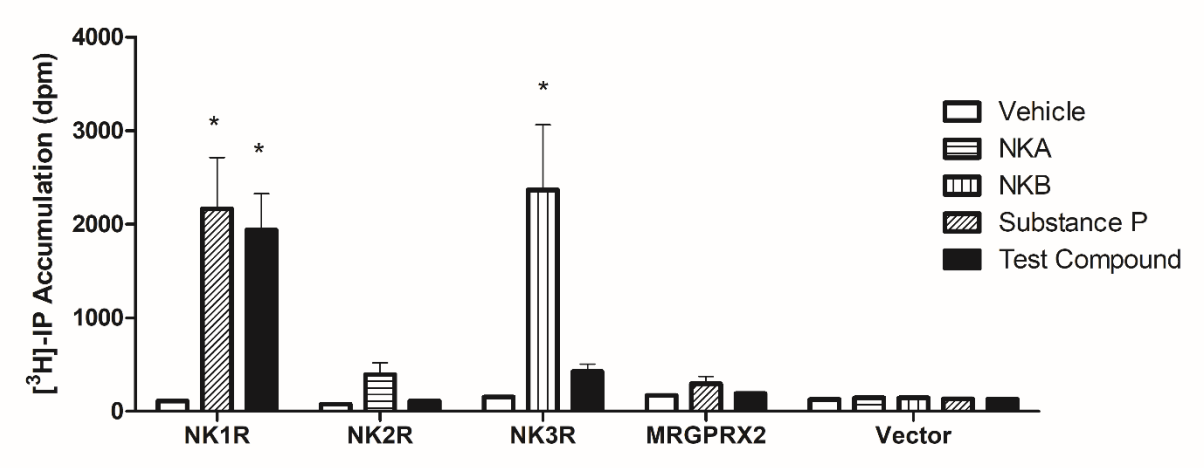


Figure 2: Activation of NK1R, NK2R, NK3R, and MRGPRX2 by known agonists and DOTA-[Thi^8,Met(O$_2$)^{11}]SP (test compound). Data are mean ± SEM from three independent experiments (n = 3).* $p < 0.05$ (one-way-ANOVA followed by Dunnett's post-hoc test) for comparison with vehicle control at the same receptor.

No agonist activity of DOTA-[Thi^8,Met(O$_2$)^{11}]SP was observed at NK2R and MRGPRX2, however there was a limited stimulation of NK3R, though this was found to be statistically insignificant. At the concentration used, endogenous SP stimulation of MRGPRX2 was also not observed. High concentrations of the ligands were used and no activity of DOTA-[Thi^8,Met(O$_2$)^{11}]SP was seen at receptors other than NK1R.

That does not exclude the possibility that at higher concentrations a different result could be obtained. Higher ligand concentrations were not evaluated because they are considered physiologically irrelevant and could be toxic in the biological system. The low-level response of NK2R to its endogenous ligand compared to the response of NK1R and NK3R to their respective endogenous ligands was not expected. However, this response was consistently obtained through all the repeat data sets and could be due to the receptor being poorly coupled to its downstream signalling partners in comparison to the other receptors in this cell model.

Dose response analyses of DOTA-[Thi^{8},Met(O$_2$)^{11}]SP and SP at the NK1R were then conducted for comparative purposes (**Figure 3**).

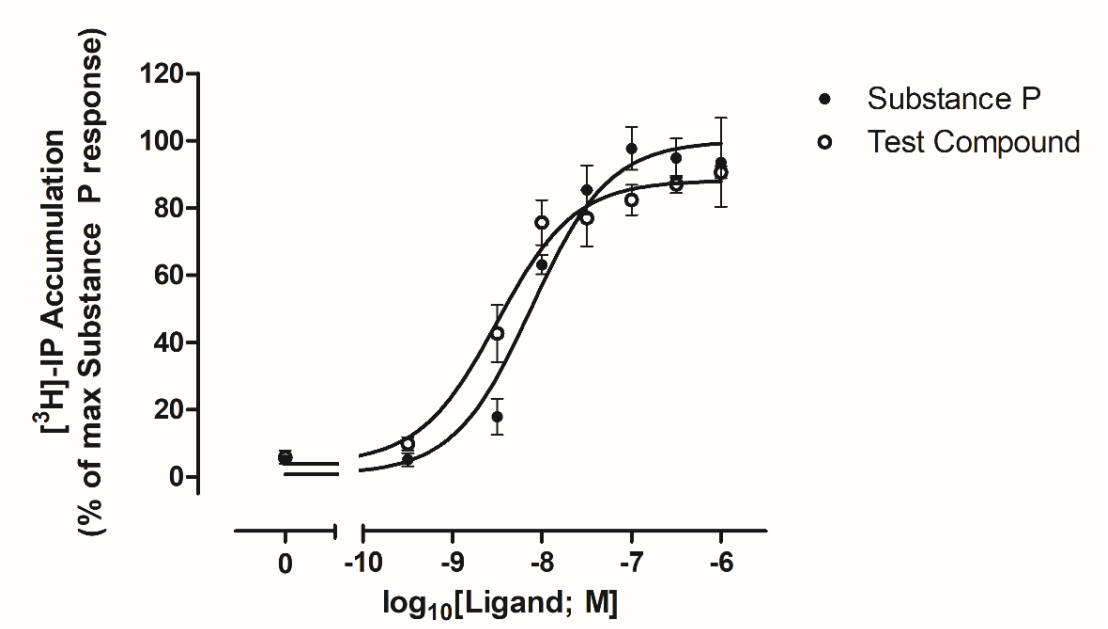


Figure 3: Dose-response analysis of NK1R activation by SP (reference) and DOTA-[Thi^{8},Met(O$_2$)^{11}]SP (test compound). Data (mean ± SEM, n = 3 independent experiments) is presented as % of the average maximal response obtained upon stimulation with SP.

DOTA-[Thi^{8},Met(O$_2$)^{11}]SP and SP activate NK1R similarly. There was no major difference in the level of response induced (as indicated by E_{max} values) nor the potency of the response (as reflected by the EC_{50} values) (**Table 1**) measured for the two ligands ($p > 0.05$; Student's t-test).

Table 1: Dose response parameters for stimulation of cells expressing NK1R with SP and DOTA-[Thi^{8},Met(O$_2$)^{11}]SP.

Ligand	pEC$_{50}$ (EC$_{50}$)	E$_{max}$ (% of maximum SP response)
SP	8.13 ± 0.07 (7 nM)	100 ± 8
DOTA-[Thi^{8},Met(O$_2$)^{11}]SP	8.48 ± 0.17 (3 nM)	89 ± 1

Due to the low response induced by the test compound at NK3R, it was not possible to conduct dose response analyses to compare the action of DOTA-[Thi^{8},Met(O$_2$)^{11}]SP to that of SP at NK3R. However, when the level of response evoked by a high concentration of DOTA-[Thi^{8},Met(O$_2$)^{11}]SP in cells expressing this receptor was compared to that elicited by SP, it was observed that the test compound was only able to elicit 23% of the level of stimulation of NK3R as induced by NKB. SP, at the same concentration, elicited 77% of the NKB response (**Figure 4**). These results suggest that the test compound acts in a very similar way to SP in cells transfected with NK1R but has increased selectivity for this receptor compared to the native SP ligand.

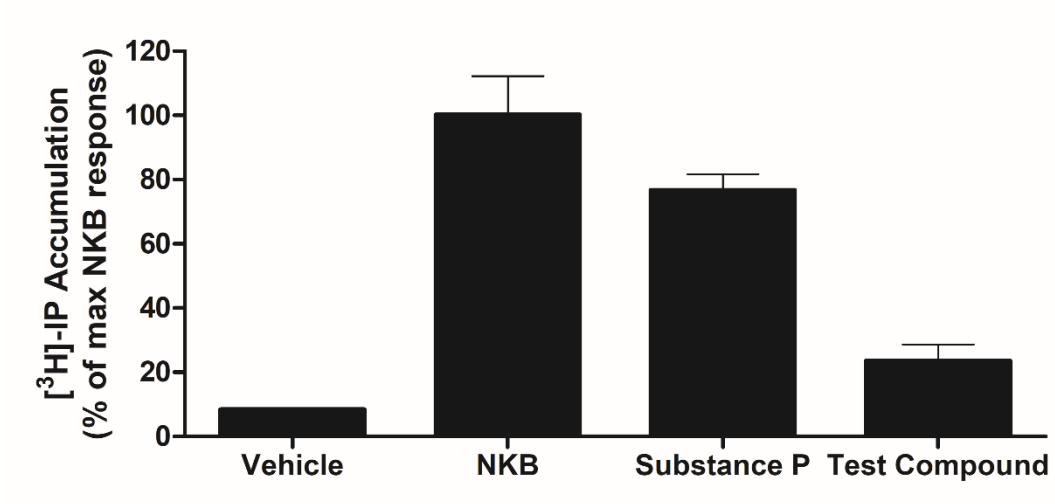


Figure 4: Activation of NK3R by SP and DOTA-[Thi^{8},Met(O$_2$)^{11}]SP (test compound). Data (mean ± SEM, n = 3 independent experiments) is presented as % of the average maximal response obtained upon stimulation with NKB.

Despite DOTA-[Thi^{8},Met(O$_2$)^{11}]SPs inability to induce a significant activation of NK2R, NK3R, and MRGPRX2, it is possible that this compound is still able to bind to these receptors and thus may act as an antagonist and inhibit the activity of the endogenous

ligands at these receptors. Antagonist activity of DOTA-[Thi^{8},Met(O_2)^{11}]SP was therefore examined. As a prerequisite, dose response analyses measuring activation of these receptors by their endogenous ligands were conducted so that the EC_{50} for each compound in this assay could be determined (**Figure 5, Table 2**).

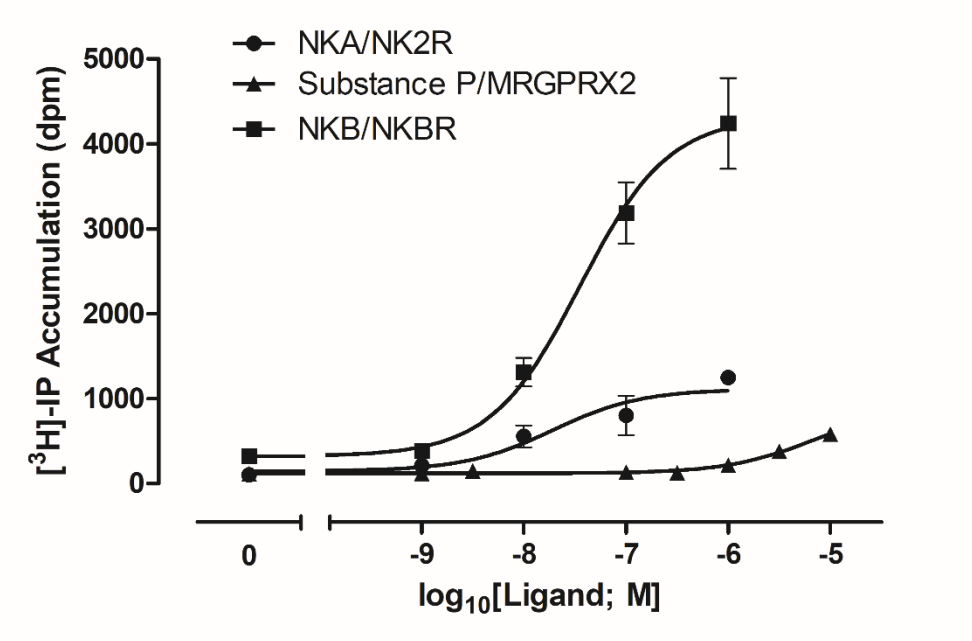


Figure 5: Dose-response analysis of NK2R, NK3R, and MRGPRX1 activation by endogenous ligands. Data are mean ± SEM from at least three independent experiments (NK2R and NK3R) or are from a single experiment (MRGPRX2).

At 1 µM of SP, no response was observed at MRGPRX2 (**Figure 5**). The concentration was increased to 10 µM for the dose response analysis therefore only one experiment at this higher concentration was conducted.

Table 2: Dose response parameters for stimulation of cells expressing NK2R, NK3R and MRGPRX2 with endogenous ligands.

Ligand/Receptor	**pEC_{50} (EC_{50})**
NKA/NK2R	8.15 ± 0.15 (7 nM)
NKB/NK3R	7.56 ± 0.12 (27 nM)
SP/MRGPRX2	5.16 (6918 nM)[1]

[1]*Plateaux not achieved, therefore EC50 is only an estimate*.

To screen for antagonist activity of the test compound at NK2R, NK2R, and MRGPRX2, cells expressing each receptor were stimulated with their endogenous ligands (at approximately their EC_{50} concentration) in the presence or absence of a high concentration (1 µM) of the test compound. Talnetant (1 µM), an existing peptide antagonist of NK3R, was utilised as a positive control (**Figure 6**).

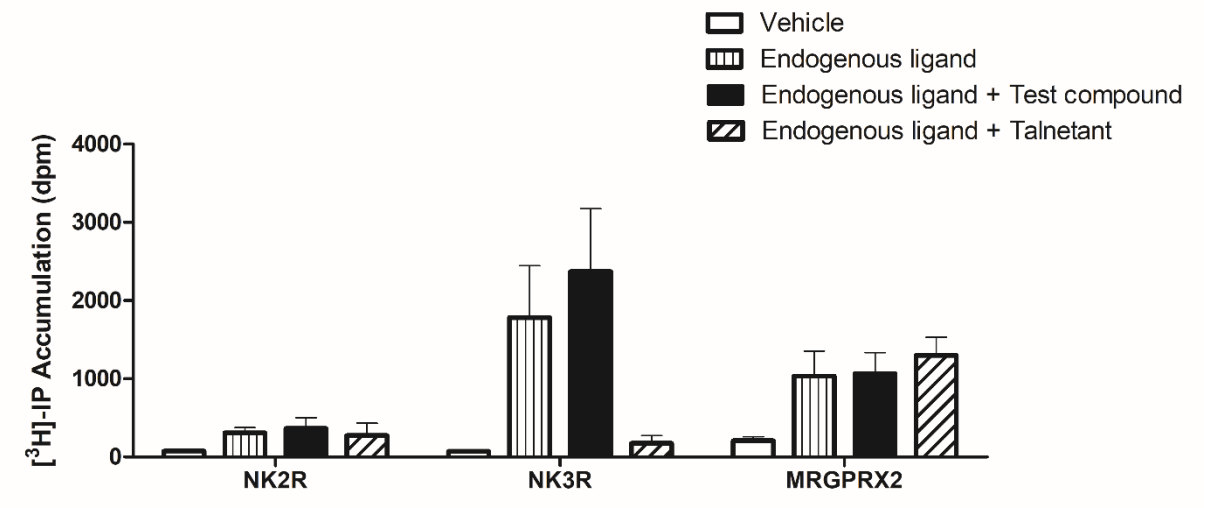


Figure 6: The effect of DOTA-[Thi^{8},Met(O_2)^{11}]SP (test compound) on activation of NK2R, NK3R, and MRGPRX2 by their endogenous ligands. Data are mean ± SEM from four independent experiments. Addition of 1 µM talnetant in place of the test compound in cells expressing NK3R served as a positive control in two experiments (therefore mean ± range).

Addition of DOTA-[Thi^{8},Met(O_2)^{11}]SP had no effect on the level of stimulation of NK2R, NK3R, or MRGPRX2 elicited by stimulation with EC_{50} concentrations of their endogenous ligands. However, Talnetant, as the positive control, induced a large reduction in NKB response at NK3R confirming that the assay was in fact appropriate for detection of antagonism. Therefore, these results indicate that the test compound does not have antagonist activity at any of the receptors.

4 CONCLUSION

[^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP is a prospective PET/CT imaging agent intended to target NK1R availability associated with chronic pain disorders. DOTA-[Thi^{8},Met(O_2)^{11}]SP is an analogue of SP with modifications to the amino acid residues in positions 8 and 11. It has previously been shown to have a longer half-life *in vivo*

than SP,[29] which is a desirable feature of a biomarker for radiopharmaceutical application. Substituting the Phe^{8} (phenylalanine) for Thi^{8} (3-thienylalanine) and modification of Met^{11} for Met(O$_2$)^{11} resulted in a reduction of lipophilicity, a property that is critical to the *in vivo* behaviour of a drug. Higher lipophilicity doesn't always correlate with improved *in vivo* behaviour. Recently synthesised modified SP fragments and derivatives, SP(5-11), SP(4-11), and [Thi^{8}, Met(O$_2$)^{11}]-SP(5-11) that were lower in molecular weight possessed a higher lipophilicity but, despite their satisfactory affinity for NK1R, the shorter SP fragments were found to be less stable in human serum due to fast enzymatic degradation of the ligand. This infers that a reasonable balance between the various factors that affect the *in vivo* behaviour of the compound, must be achieved.[30]

Literature reports that the C-terminal motif of SP, Phe^{7}–Phe^{8}–Gly^{9}–Leu^{10}–Met^{11}, is responsible for the peptides affinity for NK1R[31,32,33] whilst the N-terminal tail determines selectivity for the specific receptor subtypes,[34] and that modifications to the peptide sequence, while beneficial in respect to some ligand properties, can have large effects on tachykinin receptor selectivity of the resultant analogues.[24,25,26] It is therefore of great importance to validate the receptor-interaction profile of SP analogues.

Tachykinin receptor signalling studies with ligands NKA, NKB, SP, and modified SP (DOTA-[Thi^{8},Met(O$_2$)^{11}]SP) were carried out using an inositol phosphate accumulation assay. Results from the *in vitro* tachykinin measurements found that DOTA-[Thi^{8},Met(O$_2$)^{11}]SP has a similar efficacy and greater selectivity for NK1R compared to SP. This indicates that the SP alterations made to the amino acids in positions 8 and 11 together with the N-terminal conjugation of DOTA did not compromise the ligands affinity for NK1R or its ability to bind effectively to NK1R. Indeed, it appeared that this analogue had an improved receptor selectivity profile in comparison to native SP, which should translate to a better target to off-target ratio in [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP.

5 ACKNOWLEDGEMENTS

The authors would like to express their gratitude to the Centre of Neuroendocrinology, University of Pretoria for their contribution towards this study and the Technology Innovation Agency for funding.

6 CONFLICT OF INTEREST

The authors report no conflict of interest.

7 REFERENCES

1. Von Euler US, Gaddum JH. An unidentified depressor Substance in certain tissue extracts. J Physiol [Internet]. 1931 [cited 2019 Sep 3];72(1):74-87. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1403098/ doi:10.1113/jphysiol.1931.sp002763

2. Suvas S. Role of Substance P neuropeptide in inflammation, wound healing, and tissue homeostasis. J Immunol [Internet]. 2017 Sep [cited 2019 Dec 1];199(5):1543-52. Available from: https://doi.org/10.4049/jimmunol.1601751

3. Erin N, Ulusoy O. Differentiation of neuronal from non-neuronal Substance P. Regul Pept [Internet]. 2009 [cited 2019 Dec 1];152(1-3):108-13. Available from: https://www.sciencedirect.com/science/article/abs/pii/S0167011508001729?via%3Dihub doi: 10.1016/j.regpep.2008.10.006

4. Steinhoff MS, von Mentzer B, Geppetti P, Pothoulakis C, Bunnett NW. Tachykinins and their receptors: contributions to physiological control and the mechanisms of disease. Physiol Rev [Internet]. 2014 Jan [cited 2019 Sep 30];94(1):265–301. Available from: https://doi.org/10.1152/physrev.00031.2013

5. Harrison S, Geppetti P. Substance P. Int J Biochem Cell Bio [Internet]. 2001 [cited 2019 Sep 16];33(6):555-76. Available from:

https://www.sciencedirect.com/science/article/abs/pii/S1357272501000310?via%3Di
hub doi: 10.1016/s1357-2725(01)00031-0

6. McNeil, BD, Pundir P, Meeker S, Han L, Undem BJ, Kulka M, et al. Identification of a mast-cell-specific receptor crucial for pseudo-allergic drug reactions. Nature [Internet]. 2015 [cited 2019 Nov 23];519(7542):237-41. Available from: https://www.nature.com/articles/nature14022 doi: 10.1038/nature14022

7. Vilardaga J-P, Agnati LF, Fuxe K, Ciruela F. G-protein-coupled receptor heteromer dynamics. J Cell Sci [Internet]. 2010 [cited 2019 Nov 26];123(24):4215-20. Available from: https://jcs.biologists.org/content/123/24/4215
doi: 10.1242/jcs.063354

8. Hanson, MA, Stevens RC. Discovery of new GPCR biology: one receptor structure at a time. Structure [Internet]. 2009 Jan [cited 2019 Nov 27];17(1):8-14. Available from: https://doi.org/10.1016/j.str.2008.12.003

9. Hopkins B, Powell SJ, Danks P, Briggs I, Graham A. Isolation and characterization of the human lung NK1 receptor cDNA. Biochem Biophys Res Commun [Internet]. 1992 [cited 2019 Oct 1];182(3):1514. Available from: https://www.sciencedirect.com/science/article/pii/0006291X92919056?via%3Dihub doi: 10.1016/0006-291x(92)91905-6

10. Rosenbaum DM, Zhang C, Lyons JA, Holl R, Aragao D, Arlow DH, et al. Structure and function of an irreversible agonist-β 2 adrenoceptor complex. Nature [Internet]. 2011 [cited 2019 Nov 24];469(7329):236-40. Available from: https://www.nature.com/articles/nature09665 doi: 10.1038/nature09665

11. Rasmussen SG, Choi HJ, Fung JJ, Pardon E, Casarosa P, Chae PS, et al. Structure of a nanobody-stabilized active state of the β 2 adrenoceptor. Nature [Internet]. 2011 [cited 2019 Nov 17];469(7329):175-80. Available from: https://www.nature.com/articles/nature09648 doi: 10.1038/nature09648

12. Rasmussen SGF, DeVree BT, Zou Y, Kruse AC, Chung KY, Kobilka TS, et al. Crystal structure of the β 2 adrenergic receptor–Gs protein complex. Nature [Internet]. 2011 [cited 2019 Nov 27];477(7366):549-55. Available from: https://www.nature.com/articles/nature10361 doi: 10.1038/nature10361

13. Gilman, A. G. G proteins: transducers of receptor-generated signals. Annu Rev Biochem [Internet]. 1987 [cited 2019 Nov 11];56(1):615-49. Available from: https://www.annualreviews.org/doi/abs/10.1146/annurev.bi.56.070187.003151 doi: 10.1146/annurev.bi.56.070187.003151

14. Oldham WM, Hamm HE. Heterotrimeric G protein activation by G-protein-coupled receptors. Nat Rev Mol Cell Biol [Internet]. 2008 [cited 2019 Nov 24];9(1):60-71. Available from: https://www.nature.com/articles/nrm2299 doi: 10.1038/nrm2299

15. Lodish H, Berk A, Zipursky SL, Matsudaira P, Baltimore D, Darnell J. (2000). Molecular cell biology. 4th ed. New York: WH Freeman and Company; 2000.

16. Phiel CJ, Klein PS. Molecular targets of lithium action. Annu Rev Pharmacol Toxicol [Internet]. 2001 [cited 2019 Dec 2]41(1):789-813. Available from: https://www.annualreviews.org/doi/abs/10.1146/annurev.pharmtox.41.1.789?rfr_dat=cr_pub%3Dpubmed&url_ver=Z39.88-2003&rfr_id=ori%3Arid%3Acrossref.org&journalCode=pharmtox doi: 10.1146/annurev.pharmtox.41.1.789

17. Canning BJ. Potential role of tachykinins in inflammatory diseases. J Allergy Clin Immunol [Internet]. 1997 May [cited 2019 Nov 11];99(5):579-82. Available from: https://doi.org/10.1016/S0091-6749(97)70015-0

18. Palma C. Tachykinins and their receptors in human malignancies. Curr Drug Targets [Internet]. 2006 [cited 2019 Nov 20];7(8):1043-52. Available from: http://www.eurekaselect.com/56449/article doi: 10.2174/138945006778019282

19. Cordier D, Forrer F, Bruchertseifer F, Morgenstern A, Apostolidis C, Good S, et al. Targeted alpha-radionuclide therapy of functionally critically located gliomas with ^{213}Bi-DOTA-[Thi^{8}, Met(O_2)^{11}]-Substance P: a pilot trial. Eur J Nucl Med Mol Imaging [Internet]. 2010 [cited 2019 Nov 11];37(7):1335-44. Available from: https://link.springer.com/article/10.1007%2Fs00259-010-1385-5 doi: 10.1007/s00259-010-1385-5

20. Krolicki L, Bruchertseifer F, Kunikowska J, Koziara H, Królicki B, Jakuciński M, et al. Prolonged survival in secondary glioblastoma following local injection of targeted alpha therapy with ^{213}Bi-Substance P analogue. Eur J Nucl Med Mol Imaging [Internet]. 2018 Jul [cited 2019 Nov 12];45(9):1636-44. Available from: https://doi.org/10.1007/s00259-018-4015-2

21. Krolicki L, Bruchertseifer F, Morgenstern A, Kunikowska J, Koziara H, Krolicki B, et al. Safety and therapeutic efficacy of ^{225}Ac-DOTA-SubstanceP for therapy of brain tumors. JMIRS [Internet]. 2019 Mar [cited 2019 Dec 2];50:S22. Available from: https://doi.org/10.1016/j.jmir.2019.03.070

22. Majkowska-Pilip A, Rius M, Bruchertseifer F, Apostolidis C, Weis M, Bonelli M, et al. *In vitro* evaluation of ^{225}Ac-DOTA-Substance P for targeted alpha therapy of glioblastoma multiforme. Chem Biol Drug Des [Internet]. 2018 [cited 2019 Dec 2];92(1):1344-56. Available from: https://onlinelibrary.wiley.com/doi/abs/10.1111/cbdd.13199 doi: 10.1111/cbdd.13199

23. Song H, Guerrero-Cazares H, Horti A, Wahl RL, Quinones-Hinojosa A, Sgouros G. Synthesis and biodistribution of ^{225}Ac-substance P for intracavitary radiopharmaceutical therapy of high-grade recurrent glioma. In: Cancer Research, Proceedings of the AACR 104th annual meeting; 2013 Apr 6-10; Washington DC, USA. Philadelphia, USA: AACR:2013. Volume 73. p.4533.

24. Tousignant C, Guillemette G, Regoli D. Binding sites for [^{3}H][Sar9, Met(O_2)11]substance P in rat brain and guinea pig ileum. Brain Res [Internet]. 1991 [cited 2019 Dec 4];560(1-2):1-11. Available from:

https://www.sciencedirect.com/science/article/abs/pii/000689939191207H?via%3Dihub doi: 10.1016/0006-8993(91)91207-h

25. Clerc FF, Foucault F, Ridoux-Silly L, Fardin V. Affinity and stability of some Substance P agonists in rat and guinea-pig: A comparative study in central and peripheral tissues. Neuropeptides [Internet]. 1992 [cited 2019 Dec 4];22(1):14. Available from: https://www.sciencedirect.com/science/article/abs/pii/0143417992903757?via%3Dihub doi: https://doi.org/10.1016/0143-4179(92)90375-7

26. Wormser U, Laufer R, Hart Y, Chorev M, Gilon C, Selinger Z. Highly selective agonists for Substance P receptor subtypes. EMBO J [Internet]. 1986 Nov [cited 2019 Dec 1];5(11): 2805-8. Available from: https://doi.org/10.1002/j.1460-2075.1986.tb04571.x

27. Maggi CA. The mammalian tachykinin receptors. Gen Pharmac [Internet]. 1995 [cited 2019 Dec 4];26(5):911-44. Available from: https://www.sciencedirect.com/science/article/pii/030636239400292U?via%3Dihub doi: https://doi.org/10.1016/0306-3623(94)00292-U

28. Petitet F, Beaujouan JC, Saffroy M, Torrens Y, Chassaing G, Lavielle S, et al. Further demonstration that [Pro9]-Substance P is a potent and selective ligand of NK-1 tachykinin receptors. J Neurochem [Internet]. 1991 Mar [cited 2019 Dec 4];56(3):879-89. Available from: https://doi.org/10.1111/j.1471-4159.1991.tb02004.x

29. Majkowska-Pilip A, Halik PK, Gniazdowska E. The significance of NK1 receptor ligands and their application in targeted radionuclide tumour therapy. Pharmaceutics [Internet]. 2019 Sep [cited 2019 Dec 2];11(443):1-28. Available from: https://doi.org/10.3390/pharmaceutics11090443

30. Charron CL, Hickey JL, Nsiama TK, Cruickshank DR, Turnbull WL, Luyt LG. Molecular imaging probes derived from natural peptides. Nat Prod Rep [Internet]. 2015 [cited 2019 Dec 4];33(6):761-800. Available from:

https://pubs.rsc.org/en/content/articlelanding/2016/NP/C5NP00083A#!divAbstract doi: 10.1039/c5np00083a

31. Grady EF, Garland AM, Gamp PD, Lovett M, Payan DG, Bunnett NW. Delineation of the endocytic pathway of Substance P and its seven-transmembrane domain NK1 receptor. Mol Biol Cell [Internet]. 1995 May [cited 2019 Dec 2];6(5):509–24. Available from: https://doi.org/10.1091/mbc.6.5.509

32. Graham GJ, Stevens JM, Page NM, Grant A.D, Brain SD, Lowry PJ, et al. Tachykinins regulate the function of platelets. Blood [Internet]. 2004 Aug [cited 2019 Dec 2];104(4):1058–65. Available from: https://doi.org/10.1182/blood-2003-11-3979

33. Ho WZ, Douglas SD. Substance P and neurokinin-1 receptor modulation of HIV. J Neuroimmunol [Internet]. 2004 [cited 2019 Dec 2];157(1-2):48–55. Available from: https://www.jni-journal.com/article/S0165-5728(04)00332-7/fulltext doi: 10.1016/j.jneuroim.2004.08.022

34. Valentin-Hansen L, Park M, Huber T, Grunbeck A, Naganathan S, Schwartz TW, et al. Mapping Substance P binding sites on the neurokinin-1 receptor using genetic incorporation of a photoreactive amino acid. J Biol Chem [Internet]. 2014 [cited 2019 Dec 2];289(26):18045-54. Available from: http://www.jbc.org/content/early/2014/05/15/jbc.M113.527085 doi: 10.1074/jbc.M113.527085

PAPER 4

MANUSCRIPT FOR SUBMISSION TO THE JOURNAL

JOURNAL OF NUCLEAR MEDICINE

TRACER BIODISTRIBUTION, PHARMACOKINETIC AND TARGETED *IN VIVO* IMAGING OF CHRONIC PAIN DISORDERS IN DOGS USING GALLIUM-68 RADIOLABELED DOTA-[THI^{8}, MET(O_2)^{11}]-SUBSTANCE P-PET/CT

Janine Suthiram[1,2], Mike M. Sathekge[3], Jan Rijn Zeevaart[1,4], Thomas Ebenhan[3,5]

[1] *Radiochemistry, The South African Nuclear Energy Corporation (Necsa), Pelindaba, Pretoria, South Africa*

[2] *University of Pretoria, Department of Nuclear Medicine, Pretoria, South Africa*

[3] *University of Pretoria, Nuclear Medicine & Steve Biko Academic Hospital, Pretoria, South Africa*

[4] *Department of Science and Technology, Preclinical Drug Development Platform, North West University, Potchefstroom, North West Province, South Africa*

[5] *Nuclear Medicine Research Infrastructure/NPC, Preclinical Imaging Facility, Pretoria, South Africa*

For correspondence: Janine.Suthiram@necsa.co.za

Keywords ^{68}Ga, ^{68}Ge/^{68}Ga generator, PET/CT-imaging, peptide radiolabelling, pre-clinical, canine model, chronic pain, osteoarthritis, SUV, pain loci

ABSTRACT

The effective diagnosis and subsequent treatment of chronic pain disorders has been hampered over the years by non-specific diagnostic tools, subjective patient feedback and a lack of understanding of the underlying mechanism that gives rise to persistent pain. The relationship between Substance P (SP) and the widespread neurokinin-1 receptor (NK1R) has long been studied for its involvement in a number of different

physiological processes, including inflammation and chronic pain. Initial pre-clinical findings of blockade of NK1Rs using SP-based antagonists in rodent models did not translate successfully to a valuable application in humans. Visualisation of the NK1R expression in both healthy and diseased states could contribute to an improved understanding of the SP/NK1R pathway. [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP was evaluated as a prospective PET/CT radiotracer to assess NK1R availability in pain loci. The optimised, safe-to-administer formulation of [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP yielded injectable doses with suitable specific activity. Image-guided biodistribution of [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP was carried out in an outbreed canine model where the diseased cohort consisted of dogs with suspected osteoarthritis, characterised by chronic pain symptoms. Pharmacokinetics and biodistribution were favourable demonstrating appropriate blood pool clearance of the tracer and renal excretion. Elevated unilateral uptake was demonstrated in numerous joint and osseous tissue, synonymous with osteoarthritis.

1 INTRODUCTION

Chronic pain originating from an underlying musculoskeletal disorder is becoming an increasingly common medical diagnosis with a high estimated economic impact world-wide due to loss of productivity.[1] According to the World Health Organization, the prevalence of this class of diseases is on the rise in both the developed and developing world.[2] An estimated 33% of people, across the globe, live with a musculoskeletal disorder[3] that can affect muscle, bones and joints in all main areas of the body. The clinical challenge associated with chronic pain is the inability to accurately diagnose and localise pain generators in the biological system as the current assessment protocol is based on the patients' qualitative description and relatively non-specific diagnostic tools.[4] From a therapy perspective, the major challenges that negatively impact the development of effective chronic pain treatment is the lack of understanding of the mechanisms that convert acute tissue insult to chronic pain. The underlying pathology of the disease processes and the mechanisms available as treatment targets are not apparent.[5]

The role of Substance P (SP) in the processes of inflammation and chronic pain has made the SP/Neurokinin-1 receptor (NK1R) interaction the most investigated neurotransmitter pathway in the central nervous system.[6] SP, an 11-amino acid

tachykinin peptide, is the preferential endogenous ligand for the G-protein-coupled NK1R[7,8] that is widely expressed in both the central and peripheral nervous systems.[9] Over the last few decades, studies have alluded to the involvement of SP and NK1R in a number of different biological processes including cancer cell proliferation and angiogenesis. In relation to musculoskeletal disorders, it has been reported based on histological investigations that chronically painful tendon tissue has an elevated level of SP and NK1R.[10] In the 90s, blockade studies of NK1R were carried out in pre-clinical models[11,12,13,14] and the proposed SP-based NK1R antagonists demonstrated anti-nociceptive abilities with the potential to reduce chronic pain. Despite observing an analgesic effect in animals, a similar inhibition of nociception could not be translated to patients in clinical studies.[15,16]

Various neuroimaging modalities have been used to study pain processes, including osteoarthritis,[17] fibromyalgia,[18] chronic low back pain,[19] and chronic migraine.[20] Several cortical regions that are involved in the perception of pain were identified in humans. Chronic pain studies further revealed that there is altered structure, function, and neurochemistry in the frontal-limbic-brainstem regions.[21] Positron emission tomography/computed tomography (PET/CT) is a non-invasive molecular imaging tool that can detect molecular and biochemical pathologies often earlier than other conventional diagnostic methods.[22] Biomarkers such as SP are peptides that are naturally occurring in the human body and are therefore logical choices as potential targeting vectors in the PET tracer. There are relatively few studies in nuclear medicine that have focused on imaging of NK1R expression and of these investigations most studies have been aimed at receptor expression in the central nervous system or the effect of a proposed NK1 antagonist. Recent advances in imaging technology and radiotracer design has enabled clinicians to identify pro-nociceptive tissues involved in pain generation.[4] The development of a highly selective Sigma-1 receptor (S1R) PET agent, [^{18}F]F-6-(3-fluoropropyl)-3-(2-azepan-1-yl)ethyl)benzo[*d*]thiazol-2(3H)-one ([^{18}F]FFTC-146)[23] that targets a protein that is widely distributed in the central nervous system and peripheral tissues and has been linked to various neurological conditions,[24] including pain modulation. First-in-human studies carried out in ten healthy volunteers using PET/MRI demonstrated high uptake in S1R dense organs such as the pancreas and spleen, moderate uptake in the brain and myocardium and

only low uptake in bone and muscle. This renewed interest in the imaging of pain mechanisms highlights the value of evaluating NK1 expression on non-neural and tissue cells in an effort to gain a better understanding of the Substance P/NK1R pathway. The information obtained could contribute to improved diagnostic ability and more sound inputs to development of treatment for chronic pain disorders.

We previously reported on the radiosynthesis and the "safe-for-administration" formulation of the potential radiotracer, gallium-68-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid-[Thi^{8}, Met(O$_2$)^{11}]-Substance P ([^{68}Ga]Ga-DOTA-[Thi^{8}, Met(O$_2$)^{11}]SP). Even though DOTA-[Thi^{8},Met(O$_2$)^{11}]SP is a modified analogue of SP, evaluation of *in vitro* tachykinin activity indicated that the peptide could still activate NK1R and in fact possessed a higher selectivity for NK1R when compared to native SP. The PET isotope, ^{68}Ga (short half-life of 68 min) is commercially available from a ^{68}Ge/^{68}Ga generator[25,26] providing direct on-site access without the need of a cyclotron, to more than one usable eluate per day. This reduced cost and provided convenience together with the ability of the ^{68}Ga^{3+} cation to complex a wide variety of ligands makes ^{68}Ga an attractive option for radiopharmaceutical development. The relatively short 68 min half-life of the radioisotope matches the pharmacokinetics of many peptides and other small molecules affording improved dosimetry and repeat imaging.[27] In general, ^{68}Ga based radiopharmaceuticals must have rapid blood clearance, quick diffusion, and effective target localization.[28]

[^{68}Ga]Ga-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP co-administered with [^{213}Bi]Bi-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP has been proposed as a theranostic pair for the imaging and treatment of glioblastoma *multiforme* and is currently undergoing clinical trials.[29,30] [^{177}Lu]Lu-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP has also been previously evaluated in nude mice for pancreatic tumour cell uptake.[31] However, to our knowledge imaging of NK1R in disease states associated with chronic pain using a ^{68}Ga-based PET imaging agent has never been reported. In this study we assess the tracer biodistribution, pharmacokinetic and targeted *in vivo* PET/CT imaging of [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP for the assessment of NK1R expression in healthy dogs and in dogs with osteoarthritic conditions characterised by chronic pain symptoms. Blood and urine sampling facilitated determination of the pharmacokinetic profile of the radiotracer.

2 MATERIALS AND METHODS

2.1 CHEMICALS AND MATERIALS

Suprapure-grade 30% hydrochloric acid (HCl) and pharmaceutical-grade chemicals including, ethanol and sodium acetate were purchased from Merck & Sigma Aldrich (Johannesburg, South Africa). C18 Sep-Pak® (light) cartridges for solid-phase extraction (SPE) were obtained from Microsep (Johannesburg, South Africa). Millipore Sterile Millex GV filters for final filtration were obtained from Merck (Johannesburg, South Africa). Glass microfiber chromatography paper impregnated with silica gel (ITLC-SG) was purchased from Chemetrix (Johannesburg, South Africa). High-performance liquid chromatography (HPLC) grade water (resistivity = 18.2 MΩ.cm) was produced in-house using a Simplicity 185 Millipore system (Cambridge, Massachusetts, USA). DOTA-[Thi^{8},Met(O_2)^{11}]SP was purchased from piCHEM (Raaba-Grambach, Austria). Freeze-dried aliquots of DOTA-[Thi^{8},Met(O_2)^{11}]SP, generator maintenance and formulation of the sterile injected dose were carried out using pyrogen-free glass vials of varying volumes supplied by NTP Radioisotopes SOC Ltd. (Pelindaba, South Africa).

2.1.1 Animals

Ethical approval was obtained from the Animal Ethics Committee of the University of Pretoria (V089-14 certificates and amendment approval certificates, **Appendix 4**). Outbreed dogs (*Canis domesticus*) from private owners were recruited by veterinarians (Onderstepoort Veterinarian Institute, Pretoria or Pretoria East Vet Clinic & Reproduction Facility, Pretoria). Owners were required to sign an informed consent prior to enrolling their animals in the study. Dogs were screened for general health and against the study's inclusion/exclusion criteria before being categorised as healthy or diseased. Dogs with suspected osteoarthritic conditions characterised by chronic pain and receiving pain treatment or dogs with visible lameness in extremities were enrolled into the diseased cohort (n = 3; two male, one female). Healthy dogs were assessed according to veterinary standards for general health (n = 5; two male, three female).

2.2 PREPARATIONS

2.2.1 DOTA-[Thi^{8},Met(O$_2$)^{11}]SP

Freeze dried aliquots of DOTA-[Thi^{8},Met(O$_2$)^{11}]SP (50 µg) were stored in a freezer until required for radiolabelling. DOTA-[Thi^{8},Met(O$_2$)^{11}]SP must be buffered (2.5 M sodium acetate yielding pH 8) prior to addition of the generator eluate as the peptide could denature under the highly acidic conditions of the eluate. [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP is stable at ambient temperature for up to 150 min.

2.2.2 Animals

On the study day, dogs were transported to the Steve Biko Academic Hospital site and quarantined in an adequate holding area within the department with emphasis on alleviating the stress from the transportation. Anaesthetic administration and catheterisation for blood and urine samples were performed on site. Animals were allowed to recover from anaesthesia in the holding area and only transported once stable. Anaesthesia related chemicals, medical oxygen and catheterising equipment was dispensed from the veterinarian practices and supplied by the treating veterinarians.

2.3 RADIOLABELLING AND FORMULATION OF [^{68}GA]GA-DOTA-[THI^{8},MET(O$_2$)^{11}]SP

^{68}Ga-activity was eluted from a tin-dioxide-based ^{68}Ge/^{68}Ga generator (1.85 GBq, iThemba Labs, South Africa) using HCl (0.6 M, 10 ml) via eluate fractionation (first 1 ml – waste; followed by collection of up to 2 ml ^{68}Ga-eluate for radiolabelling; last 7 ml - waste). DOTA-[Thi^{8},Met(O$_2$)^{11}]SP was radiolabelled directly using an optimised radiolabelling method, an amendment from a previously reported method of Rossouw et al.[32] Briefly, DOTA-[Thi^{8},Met(O$_2$)^{11}]SP was reconstituted with sodium acetate (250 µl, 2.5 M) to yield pH 8. The ^{68}Ga-activity (990 µl) was added to the peptide (solution pH range 3.5-4), vortex mixed and incubated at 95 ˚C for 15 min. Two ITLC methods were performed as quality control (for determination of the percentage radiochemical purity and for the presence of colloidal-^{68}Ga). For radiosyntheses with labelling efficiency < 95%, the crude radiolabelled solution was purified using a pre-conditioned C18 cartridge (4 ml absolute ethanol followed by 2 ml of Millipore

deionised water). Following immobilisation of the ^{68}Ga-labelled peptide solution on the cartridge, 6 ml saline was used as a rinse agent followed by desorption of the product utilising 10, 20, 30 and 40% ethanol/saline solutions (1 ml) in sequence. Majority of the ^{68}Ga-labelled peptide solution with no free $^{68}Ga^{3+}$ was obtained in the 30% ethanol/saline mixture. About 1 to 1.2 ml of [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP was passed through a pre-soaked sterile filter (Millex-GV, 0.22 µm) into a sterile vial of pre-buffered saline solution (5.5 ml 0.9% sodium chloride, 2 ml 2.5 M sodium acetate).

2.4 IMAGE ACQUISITION, RECONSTRUCTION AND ANALYSIS

Animals were not starved and allowed access to water *ad libitum*. Diseased dogs that were being treated with pain medication were weaned off their medication two weeks prior to the imaging session. Anaesthesia was induced by bolus injection of 6% sodium pentobarbitone solution until the surgical plane was achieved, maintained by a pre-calculated flow rate of anaesthetics that was administered via an infusion pump. Electrocardiographic and pulse oximetry equipment was placed on the dog to monitor heart rate; controlled breathing was facilitated by intubation. The animals were positioned on the camera bed laterally. [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP was administered intravenously. Image acquisition and analysis was carried out as reported by Ebenhan et al.;[33] each animal was allowed three static whole-body PET/CT scans at 30, 60 and 120 min post injection. During image acquisition, the blood pressure of the animal was monitored with the use of a femoral artery port.

2.5 PHARMACOKINETICS OF [^{68}GA]GA-DOTA-[THI^{8},MET(O_2)^{11}]SP

Following administration of the radioactive tracer, blood samples (0.5-1.5 mL) were collected from the jugular vein in pre-weighed, heparinised vacutainer tubes (2 ml) (Becton Dickinson, Johannesburg, South Africa) at different time intervals up to 60 min. Blood fractionation was achieved by spinning the whole blood sample in a centrifuge for ~10 min at 4000 rpm using a bench top centrifuge (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode Am Harz, Germany), separating the sample into blood cells (bottom) and a clear plasma fraction (top). The plasma fraction was transferred to a pre-weighed tissue culture container. Urine (volume available at point in time ≤ 25 mL) was collected in pre-weighed urine collection cups (Becton Dickinson, Johannesburg, South Africa) from catheterized bladders at different time

intervals up to 120 min. All blood and urine samples were weighed. Radioactivity was measured using a CRC 25 dose calibrator (Capintec Inc., USA) and a semi-automated well counter (Biodex, Shirley, NY, USA) and data was corrected for radioactive decay and sample volume. Time-activity curves to determine the blood clearance (pharmacological half-life) and excretion (urinary elimination rate) of the radiopharmaceutical were constructed. The total urine radioactivity recovery was calculated by accumulating all samples yielded within 150 min.

2.6 STATISTICAL ANALYSIS

Outliers were identified using an online test (Grubbs Test), if systematic errors were non-existent. If applicable, data sets were averaged and expressed as mean values ± standard deviation (SD) using Microsoft Office Excel Software (Microsoft, Albuquerque, USA); the standard error of mean (SEM) was used to express populations of individual radiolabelling data sets. The significance of two mean values (comparing time points, pharmacokinetic tracer behaviour) was calculated by a Student's-*t*-test (paired and unpaired) and the significance level was set at a value $p \leq 0.05$. Blood and urine concentration results were analysed using regression analysis, and the agreement on correlation of tracer time activity curves of urine and blood was verified through the correlation coefficient (R^2).

3 RESULTS

3.1 RADIOLABELLING FORMULATION OF [^{68}GA]GA-DOTA-[THI^{8},MET(O$_2$)^{11}]SP

Freeze dried aliquots of DOTA-[Thi^{8},Met(O$_2$)^{11}]SP were radiolabelled with ^{68}Ga-eluates (616 ± 148; n > 3). Reliably-high radiolabelling efficiencies were achieved (> 90%) with colloids < 6% and uncomplexed ^{68}Ga < 3% (n > 3). High radiochemical purity > 99% could be achieved following a rapid C18-based solid purification.[32] The final formulation of [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP resulted in a specific activity of 13.5 ± 3.9 MBq/nmol. The final formulated products (in saline) were close to neutral (pH of 6.5) and may contain ethanol < 5% (only in purified samples) which was deemed safe for administration.

Table 1: Summary of parameters evaluated to achieve injectable doses.

Parameter	**[^{68}Ga]Ga-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP**	
	Healthy dogs	**Diseased dogs**
Radiosyntheses (n)	5	3
Yield (MBq)	493 ± 89	468 ± 223
Available	405 ± 121	443 ± 165
Total activity (MBq) / dog	139 ± 49	158 ± 52
Injected dose (MBq/kg)	4.6 ± 0.7	4.6 ± 2.7
Injected mass (µg)	16.1 ± 8.0	16.6 ± 5.3
Molar activity (MBq/nmol)	17.6± 3.2	12.6 ± 5.3

3.2 [^{68}GA]GA-DOTA-[THI^{8},MET(O$_2$)^{11}]SP PET/CT IMAGING

Healthy dogs (n=6) of 20-40 kg received a 4-7 ml intravenous bolus dose of 2.3 – 5.2 mCi (4.6 ± 0.7 MBq/kg) [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP. Similar doses ranging from 3.6 – 6.2 mCi (4.6 ± 2.1 MBq/kg) of [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP were administered to three dogs (29-55 kg) suffering from suspected osteoarthritis characterised by chronic pain symptoms (diseased dogs). Tracer biodistribution was demonstrated (**Figure 1**) using three static qualitative PET/CT image acquisitions at 29 ± 6, 65 ± 3 and 120 ± 4 min. achieved by 3D-VOI (volume of interest) analysis for further quantification of the activity concentration (SUV_{mean}) in relevant organs and tissues (**Table 2**).

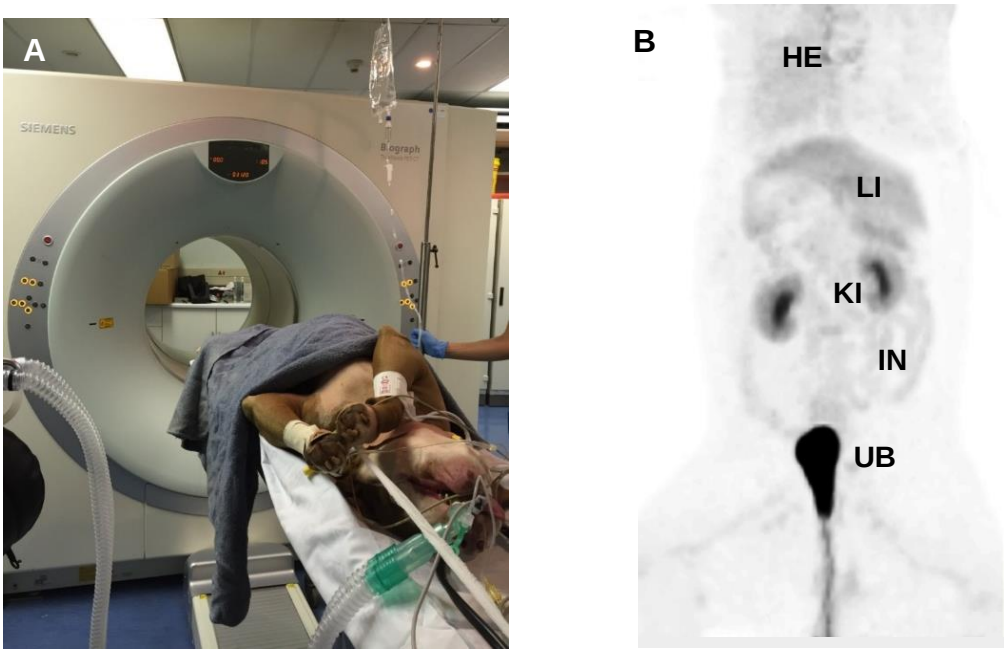


Figure 1: A) Veterinary-approved positioning of the animal on the PET/CT scanner bed for ease of access. B) Maximum intensity projection PET image of the *in vivo* biodistribution in a healthy dog at 60 min after injection of [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP (urinary bladder (UB)> kidneys (KI)> liver (LI)> intestine (IN)> heart (HE)).

Table 2: Image-guided tracer concentration of [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP. Pharmacokinetic tracer profile over 120 min; normal organ/tissue uptake using semi-quantitative image analysis (SUV_{mean}).

Organ / Tissue	**[^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP concentration (SUV)$^{\#}$**							
Time post tracer injection	**29 ± 6 min**		**65 ± 3 min**			**120 ± 4 min**		
	Mean	***SD***	***Mean***	***SD***	***P 1)***	***Mean***	***SD***	***P 1)***
Blood (*arcus aortae*)	1.05	0.44	0.87	0.46	*0.02*	0.66	0.38	*0.0003*
Heart	1.10	0.43	0.88	0.44	*0.02*	0.62	0.34	*0.003*
Liver	3.10	0.54	3.17	0.57		2.33	0.60	
Spleen	0.66	0.48	0.57	0.51		0.39	0.36	*0.01*
Gallbladder	1.39	0.78	1.49	0.70		1.15	0.63	*0.04*
Lung	0.40	0.13	0.37	0.14	*0.08*	0.27	0.11	*0.004*
Salivary glands	1.28	0.50	1.09	0.45		0.81	0.42	*0.01*
Brain	0.20	0.07	0.15	0.06	*0.03*	0.12	0.06	*0.02*
Thyroid	1.01	0.40	0.77	0.37	*0.001*	0.57	0.31	*0.004*
Stomach mucosa	2.41	0.92	1.84	0.71		1.54	0.55	*0.04*
Bone (femur)	0.38	0.09	0.30	0.12	*0.01*	0.25	0.06	*0.001*
Genitals (testes, ovaries)	0.93	0.43	0.71	0.30		0.50	0.20	*0.03*
Muscle (reference tissue)	0.24	0.13	0.19	0.09		0.12	0.07	*0.03*
Pancreas	1.40	0.36	1.30	0.25		0.98	0.26	*0.03*
Intestines (small / large)	1.20	0.50	1.01	0.47		0.74	0.37	*0.01*
Kidneys $^{\$)}$	5.29	3.68	4.63	4.36		3.14	2.87	
Bladder, urinary tract $^{\$)}$	16	14	37	47		15	14	

Mean and SD are presented for n = 4-7 animals; #) Standard uptake value (g/mL); $) bladder was drained at times for further urine analysis; 1) Student's t test (2-way-2-tailed) was used to determine the significance ($P<0.05$) of the SUV values.

3.2.1 Side effects

Some animals experienced mild, transient physiological effects including increased heart rate upon injection of the tracer which normalised very quickly thereafter. Other observed effects included salivation, which is a known effect of SP, and defecation (one animal only). The faeces was collected and analysed and no activity was present.

No animals were sacrificed as a result of the study other than one dog that was diagnosed through imaging as being in renal failure.

3.2.2 Qualitative and quantitative image analysis

Maximum intensity projection of [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP PET/CT images showed expected organ/tissue biodistribution with the highest tracer presence in the urinary bladder (SUV = 37, 60 min) and kidneys because of predominant renal tracer excretion. Considerable tracer uptake was noted in liver and stomach mucosa decreasing over 120 min. Moderate uptake (SUV = 1.0 – 1.5, 60 min) was observed in the gallbladder, salivary glands, pancreas and intestines. All other organs showed negligible uptake (SUV ≤ 1.0). Tracer washout was observed for all organs between the 60 and 120 min imaging time points.

3.3 PHARMACOKINETICS OF [^{68}GA]GA-DOTA-[THI^{8},MET(O_2)^{11}]SP

3.3.1 Blood activity

At nine different time points blood samples were taken and separated by centrifugation into red blood cells (RBC) and plasma. Both fractions were weighed and measured. Peak blood activity of the tracer was observed in the sample taken in the first few minutes post injection. Time activity curves from both the erythrocyte and plasma fraction (**Figure 2**), indicated rapid clearance of the tracer from the blood pool typically following an exponential decrease of the initial activity within the first hour. The decay corrected peak activity (kBq/g) levels in healthy dogs were 4.8 ± 1.8 (RBC) and 6-11 times higher in plasma (44.9 ± 17.4). Diseased dogs showed no significant differences in peak activity (kBq/g) levels of 5.7 ± 3.5 (RBC; p = 0.375); 6-10-times higher in plasma (43.4 ± 27.8; p = 0.467). The pharmacological half-life (min) calculated from regression analysis for [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP in healthy dogs was the same for RBC 14 ± 3 and plasma (14 ± 4). Interestingly, [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP tended to reside longer (19 ± 3; p = 0.063) in RBC and shorter in plasma (9 ± 3, p = 0.103) of the three diseased dogs (weak statistical power).

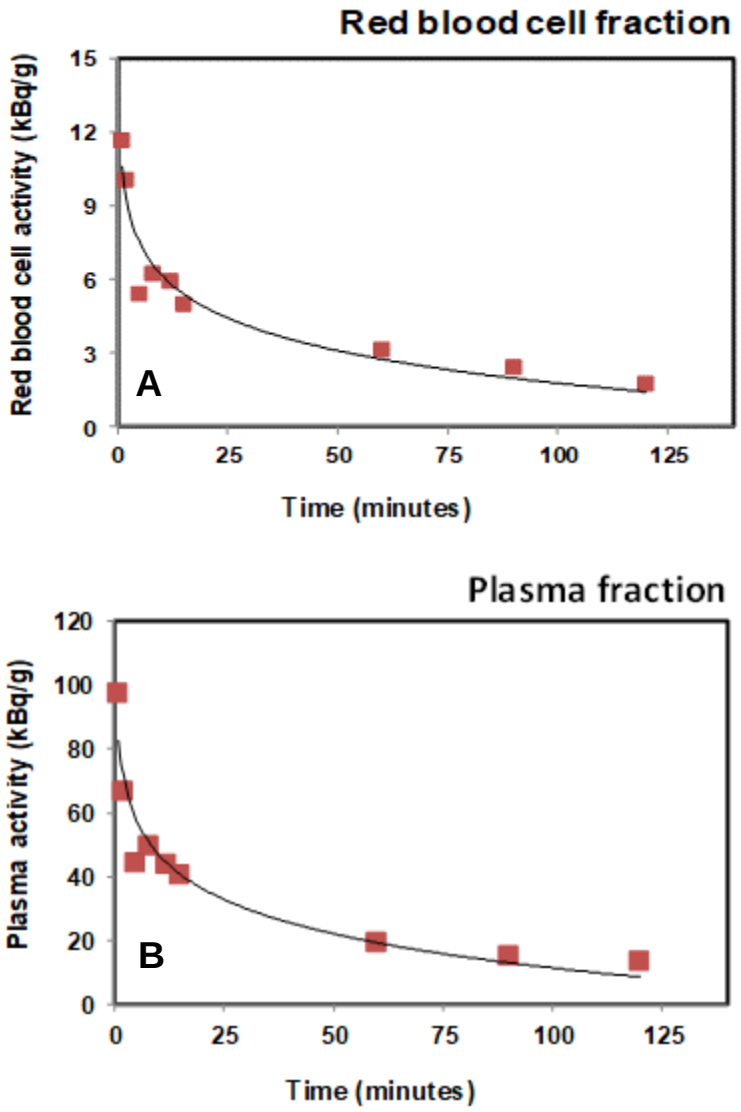


Figure 2: Representative time-activity-curves yielded from **A**) red blood cells and **B**) plasma fraction using regression analysis (exponential fit). Data is corrected for volume and radioactive decay.

3.3.2 Urinary excretion

The dominant excretion route of the tracer was renal. Time activity curves from urine samples showed accumulating levels of [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP following a linear incline (**Figure 3**). [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP-based activity was excreted (MBq/h) with varying rates (healthy dogs: 21.6 ± 4.2; diseased dogs: 22.7 ± 14.4) in both populations. The total tracer activity recovery (%ID) in healthy dogs was higher (33 - 46) than in the three diseased dogs (17 - 48; p = 0.105) analysing all collected urine over 150 min.

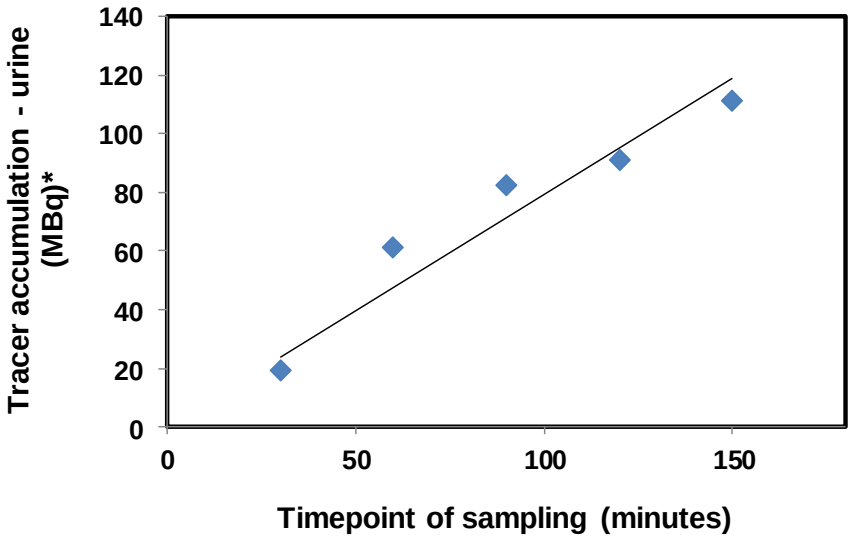


Figure 3: Representative time-activity-curve yielded from urine samples (~5 samples depending on availability) using regression analysis (linear fit). Data is corrected for volume and radioactive decay.

3.4 TARGETING PAIN WITH [^{68}GA]GA-DOTA-[THI^{8},MET(O_2)^{11}]SP

Abnormal tracer uptake was observed in 3/3 dogs that were classified as “diseased” during pre-screening. These animals were treated with pain medication and had to be weaned off for the purpose of the study. One animal suffered from lameness in the back legs, which was visible on the day of study. Tracer uptake was observed in several areas including the legs, paws, hips, and shoulders. Areas presenting with unilateral uptake of the tracer could be seen in soft tissue (**Figure 4**) and in bone (**Figure 5, Figure 6**).

Intense unilateral [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP accumulation was particularly noted in the hip region of a dog presenting with chronic pain and lameness in the leg (**Figure 7**). The target-to-non-target (T/NT) ratios for the diseased cohort increased from 2.15 ± 0.52 at 60 min p.i. to 2.76 ± 0.72 at 120 min p.i.

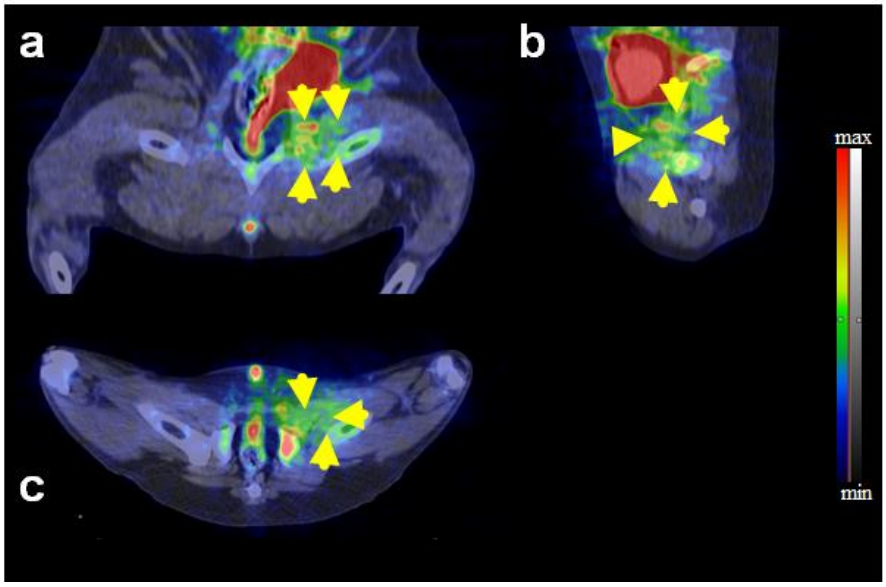


Figure 4: Representative PET/CT image slices in a) coronal b) sagittal and c) axial orientation from a dog with symptoms of chronic pain. Unilateral tracer uptake determined in the thigh soft tissue (arrows) at 60 min p.i. of [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP.

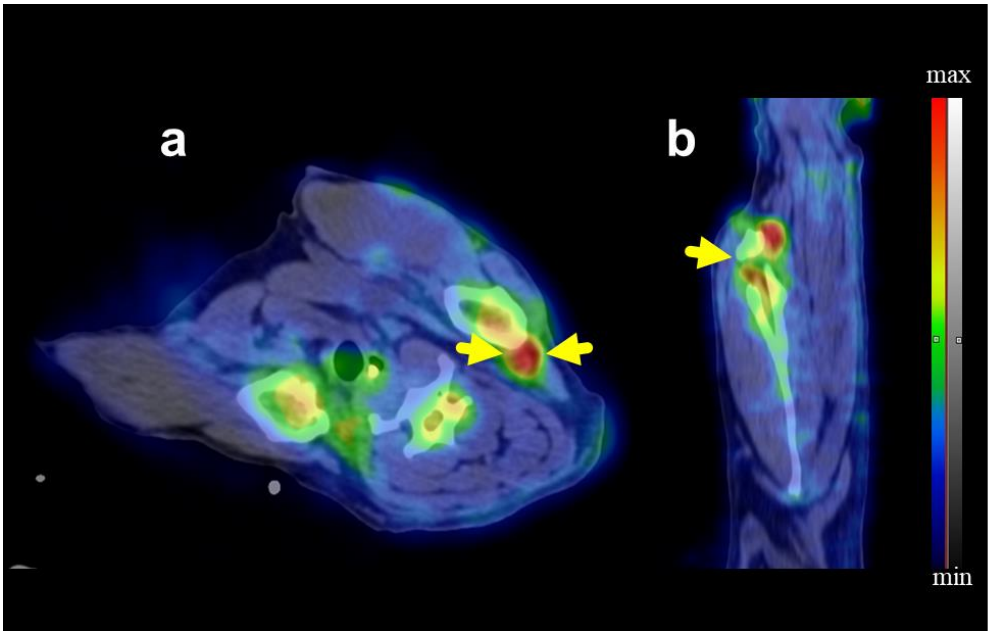


Figure 5: Representative PET/CT image slices in a) axial taken from a dog with symptoms of chronic pain. Unilateral tracer uptake determined in the bone and soft tissue of the shoulder (arrows) at 60 min post administration of [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP.

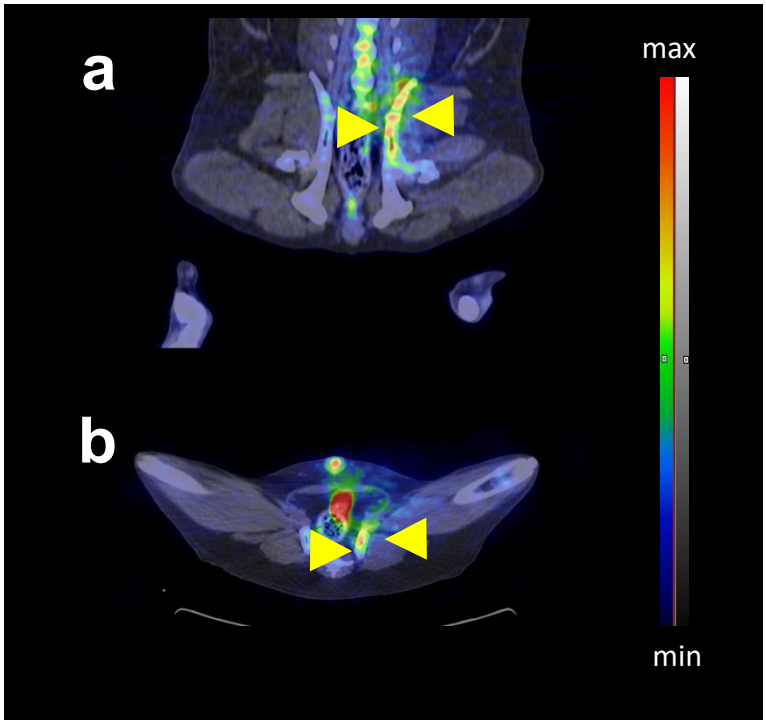


Figure 6: Representative PET/CT image slices in a) coronal and b) axial orientation taken from a dog with symptoms of chronic pain. Unilateral tracer uptake determined in the pelvic bone (arrows) at 60 min post administration of [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP.

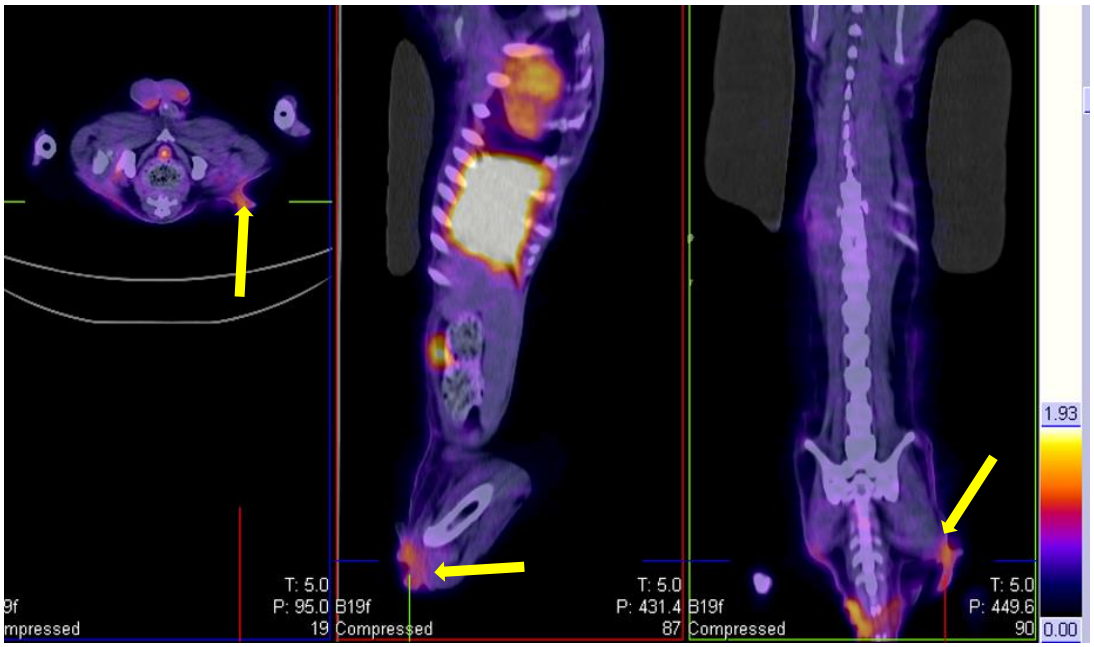


Figure 7: PET/CT image projections (120 min p.i.) of a dog with lameness in the back leg (ID: 166.5 MBq; [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP) showing intense unilateral uptake in the hip region (arrows).

3.5 IMAGE-GUIDED QUANTIFICATION OF PAIN-RELATED UPTAKE OF [^{68}GA]GA-DOTA-[THI^{8},MET(O$_2$)^{11}]SP

PET/CT imaging of the biodistribution of [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP in diseased dogs afforded targeting of painful loci. These painful sites were observed in several of the joint and bone areas that could be associated with musculoskeletal conditions such as osteoarthritic pain. SUV-based analysis of target areas (T) assessed in relation to contralateral reference tissue (NT), demonstrated significant accumulation of [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP over time (ratio ≥ 2), in areas including the ankle, paw joint, humerus and pelvic bone (**Figure 8**).

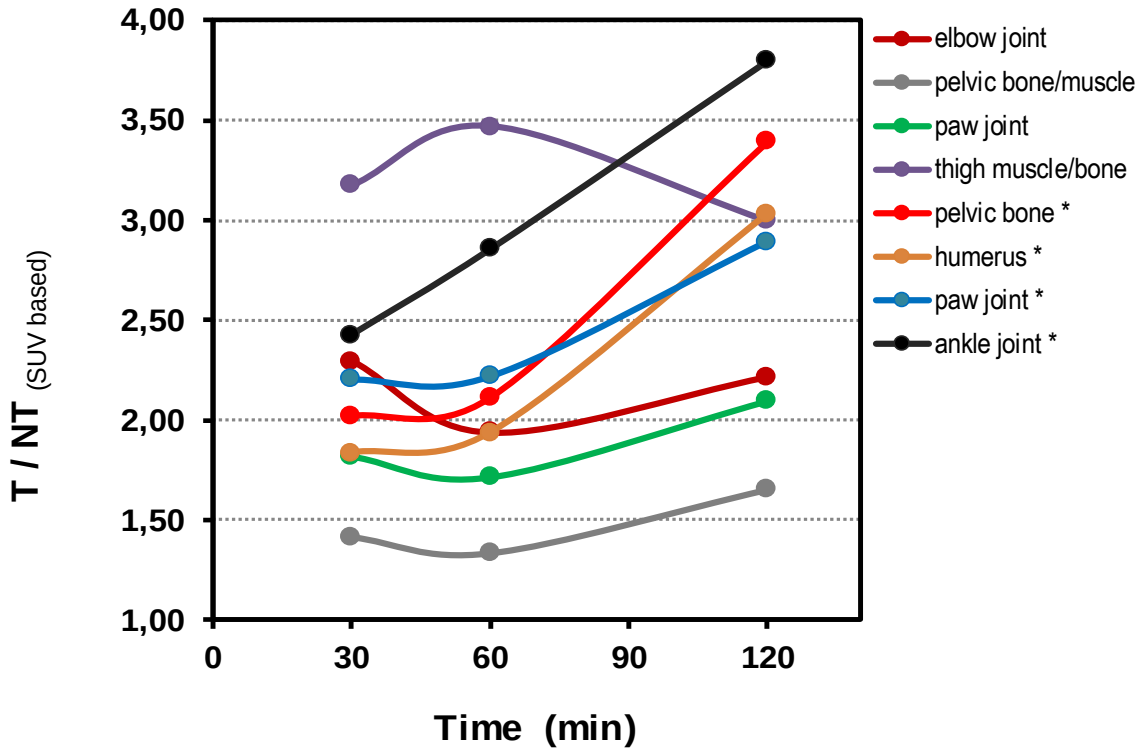


Figure 8: [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP-PET/CT image guided quantification in pathological tissues and includes a 3D area under the curve. PET information was used to delineate the pathological tissue and the identical contralateral reference tissue. The T/NT ratio was obtained by dividing the SUV (decay corrected) of the pathological tissue (T) by the SUV of the contralateral reference tissue (NT). *) significant [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP T/NT ratio increase (p > 0.001) over 120 min.

4 DISCUSSION

The SP/NK1R pathway has been implicated as playing a role in inflammation and chronic pain related disorders[6] yet blockade studies using SP based NK1R antagonists that were intended to have an anti-nociceptive effect, have proven to have no analgesic effect in humans. Molecular imaging modalities such as PET/CT have the ability to visualise and provide a quantitative measurement *in vivo* of the function of cellular and biological processes.[34] This non-invasive assessment of NK1R expression related to non-neuronal tissue pathology could markedly contribute to a better understanding of SP/NK1R associated musculoskeletal disorders such as osteoarthritis. A great advantage of PET imaging is the ability to evaluate the image-guided *in vivo* biodistribution of [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP. Comparison of healthy and diseased dogs may reveal the potential of [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP as an imaging tool for the visualisation of NK1R-mediated pain processes. A successful tracer assessment, based on the results obtained from this investigation would allow for prospective clinical translation of [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP-PET into pain-suffering humans.

The use of animals as a comparative model for the human biological system is based on the concept that humans and other animal species share physiological, behavioural and other characteristics.[35] As the emphasis on rapid and robust translation from animal model to human increases, it is likely that the concept of animal modelling will move even more towards using models that very closely mimic human conditions. This is best observed in the use of domesticated animal species in which a specific target disease is naturally occurring. Known as One Medicine,[36,37] the aim of this approach is to promote the sharing of knowledge and resources and to have a common goal of improving the health of all species. The use of a naturally occurring disease model in companion animals has the benefit that the animal might better reflect the complex genetic, physiological and environmental variation that is present in humans compared to an inbreed laboratory animal model. This implies that a canine model would be more representative but at the same time complex in design and interpretation. These genetically variable subjects would provide more generalised findings compared to the inbred rodent strains that are currently being used in pain research.[38] For this study the domesticated canine model was selected because osteoarthritis is a commonly

occurring condition in large breed dogs, particularly working dogs and would therefore provide a close mimic of human osteoarthritis.[39] The healthy and diseased cohorts are comprised of both male and female subjects to ensure that sex bias is eliminated. This is because many studies regarding pain perception in humans have indicated that in general females are afflicted with chronic pain at a higher rate than men.[40] The evaluation of both sexes also allows for quantification of the radiotracer uptake in the genitals.

Even though rat and mice model is considered the workhorse of drug discovery in reference to pre-clinical imaging, there are many ways the biology of rodents are limited in accurately predicting the biology and pharmacology of painful conditions in humans.[41] Studies have found that the canine gastrointestinal tract for example corresponds closely to human tissue, both histologically and functionally. This provides anatomical resolution that is not attainable in a rodent model.[42] The development of behavioural expressions to pain differs among species and is affected by the different evolutionary pressures experienced. It is also common knowledge that different species metabolize compounds differently and at differing rates.[41] The larger animal model is a more suitable fit to a clinical PET/CT camera[43] and also afforded the opportunity to determine pharmacokinetic data from the collection of blood and urine samples which provides valuable information on the behaviour of the tracer in the blood and renal system. This is more difficult to achieve for small animal models.

Published approaches for the radiosynthesis of similar ^{68}Ga-labelled peptides[32,44] set the scientific foundation for the development of a robust radiolabelling method for the preparation of [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP using freeze-dried aliquots of DOTA-[Thi^{8},Met(O_2)^{11}]SP and radioactivity from a tin-dioxide-based ^{68}Ge-/^{68}Ga-generator. A C18-SPE-based purification yielded suitable injectable doses with an excellent specific activity. The optimised method and formulation were used to routinely prepare the safe-to-administer radiotracer at a near-physiological pH with minimum salt and ethanol contents. Injected doses for both, healthy and diseased dogs, were deemed safe and high enough to consequently yield PET images of superior quality.

[^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP-PET/CT in healthy outbreed canines demonstrated favourable tracer pharmacokinetics and biodistribution allowing for further investigation in canines with suspected pain disorders. Elevated unilateral uptake of [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP, which increases over time, was observed in bone and soft tissue areas that are synonymous with osteoarthritis, including the legs, paws, hips and shoulders. SUV-based quantification of target areas (T) were assessed in relation to contralateral reference tissue (NT). Areas identified as having a T/NT ratio ≥ 2 represented significant accumulation of [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP over time of imaging. This was observed in areas including the ankle, paw joint, humerus and pelvic bone. For areas with T/NT ratio < 2, the tracer uptake was not cumulative over time and could therefore be attributed to an inflammatory process or tissue perfusion. This quantitative analysis confirms the qualitative observations demonstrating that [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP is capable to visualize unilateral pain-mediated uptake in bone- and soft tissue.

The radiotracer demonstrated elevated and persisting uptake in characteristic NK1R-dense tissues such as the gut mucosa[42] and salivary glands. This could be considered an indirect confirmation that substantial amounts of tracer remain intact within human serum but requires confirmation through enzymatic stability analysis in blood. The activity loaded organs such as kidneys, liver, gut and bladder are mostly located in the abdominal body cavity which would be unfavourable to target NK1R expression in the vicinity of these organs. However, pain imaging of osteoarthritic painful loci being peripherally located, will benefit significantly from the use of this tracer as bone and soft tissue uptake was very low.

The PET biodistribution of [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP may be different in anesthetised *versus* conscious dogs. Anaesthesia can have a significant impact on the cardiovascular, central nervous and respiratory system.[45] [^{18}F]FDG studies in pigs[46] and mice[45] demonstrated deviation of the respective SUVs based on the anaesthetic used and the organ being evaluated. Particular variations were observed in different parts of the brain. These findings are specifically related to the impact of anaesthesia on glucose metabolism which affects the [^{18}F]F-FDG uptake. Even though this [^{18}F]FDG uptake is not a receptor mediated process a probable influence

of anaesthesia should be looked into for [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP regarding its prospective bench to bedside translation.

5 CONCLUSION

The radiosynthesis of [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP yielded a safe-to-administer prototype radiopharmaceutical which was successfully tested *in vivo* using outbreed dogs. Favourable [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP pharmacokinetics and biodistribution was determined which deemed this compound suitable for imaging of osteoarthritic pain. [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP-PET/CT imaging may be a powerful tool to detect NK1R-mediated tissue pain. However further investigations should focus on correlating the NK1R expression in non-neuronal tissues with the accumulation of [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP *in vivo*.

6 ACKNOWLEDGEMENTS

The authors would like to express their gratitude to The South African Nuclear Energy Corporation SOC Limited (Necsa) and The Nuclear Technologies in Medicine and the Biosciences Initiative (NTeMBI) for funding the project and Steve Biko Academic Hospital for access to the hospital radiopharmacy ^{68}Ge-/^{68}Ga-generator and other equipment. Thank you to Mrs Delene van Wyk for her excellent support on the project as the radiographer.

7 CONFLICT OF INTEREST

The authors report no conflict of interest.

8 REFERENCES

1. Brooks PM. The burden of musculoskeletal disease – a global perspective. Clin Rheumatol [Internet]. 2006 [cited 2019 Oct 9];25(6):778-81. Available from: https://link.springer.com/article/10.1007%2Fs10067-006-0240-3 doi: 10.1007/s10067-006-0240-3

2. World Health Organization: The burden of musculoskeletal conditions at the start of the new millennium (WHO Technical Report Series 919). World Health Organization 2003.

3. World Health Organization. Musculoskeletal conditions [Internet].Geneva:WHO;2019 Nov 26 [cited 2019 Dec 5]. Available from: https://www.who.int/news-room/fact-sheets/detail/musculoskeletal-conditions

4. Cipriano PW, Lee S-W, Yoon D, Shen B, Tawfik VL, Curtin CM, et al. Successful treatment of chronic knee pain following localization by a sigma-1 receptor radioligand and PET/MRI: a case report. J Pain Res [Internet]. 2018 Oct [cited 2019 Dec 5];11:2353-7. Available from: https://doi.org/10.2147/JPR.S167839

5. Gold MS & Gebhart GF. Nociceptor sensitization in pain pathogenesis. Nat Med [Internet]. 2010 [cited 2019 Sep 18];16(11):1248-57. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5022111/ doi: 10.1038/nm.2235

6. Zieglgänsberger W. Substance P and pain chronicity. Cell Tissue Res [Internet]. 2019 [cited 2019 Oct 28];375(1):227–41. Available from: https://link.springer.com/article/10.1007/s00441-018-2922-y doi: 10.1007/s00441-018-2922-y

7. Otsuka M, Yoshioka K. Neurotransmitter functions of mammalian tachykinins. Physiol Rev [Internet]. 1993 [cited 2019 Sep 9];73(2):229-307. Available from: https://europepmc.org/article/med/7682720 doi: 10.1152/physrev.1993.73.2.229

8. Maggi CA, Patacchini R, Rovero P, Giachetti A. Tachykinin receptors and tachykinin receptor antagonists. J Auton Pharmacol [Internet]. 1993 [cited 2019 Nov 25];13(1):23–93. Available from: https://onlinelibrary.wiley.com/doi/abs/10.1111/j.1474-8673.1993.tb00396.x?sid=nlm%3Apubmed doi: 10.1111/j.1474-8673.1993.tb00396.x

9. Quartara L, Maggi CA. The tachykinin NK1 receptor. Part I: ligands and mechanisms of cellular activation. Neuropeptides [Internet]. 1997 Dec [cited 2019 Nov 15];31(6):537-63. Available from: https://www.sciencedirect.com/science/article/abs/pii/S0143417997900019?via%3Dihub doi: 10.1016/s0143-4179(97)90001-9

10. Babenko VV, Graven-Nielsen T, Svensson P, Drewes AM, Jensen TS, Arendt-Nielsen L. Experimental human muscle pain induced by intramuscular injections of bradykinin, serotonin, and substance P. Eur J Pain [Internet]. 1999 [cited 2019 Aug 30];3:93–102. Available from: https://onlinelibrary.wiley.com/doi/abs/10.1053/eujp.1998.0103?sid=nlm%3Apubmed doi: 10.1053/eujp.1998.0103

11. Murray CW, Cowan A, Larson AA. Neurokinin and NMDA antagonists (but not a kainic acid antagonist) are antinociceptive in the mouse formalin model. Pain [Internet]. 1991 [cited 2019 Dec 1];44(2):179-85. Available from: https://insights.ovid.com/article/00006396-199102000-00013 doi:10.1016/0304-3959(91)90135-K

12. Seguin L, Le Marouille-Girardon S, Millan MJ. Antinociceptive profiles of non-peptidergic neurokinin1 and neurokinin2 receptor antagonists: a comparison to other classes of antinociceptive agent. Pain [Internet].1995 [cited 2019 Dec 1];61(2):325-43. Available from: https://journals.lww.com/pain/Abstract/1995/05000/Antinociceptive_profiles_of_non_peptidergic.20.aspx doi:10.1016/0304-3959(94)00194-J

13. Sakurada T, Katsumata K, Yogo H, Tan-No K, Sakurada S. Ohba M, et al. The neurokinin-1 receptor antagonist, sendide, exhibits antinociceptive activity in the formalin test. Pain [Internet]. 1995 [cited 2019 Nov 11];60(2):175-80. Available from: https://insights.ovid.com/article/00006396-199502000-00009 doi: 10.1016/0304-3959(94)00107-P

14. Traub RJ. The spinal contribution of substance P to the generation and maintenance of inflammatory hyperalgesia in the rat. Pain [Internet]. 1996 [cited 2019 Dec 1];67(1):151-61. Available from: https://insights.ovid.com/article/00006396-199609000-00020 doi: 10.1016/0304-3959(96)03076-X

15. Hill R. NK1 (substance P) receptor antagonists--why are they not analgesic in humans? Trends Pharmacol Sci [Internet]. 2000 [cited 2019 Dec 2];21(7):244-6. Available from: https://www.sciencedirect.com/science/article/abs/pii/S0165614700015029 doi:10.1016/S0165-6147(00)01502-9

16. Boyce S, Hill R. Discrepant results from precinical and clinical studies on the potential of substance P-receptor antagonist compounds as analgesics. In: Mea D, editor. Proceedings of the 9th World Congress on Pain: Seattle. IASP Press; 2000; p. 313-24.

17. Howard MA, Sanders D, Krause K, O'Muircheartaigh J, Fotopoulou A, Zelaya F, et al. Alterations in resting-state regional cerebral blood flow demonstrate ongoing pain in osteoarthritis: an arterial spin-labeled magnetic resonance imaging study. Arthritis Rheumatol [Internet]. 2012 Dec [cited 2019 Aug 4];64(12):3936-46. Available from: https://doi.org/10.1002/art.37685

18. Julien N, Goffaux P, Arsenault P, Marchand S. Widespread pain in fibromyalgia is related to a deficit of endogenous pain inhibition. Pain [Internet]. 2005 [cited 2019 Oct 16];114(1-2):295-302. Available from: https://insights.ovid.com/article/00006396-200503000-00033 doi: 10.1016/j.pain.2004.12.032

19. Ung H, Brown JE, Johnson KA, Younger J, Hush J, Mackey S. Multivariate classification of structural MRI data detects chronic low back pain. Cereb Cortex [Internet]. 2014 Apr [cited 2019 Aug 12];24(4):1037-44. Available from: https://doi.org/10.1093/cercor/bhs378

20. Chiapparini L, Ferraro S, Grazzi L, Bussone G. Neuroimaging in chronic migraine. Neurol Sci [Internet]. 2010 Jun [cited 2019 Aug 4];31(S1):19-22. Available from: https://doi.org/10.1007/s10072-010-0266-9

21. Lee MC, Tracey I. Imaging pain: a potent means for investigating pain mechanisms in patients. Br J Anaesth [Internet]. 2013 Jul [cited 2019 Aug 4];111(1):64-72. Available from: https://doi.org/10.1093/bja/aet174

22. Majkowska-Pilip A, Krzysztof P, Halik PK, Gniazdowska E. The significance of NK1 receptor ligands and their application in targeted radionuclide tumour therapy. Pharmaceutics [Internet]. 2019 Sep [cited 2019 Dec3];11(9):443. Available from: https://doi.org/10.3390/pharmaceutics11090443

23. Hjornevik T, Cipriano PW, Shen B, Park JH, Gulaka P, Holley D, et al. Biodistribution and radiation dosimetry of 18F-FTC-146 in humans. J Nucl Med [Internet]. 2017 [cited 2019 Nov 30];58(12):2004-9. Available from: http://jnm.snmjournals.org/content/58/12/2004 doi: 10.2967/jnumed.117.192641

24. Shen B, Park JH, Hjornevik T, Cipriano PW, Yoon D, Gulaka PK, et al. Radiosynthesis and first-in-human PET/MRI evaluation with clinical-grade [18F]FTC-146. Mol Imaging Biol [Internet]. 2017 Oct [cited 2019 Nov 13];19(5):779-86. Available from: https://doi.org/10.1007/s11307-017-1064-z

25. Dash A, Chakravarty R. Radionuclide generators: the prospect of availing PET radiotracers to meet current clinical needs and future research demands. Am J Nucl Med Mol Imaging [Internet]. 2019 Feb [cited 2019 Dec 2];9(1):30-66. Available from: www.ajnmmi.us /ISSN:2160-8407/ajnmmi0086228

26. Velikyan, I. Prospective of 68Ga-radiopharmaceutical development. Theranostics [Internet]. 2014 [cited 2019 Nov 30];4(1):47-80. Available from: http://www.thno.org/v04p0047.htm

27. Banerjee SR, Pomper MG. Clinical applications of gallium-68. Appl Radiat Isot [Internet]. 2013 Jun [cited 2019 Nov 21];76:2-13. Available from: https://doi.org/10.1016/j.apradiso.2013.01.039

28. Smith DL, Breeman WAP, Sims-Mourtada J. The untapped potential of gallium-68 PET: the next wave of 68Ga agents. Appl Radiat Isot [Internet]. 2013 Jun [cited 2019 Nov 19];76:14-23. Available from: https://doi.org/10.1016/j.apradiso.2012.10.014

29. Merlo A, Macke H, Reubi J-C, Good S. Radiolabeled conjugates based on Substance P and the uses thereof. Universitätsspital Basel, Universität Bern; EP 1603598 B1, 2008.

30. Merlo A, Macke H, Reubi J-C, Good S. Radiopharmaceuticals for cancer diagnosis and treatment. Kantonsspital Basel, Universität Bern; US 2007/0053837 A1, 2007.

31. De Araújo EB, Pujatti PB, Mengatti J. Radiolabeling of Substance P with 177Lu and in vivo evaluation of tumor cell uptake in nude mice: Preliminary results. Cell Mol Biol (Noisy-le-grand) [Internet]. 2010 May [cited 2018 May 12];56(2):12-7. Available from: https://www.ipen.br/biblioteca/2008/eventos/13820.pdf

32. Rossouw DD, Breeman WA. Scaled-up radiolabelling of DOTATATE with 68Ga eluted from a SnO2-based 68Ga/68Ge generator. Appl Radiat Isot [Internet]. 2012 [cited 2019 Nov 23];70(1):171-5. Available from: https://www.sciencedirect.com/science/article/abs/pii/S0969804311003988?via%3Dihub doi: 10.1016/j.apradiso.2011.07.016

33. Ebenhan T, Schoeman I, Rossouw DD, Grobler A, Marjanovic-Painter B, Wagener J, et al. Evaluation of a flexible NOTA-RGD kit solution using gallium-68

from different 68Ge/68Ga-generators: pharmacokinetics and biodistribution in nonhuman primates and demonstration of solitary pulmonary nodule imaging in humans. Mol Imaging Biol [Internet]. 2016 Oct [cited 2019 Dec 1];19:469-82. Available from: https://doi.org/10.1007/s11307-016-1014-1

34. Pysz MA, Gambhir SS, Willmann JK. Molecular Imaging: Current Status and Emerging Strategies. Clin Radiol [Internet]. 2010 Jul [cited 2019 Dec 10];65(7):500-16. Available from: https://doi.org/10.1016/j.crad.2010.03.011

35. Ericsson AC, Crim MJ. Franklin CL. A brief history of animal modelling. Mo Med [Internet]. 2013 May-Jun [cited 2019 Dec 10];110(3):201-4. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3979591/

36. Sundberg JP, Schofield PA. One medicine, one pathology, one concept. J Am Vet Med Assoc [Internet]. 2009 Jun [cited 2019 Dec 10]; 234(12):1530-1. Available from: https://doi.org/10.2460/javma.234.12.1530

37. Gyles C. One medicine, one health, one world. Can Vet J [Internet]. 2016 Apr [cited 2019 Dec 10];57(4):345-6. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4790223/

38. Mogil JS. Animal models of pain: progress and challenges. Nat Rev Neurosci [Internet]. 2009 [cited 2019 Dec 11];10(4):283-94. Available from: https://www.nature.com/articles/nrn2606 doi: 10.1038/nrn2606

39. Carrig CB. Diagnostic imaging of osteoarthritis. Vet Clin North Am Small Anim [Internet]. 1997 Jul [cited 2019 Dec 16];27(4):777-814. Available from: https://doi.org/10.1016/S0195-5616(97)50080-5

40. Da Silva JT, Seminowicz DA. Neuroimaging of pain in animal models: a review of recent literature. Pain Rep [Internet]. 2019 [cited 2019 Dec 9];4(4):732. Available from: https://journals.lww.com/painrpts/Fulltext/2019/08000/Neuroimaging_of_pain_in_animal_models__a_review_of.9.aspx doi: 10.1097/PR9.0000000000000732

41. Henze DA, Urban MO. Translational pain research: From mouse to man. Boca Raton FL:CRC Press/Taylor & Francis;2010.

42. Vigna SR, Mantyh CR, Soll AH, Maggio JE, Mantyh PW. Substance P receptors on canine chief cells: localisation, characterisation and function. J Neurosci Res [Internet]. 1989 Aug [cited 2019 Dec 9];9(8):2678-86. Available from: https://doi.org/10.1523/JNEUROSCI.09-08-02878.1989

43. El-Sherif O, Xhaferllari I, Sykes J, Butler J, deKemp R, Renaud J, et al. 18F-FDG cardiac PET imaging in a canine model of radiation induced cardiovascular disease associated with breast cancer radiography. Am J Physiol Heart Circ Physiol [Internet]. 2019 [cited 2019 Dec 11];316(3):H586-H595. Available from: https://www.physiology.org/doi/abs/10.1152/ajpheart.00273.2018?rfr_dat=cr_pub%3Dpubmed&url_ver=Z39.88-2003&rfr_id=ori%3Arid%3Acrossref.org&journalCode=ajpheart doi: 10.1152/ajpheart.00273.2018

44. Ebenhan T, Chadwick N, Sathekge MM, Govender P, Govender T, Kruger HG, Marjanovic-Painter B, Zeevaart JR. Peptide synthesis, characterization and 68Ga-radiolabeling of NOTA-conjugated ubiquicidin fragments for prospective infection imaging with PET/CT. Nucl Med Biol [Internet]. 2014 Feb [cited 2019 Dec 5];41:390-400. Available from: https://doi.org/10.1016/j.nucmedbio.2014.02.001

45. Toyama H, Ichisea M, Liowa JS, Vinesa DC, Senecaa NM, Modella KJ, et al. Evaluation of anesthesia effects on [18F]F-FDG uptake in mouse brain and heart using small animal PET. Nucl Med Biol [Internet]. 2004 Feb [cited 2019 Nov 30];31:251–56. Available from: https://doi.org/10.1016/S0969-8051(03)00124-0

46. Lee YA, Kim J-I, Lee JW, Cho YJ, Lee BH, Chung HW, et al. Effects of various anesthetic protocols on 18F-Flurodeoxyglucose uptake into the brains and hearts of normal miniature pigs (Sus scrofa domestica). J Am Assoc Lab Anim Sci [Internet]. 2012 Mar [cited 2019 Dec 11];51(2):246–52. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3314529/#bib43

7 STUDY OUTCOMES, LIMITATIONS AND RECOMMENDATIONS

7.1 SUMMARY OF STUDY OUTCOMES

This study set out to use a suitable DOTA-conjugated Substance P(1-11)-derivative (DOTA-[Thi^{8},Met(O_2)^{11}]SP) for the development of an easy to perform radiolabelling using the convenient ^{68}Ge/^{68}Ga generator and safe-to administer prototype radiopharmaceutical [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP. In parallel, DOTA-[Thi^{8},Met(O_2)^{11}]SP was evaluated *in vitro* for its potential to target and activate NK1R and gain an understanding of its receptor selectivity, which is still lacking from previous literature reports. Consequently, [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP-PET/CT imaging was performed with the aim to investigate the *in vivo* pharmacokinetics, biodistribution and potential to target NK1R-mediated uptake in osteoarthritic foci, non-invasively. The main results can be summarized as follows:

- DOTA-[Thi^{8},Met(O_2)^{11}]SP is able to activate NK1R with a similar potency compared to the native SP and also has greater selectivity to NK1R compared to NK2R and NK3R.
- The structural modifications required for SP to achieve the precursor DOTA-[Thi^{8},Met(O_2)^{11}]SP (for ^{68}Ga-radiolabelling) did not compromise its receptor interaction.
- An appropriate radiolabelling solution, purification method and formulation of [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP was developed that provided an injectable dose for a small population of outbred dogs, which was suitable for non-invasive PET/CT imaging.
- [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP was rapidly cleared from the blood pool (half-life of 10 to 15 min), followed by renal excretion, predominantly.
- Elevated unilateral uptake of [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP, which increases over time, was observed in bone and soft tissue, particularly areas that are synonymous with osteoarthritis.

7.2 STUDY LIMITATIONS

7.2.1 ^{68}Ga radiolabelling and formulation of DOTA-[Thi^{8},Met(O$_2$)^{11}]SP

DOTA was chosen as a chelating agent as it is known to allow universal radiolabelling with various radiometal isotopes. However, heat incubation is required to complex the radioisotope and this can limit heat-vulnerable peptides from being used in the preparation as prospective radiopharmaceuticals. In this study, DOTA-[Thi^{8},Met(O$_2$)^{11}]SP was able to withstand the heating period for effective labelling. Should a better radiolabelling performance be required however, SP derivatives should rather be conjugated to other chelating agents such as acyclic chelators. On the other hand, DOTA has the advantage of enabling theranostic labelling of therapeutic radionuclides such as ^{177}Lu and ^{213}Bi.

Identification of possible degraded SP-derivatives (mainly suspected for [^{68}Ga]Ga-DSP-A and [^{68}Ga]Ga-DSP-B) was restrained; the challenge came about analysing cold samples of the components using HPLC-Mass Spectrometry (MS) to first verify the molar masses. Preliminary MS investigations yielded results of DOTA (MW = 687 g.mol^{-1}) and SP (MW = 1347.6 g.mol^{-1}) corresponding accurately to their respective masses. Due to the large molar mass of DSP-A/B and DOTA-[Thi^{8},Met(O$_2$)^{11}]SP (> 1500 m/z), their masses could not be confirmed on our instrument. During the MS analysis, the concentration of TFA in the mobile phase was lowered to reduce existing MS signal suppression but this also contributed to significant peak tailing.

Since the scope of the study included the ITLC analysis of blood and urine samples for eventual metabolites, the enzymatic stability of [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP was not considered to be tested in a separate blood-based assay. Unfortunately, logistical challenges made the latter sample analyses impossible.

7.2.2 *In vitro* investigations for DOTA-[Thi^{8},Met(O$_2$)^{11}]SP

The inability to mimic NK1R expression relevant to osteoarthritic foci *in vitro* implies that the results obtained from the *in vitro* study could not be directly linked to NK1R expression in pain disorders. However, fundamental *in vitro* work was necessary to gain a better understanding of the DOTA-[Thi^{8},Met(O$_2$)^{11}]SP analogues behaviour in

relation to the tachykinin receptor family. Literature reports *in vitro* studies of DOTA-[Thi^{8},Met(O$_2$)^{11}]SP but these were performed using isolated cells from tumours which could have a number of different receptor-mediated processes that illicit a response in the presence of the analogue. Furthermore, no comparison was made between the analogue's affinity for NK1R in relation to NK2R and NK3R. This is relevant since interaction with the other NKRs would result in a reduced target to non-target ratio. The benefit of the strategy employed in this study is that transfection of the receptor into a blank, "unbiassed" vehicle provides confidence that the measured response can only be attributed to interaction between the receptor and the ligand.

The study was not a classical binding study however the use of a transfected receptor model was sufficient to confirm the ability of DOTA-[Thi^{8},Met(O$_2$)^{11}]SP to target and activate NK1R. The DOTA conjugated peptide functions as an agonist at the receptor with no antagonistic behaviour. Activation of the receptor may result in undesired pharmacological effects. This will have to be carefully considered when attempting the translation into a clinical setting (nuclear medicine in particular). Thus, the characteristics of the injected dose will decide the severity of the agonistic effect.

7.2.3 *In vivo* application of [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP

The inability to verify radiolabelling efficiency using HPLC can be considered a limitation as HPLC is more sensitive than ITLC. However, during the development phase of the tracer, the results obtained from HPLC and ITLC was checked for their compatibilities. The variation in the data obtained from the two systems was limited to a maximum of 15%.

Enzymatic stability (based on degradation of the peptide in the biological system due to proteolytic enzymes) and the HPLC analysis of blood was not done. Addition of the tracer to fresh plasma would enable determination of stability over time giving an indication if the radiolabel would exchange with plasma proteins. Although literature suggests that the ^{99m}Tc-labeled DOTA-[Thi^{8},Met(O$_2$)^{11}]SP was stable in serum, this finding may be also confirmed for [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP. That being said, the *in vivo* biodistribution of the tracer demonstrated elevated and persisting uptake in characteristic NK1R-dense tissues such as the gut mucosa. This can be

considered an indirect confirmation that substantial amounts of tracer remain intact within human serum.

7.3 RECOMMENDATIONS

7.3.1 Radiolabelling and formulation of [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP

Pre-purification of the generator-derived ^{68}Ga-activity was not carried out prior to preparation of [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP. Including this step is a more frequently used approach to improve the specific activity of the final product as well as eliminate any co-eluted metals from the generator. The available procedures to prepurify ^{68}Ga-activity may be worth implementing, especially for future clinical investigations. In the clinical setting, radiopharmaceutical preparations will be undertaken using a generator that is within specification, that is, within its 9 month GMP shelf life. Despite this, pre-purification of the generator eluate is still an improvement that would ensure removal of unwanted metal ions and germanium-68 to assure patient safety. Even though our investigation provided a potent purification segment, other solid phase extraction material could also be considered. A rapid and efficient purification of the crude radiolabelling mixture is essential to improve the specific activity of the final product.

In the radiolabelling optimization process it is proposed that freeze-dried aliquots of the peptide is used to ensure accuracy with respect to the peptide molarity and increase the shelf-life of the peptide. The use of this “mini-kit” also facilitated a more straightforward radiolabelling solution. Going forward, the addition of a buffer to the peptide in the preparation of the freeze-dried aliquot would constitute a one vial radiolabelling kit that facilitates a more convenient radiosynthesis. Kit based radiopharmaceuticals (similar to the ^{99m}Tc based ‘shake-and-shoot’ kits and subsequent compounding) are favourable for routine use in hospitals and radio pharmacies.

7.3.2 *In vitro* investigations for [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP

A radioligand-based binding assay should be considered to evaluate the affinity of [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP for NK1R.

7.3.3 *In* vivo application of [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP

An NK1R blockade study should be carried out by way of co-administering an NK1R antagonist prior to the administration of [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP to determine the difference in tracer uptake (i.e. a reduction of uptake would indicate specific binding to NK1R).

If available, the use of a small animal model such as an osteoarthritic mouse model should be considered to afford an evaluation of the *ex vivo* biodistribution of [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP, because unlike a dog, a mouse can be sacrificed at certain time points to yield tissue samples for further *ex vivo* exploration (autoradiography, tissue staining for NK1R). The use of a few pre-clinical models can be beneficial to the process of translating pre-clinical findings to successful application in patients, including drug safety. In the case of imaging non-neuronal pain loci, the complexity of the underlying mechanism could warrant this non-invasive, holistic approach.

The PET/CT data that has been calculated from the image-guided biodistribution can be used to determine the radiation dosimetry of [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP that will provide an estimate of organ residence times and absorbed doses to critical organs. This is a significant parameter that would contribute towards establishing [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O$_2$)^{11}]SP as a routinely acceptable nuclear medicine tool and essential if therapeutic radionuclides were to be used in future.

APPENDIX 1A

ANIMAL SELECTION

The study included male or female large breed young adolescent and mature canines suffering from acute or chronic osteoarthritic pain symptoms as diagnosed in the Onderstepoort Veterinary Academic Hospital as well as a cohort of healthy dogs with no pre-existing health conditions. The dog owners were invited by their current treating veterinarian to participate in the study. They were given an information leaflet to read and an informed consent document which they had to sign if they were willing to allow their dog to be involved in the study. Thereafter the dogs were assessed and only dogs that appropriately fulfilled all the inclusion criteria and none of the exclusion criteria were enrolled into the study.

1. INCLUSION CRITERIA

In order for an animal to be included in this research, the animal had to meet the following requirements:

1.1 Dogs had to possess the clinical symptoms consistent with chronic osteoarthritic pain.

1.2 Female dogs should not have been pregnant or nursing puppies and owners were compelled to do their best to ensure that the dog did not fall pregnant during the course of the study.

1.3 Female dogs were subjected to a canine pregnancy test at screening and prior to imaging.

1.4 The dog owners were required to discontinue their dog's pain medication from a few days prior to the procedure.

1.5 The dog owners were required to consent to their dog undergoing the following standard procedures:

- Receive an intravenous bolus injection of the imaging agent into a vein in the leg.

- A central line for the administration and maintenance of anaesthetic throughout the procedure, as well as for the collection of blood samples.
- A catheter insertion for the collection of urine at the time intervals previously mentioned.
- Three PET/CT image acquisitions following [^{68}Ga]Ga-DOTA-Substance P injection (30, 60 and 120 min) including anaesthesia.

1.6 The dog owner was required to keep to the scheduled appointments for the procedures.

2. EXCLUSION CRITERIA

Dogs were excluded from participating in this study if they presented any of the following:

2.1 Female dogs with known or suspected pregnancy, lactation or intended pregnancy.

2.2 Dogs receiving treatment for cancer (radiation therapy, surgery, or chemotherapy) at the time of the study screening.

2.3 Animal owners who indicated that they have not strictly adhered to the instruction to discontinue their dog's medication prior to the first imaging session.

2.4 Animal owners whose dogs were participating in any other continuing pre-clinical study at the time of this study.

2.5 Dogs with any clinical and diagnostic indications of concern (laboratory, physical examination, other) that, in the judgement of the current treating veterinarian, would expose the dog to unnecessary risks if the dog were to be included in the study.

2.6 The following had also been evaluated by the current treating veterinarian and if found to be relevant resulted in exclusion of the dog from the study:

- Current condition of the dog - health status, body condition scores
- Any pre-existing condition that might put the dog at undue risk

3. WITHDRAWAL CRITERIA

An animal owner could decide at any time before or during the study to withdraw their informed consent for their dog and thereby withdraw their dog from the study for any reason. This had no impact on their treatment or management by the current treating veterinarian. The investigator of the study could also withdraw dogs for the following reasons:

a) Failure to comply with the protocol requirements.

b) Failure to complete the protocol specified evaluations.

c) Undesirable and unacceptable adverse reaction that in the judgement of the investigator will pose an unacceptable risk to the dog.

d) Detection of a condition or disease during imaging that could be adversely affected if the dog continues with the study. (In our study, one dog was diagnosed with pre-existing kidney failure and had to be euthanized as a result).

APPENDIX 1B

ANIMAL OWNER INFORMATION LEAFLET AND INFORMED CONSENT FORM FOR PILOT STUDY / NON-INTERVENTION STUDY

TITLE OF STUDY: An imaging study of [^{68}Ga]Ga-DOTA-(8,11)SP with dogs suffering from chronic pain

PART 1: INFORMATION LEAFLET

Dear Mr./Mrs. ..... Date …...../…...../…....

You are invited to volunteer your dog for a research study. This information leaflet is to help you decide if you would like your dog to participate. Before you agree to participate you should understand what is involved. If you have any questions, which are not fully explained in this leaflet, ask your dog's current veterinarian who has referred you to this study.

The aim of this study is to evaluate ^{68}Ga-Substance P as an imaging agent for chronic pain disorders. The biological part of the test compound is already present in the nervous systems of humans and dogs.

You will be required to take your dog off any pain treatment two weeks before the imaging study. You must also fast your dog at least 12 h prior to the imaging session.

When your dog arrives at the Nuclear Medicine Department, your dog will be anaesthetised and a catheter will be insertedin the vein of the leg in order to keep your dog under anaesthetics for the duration of the procedure. Your dog will also receive an injection of an anti-anxiety medication. A catheter will be inserted for the collection of urine and a second catheter for the collection of small blood samples (less than half a

teaspoon – 2 ml). Once your dog is stabilised on the camera bed, an injection of a small dose of ^{68}Ga-Substance P will be administered. After the injection your dog will be scanned using a PET-CT camera that is able to detect the signals from the ^{68}Ga that has been injected into your dog's body. The camera does not produce any radiation, so your dog will not be exposed to any additional radiation while the scan is being done. Images will be taken at 30, 60 and 120 min.

Small blood samples (2 ml each) will be taken at 5, 10, 20, 30, 60, and 120 minutes intervals after injection. Once each scan is complete, your dogs' bladder will be emptied using the catheter and the urine will be collected for analysis.

Once the procedure is complete, your dog will be monitored whilst recovering from the anaesthetic.

Female dogs must not be pregnant/nursing puppies for the duration of the study and will be required to undergo a canine pregnancy test before each imaging procedure.

The dose of radiation given is of a tracer level and less than the dose used routinely for imaging studies in humans in a nuclear medicine ward. ^{68}Ga has a short half-life of 68 min and is rapidly excreted from the body, therefore the exposure to radiation is minimal. The radioactivity is therefore not a concern and neither is the toxicity of the compound which is given at doses far below toxicity levels.

The information obtained from your animal will be useful for further human clinical studies.

A detailed protocol of this study has been submitted to the Animal Sciences Ethics Committee of the University of Pretoria and has received full approval.

I understand that if I do not consent for my dog to participate in this study it will not alter the management and treatment of my dog's existing condition by my dog's current treating veterinarian in any way. I may at any time withdraw my dog from this study.

You will not be responsible for any costs directly associated with the study. You will receive a travel allowance of R500 to cover your expenses in bringing your dog to Steve Biko Academic Hospital and returning home once the study is complete. Participation in the study is entirely on a voluntary basis. Aside from the travel allowance, you will receive no payment for involving your dog in the study.

PART 2: INFORMED CONSENT

I have read or had read to me in a language that I understand the above information before signing this consent form for my dog to participate in the study. The content and meaning of this information has been explained to me. I have been given the opportunity to ask any questions and am happy that they have been answered to my satisfaction. I was given enough time to carefully consider my decision to allow my dog to participate.
I confirm that I am over 21 years of age and the rightful owner of the dog in question and therefore am capable of giving my consent for my dog to participate in this study. I understand that if my dog does not participate it will not alter my dog's current management in any way. I understand that I can withdraw my dog from the study at any time.
Female dogs: I confirm that to the best of my knowledge my dog is currently not pregnant or nursing puppies. I understand that I should ensure to the best of my ability that my dog does not fall pregnant during the study due to the possible radiation risk to an unborn puppy. I agree that if my dog falls pregnant that I will let the investigator know immediately.
I hereby volunteer for my dog to take part in this study. I acknowledge that I have received a signed copy of this informed consent agreement for my own records.

Personal details of my dog:

Name: _____

Breed: _____

Age: _____

Sex: _____

Colour: _____

.....

Dog Owners name (Please print)

....

Dog Owners signature Date

.....

Referring Veterinarians name (Please print)

....

Referring Veterinarians signature Date

.....

Name of the Veterinarian responsible for animal care during the study (Please print)

....

Veterinarian responsible for animal care during the study Date

.....

Principle Investigator's name (Please print)

....

Principle Investigator's signature Date

APPENDIX 1C

SCHEDULE OF STUDY EVENTS FOR PRE-CLINICAL IMAGING STUDY WITH DOGS

Study Period	**Screening**	**Weaning**		**Imaging session**
Study Week	**-4**	**-2**		**0**
Day		**1**	**14**	**1**
Informed Consent	x			
Medical History	x			
Clinical Assessment*	x			x
Inclusion/Exclusion	x			
Concomitant Medication	x			
Pregnancy test	x			x
Discontinue treatment regime (dysplastic dogs)		x		
Fasting			x+	
Imaging Agent Administration				x
Urine collection				x
Blood samples				x

*Temperature, pulse, respiratory rate, weight/body condition

METHODOLOGY

1.1. Dog owners with dogs that possess the clinical symptoms that are consistent with chronic osteoarthritic pain are invited by their treating veterinarian to participate in the study during Study Week -4. The animal owners are given the information leaflet and informed consent document for review. They are given sufficient time to ask questions and consider the participation of their dog in the study.

1.2. Once the animal owners sign the informed consent, their dog is assessed for suitability against the inclusion and exclusion criteria by the principle investigator. Only the dogs which meet the criteria are enrolled into the study. This is within Study Week -4.

1.3. The animal owners of the enrolled dogs will be asked to discontinue the pain medication that they are currently taking for their condition. This is Day 1 of Study week -2 which will be on a Saturday.

1.4. On Day 14 of Study Week -2 the dogs will need to be fasted from food starting at 10 pm that evening.

1.5. On Day 1 of Study Week 0 the dogs will be received at the Nuclear Medicine Department of the Steve Biko Academic Hospital where they will be anaesthetised for 150 min. During this period the following will take place:

- A catheter will be inserted for collection of urine which will take place immediately after each scan.
- A central line inserted for the maintenance of anaesthetic and collection of blood samples.
- Monitoring apparatus for pulse and SpO_2.
- Dogs will be injected with a small dose of ^{68}Ga-Substance P.
- Dogs will undergo PET/CT imaging 30, 60 and 120 min post injection.

1.6. On Day 2 of Study Week 0 the dogs may resume with their usual pain treatment.

APPENDIX 2

RADIOLABELLING DATA CAPTURE SHEET

Radiolabelling of DOTA-(8,11)SP with Ga-68 **DATE:** _____

DOTA Substance P: 50 µg (freeze dried in 100 µl of 0.01 M HCl)

Reconstituted with: 250 µl Na Acetate buffer (2.25 M, prepared _____) (vortex to mix) pH = _____ (measure with pH strip)

Fractionated elution: 2 ml fraction activity:_____MBq @_____(time)

8 ml fraction activity (unused): _____MBq @_____ (time)

Add _____µl Ga-68 and mix (vortex for a few seconds) pH = _____ Activity: _____MBq @_____(time)

Heat for 15 mins at 95 ˚C Time period: _____

Activity after heating: _____MBq @_____(time)

Crude ITLC result: Product ___ % Free Ga-68 ___% Colloid ITLC result:___%

Activity before loading: _____@_____ (time)

Purification (Sep-Pak C18 light cartridge):

Conditioned with 4 ml EtOH, 2 ml MilliQ H2O

Load & rinse (1 ml saline) + 6 ml saline rinse activity: _____MBq @_____ (time)

Empty vial & syringe activity: _____ MBq @_____ (time)

Empty Cartridge activity: _____ MBq @_____ (time)

10 % EtOH fraction (1 ml): _____ MBq @_____ (time) ITLC:_____ %

20 % EtOH fraction (1 ml): _____ MBq @_____ (time) ITLC:_____ %

30 % EtOH fraction (1 ml): _____ MBq @_____ (time) ITLC:_____ %

40 % EtOH fraction (1 ml): _____ MBq @_____ (time) ITLC:_____ %

Formulation: 1ml 30 % EtOH + 5 ml saline = 5 % EtOH, 6ml total volume. Wet filter with little saline, Filter (Filter: Millex GV) all 6 ml into vacu vial and then pass remainder of 1 ml saline to rinse filter, Activity of solution: _____MBq @ _____ (time) therefore _____ MBq/ml

Activity in syringe_____ MBq @ _____ (time) in _____ ml

Activity in empty syringe after injection: _____ MBq @ _____ (time)

APPENDIX 3

SUPPLEMENTARY DATA

Supplementary Table 1: Summary of the %LE obtained from HPLC and ITLC analysis of crude preparations of [^{68}Ga]Ga-DOTA-[Thi^{8},Met(O_2)^{11}]SP whilst varying reaction parameters.

PARAMETERS n ≥ 3	% LABELLING EFFICIENCY (%LE)							
	HPLC	ITLC	HPLC	ITLC	HPLC	ITLC	HPLC	ITLC
^{68}Ga-PEPTIDE ACIDITY (pH)*	*< 3.5*		*3.5 - 4.2*		-		-	
	48.1±16.4	58.5±30.4	65.8	78.0 ±1.2				
TEMPERATURE (°C)	*25***		*60*		*95*		-	
	0	0	57.8±1.3	75.0±0.5	93.2±1.7	100±0		
TIME (min)	*5*		*10*		*15*		-	
	91.2±2.1	97.0±2.1	92.9±1.0	98.6±1.8	93.2±1.7	98.8±1.2		
PEPTIDE MOLARITY (nmol)	*9*		*14*		*28*		*46*	
	15.7±1.7	27.7±8.9	16.0±3.6	32.3±7.9	93.2±1.7	98.8±1.2	87.7±5.0	95.7±1.9

*pH of solution prior to heating.

*At ambient temperature and t=0 there was zero radiolabelled product. The solutions were re-analysed 180 min later and there was ~3 % radiolabelled product.

APPENDIX 4

ETHICS APPROVAL V089-14

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Evaluation of an inflammation imaging agent for use in the detection of fibromyalgia**
PROJECT NUMBER	**V089-14**
RESEARCHER/PRINCIPAL INVESTIGATOR	**J Suthiram**

STUDENT NUMBER (where applicable)	**144 645 932**
DISSERTATION/THESIS SUBMITTED FOR	**PhD**

ANIMAL SPECIES	**Canine**	
NUMBER OF ANIMALS	**18**	
Approval period to use animals for research/testing purposes		**November 2014 – November 2015**
SUPERVISOR	**Prof. MM Sathekge**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date	24 November 2014
CHAIRMAN: UP Animal Ethics Committee	Signature	

ETHICS APPROVAL V089-14 (AMENDMENT 1)

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Evaluation of an inflammation imaging agent for use in the detection of fibromyalgia**
PROJECT NUMBER	**V089-14 (Amendment 1)**
RESEARCHER/PRINCIPAL INVESTIGATOR	**J Suthiram**

STUDENT NUMBER (where applicable)	**144 645 932**
DISSERTATION/THESIS SUBMITTED FOR	**PhD**

ANIMAL SPECIES	**Canine**	
NUMBER OF ANIMALS	**18**	
Approval period to use animals for research/testing purposes		**May 2016 – May 2017**
SUPERVISOR	**Prof. MM Sathekge**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 30 May 2016
CHAIRMAN: UP Animal Ethics Committee	Signature

ETHICS APPROVAL V089-14 (AMENDMENT 2)

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Evaluation of an inflammation imaging agent for use in the detection of fibromyalgia**
PROJECT NUMBER	**V089-14 (Amend 2)**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Mrs. J Suthiram**

STUDENT NUMBER (where applicable)	**UP_144 645 932**
DISSERTATION/THESIS SUBMITTED FOR	**PhD**

ANIMAL SPECIES	**Canine**	
NUMBER OF ANIMALS	**16**	
Approval period to use animals for research/testing purposes		**July 2017 – July 2018**
SUPERVISOR	**Prof. MM Sathekge; Prof. JR Zeevaart**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 27 July 2017
CHAIRMAN: UP Animal Ethics Committee	Signature