

**Gallium-68 and fluorine-18 labeling of a peptide binding
to the human transferrin receptor and determination of
its uptake into transferrin receptor-expressing human cell
lines**

by

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“A thesis submitted to McGill University in partial fulfillment of the requirements
of the degree of Master of Science”

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ABSTRACT

An often proposed mechanism to overcome the system of efflux transporters and tight junctions present at the blood brain barrier (BBB) is the receptor-mediated transport via endocytosis. Among the proposed transport vehicles, the transferrin receptor (TfR) has been considered to be one of the most attractive targets. Besides transferrin itself and the antibody OX26 (anti-rat TfR-antibody), it has recently been shown that peptides are also able to bind to the transferrin receptor and are internalized into TfR expressing cells. In this study we evaluated the ability of the ^{68}Ga -labeled peptide THRPPMWSPVWP to become internalized into human TfR bearing cells to pre-evaluate its potential to act as a carrier system for small molecules across the BBB utilizing the TfR receptor located on the endothelial cells. To prove the validity of this concept we first synthesized the peptide THRPPMWSPVWP, and then a conjugate consisting of the D2 receptor ligand fallypride and the peptide which was synthesized by our collaborators and evaluated as to its binding affinity towards the D2 receptor. The conjugate still showed a high receptor affinity ($K_i = 27 \text{ nM}$) despite the strong structural alteration exerted by the peptide.

For radioactive-labeling, the reported TfR-binding peptide THRPPMWSPVWP was derivatized with p-SCN-Bn-NOTA. It was then labelled with ^{68}Ga by our collaborators. The uptake of the ^{68}Ga -labelled peptide into TfR-bearing cells was investigated using two human TfR-expressing cell lines, the human glioblastoma cell line U87MG and the human colon adenocarcinoma cell line HT-29 to assess the peptides' potential as a carrier molecule targeting the TfR

receptor . The binding was found to be less than 1% after 60 min incubation at 37°C.

These results suggest that the concept of using peptide THRPPMWSPVWP to act as a carrier system for short lived PET radio-pharmaceuticals across the BBB within a reasonable time span is doubtful. However, further experiments have to be carried out to determine the potential of this peptide as transferrin receptor targeting carrier molecule for therapeutic agents.

Besides, and owing to the need for optimization of the synthetic procedures for ^{18}F labeling of biologically-active peptides for clinical PET, we also investigated the possibility to synthesize a glucose based labeling synthon 2,3,4-tri-O-acetyl-1-azido-6- ^{18}F fluoro- β -D-glucopyranoside, to radiolabel alkyne derivatized peptides with ^{18}F using click chemistry. Our strategy would combine the advantages of carbohydration of peptides with the fast and efficient click reaction. We synthesized 2,3,4-tri-O-acetyl-1-azido-6-O-p-toluene sulfonyl- β -D- glucopyranoside in an overall chemical yield of 80%, which served as a labeling precursor bearing an azide group ready for coupling to an alkyne derivative of a biologically active peptide by click chemistry and a p-toluene sulfonyl group ready for radio-labeling with ^{18}F by a nucleophilic substitution reaction. The second compound was 2,3,4-tri-O-acetyl-1-azido-6-fluoro- β -D-glucopyranoside in an overall chemical yield of 77%, which served as a reference compound to establish HPLC and TLC conditions for the radio-labeling experiments. Initial labeling experiments with nucleophilic ^{18}F did not yield the

desired ^{18}F -labeled glucose derivative so far. We are currently taking efforts to radiolabel the precursor with ^{18}F by re-crystallizing the precursor several times as it was proven by HPLC that it still contains some impurities. These impurities can compromise the intended labeling reaction with ^{18}F .

Résumé

Un mécanisme proposé expliquant le transport par efflux présent dans la 'blood brain barrier' consiste en un système de transport membranaire assisté faisant intervenir des récepteurs spécifiques, par d'endocytose. Parmi les vésicules de transport proposées, les récepteurs de transferrine (TfR) sont considérés comme les plus attractives. A part la transferrine et l'anticorps pour clone MRC OX-26 (anticorps TfR anti-rat), il a été montré que des peptides sont aussi capables de se lier spécifiquement au récepteur de la transferrine, puis d'y être internalisé dans les cellules possédant les récepteurs à transferrine. Dans cette étude, nous avons évalué la possibilité du peptide 'THRPPMWSPVWP' marqué avec du ^{68}Ga de s'internaliser dans des cellules humaines présentant les récepteurs TfR. Nous avons aussi évalué son potentiel de se comporter comme vecteur de transport de petites molécules à travers la 'blood brain barrier'. Nous avons utilisé des cellules endothéliales avec des récepteurs TfR. Pour prouver la validité du concept, nous avons synthétisé le peptide THRPPMWSPVWP, puis conjugué au fallypride de type D2, qui est un ligand avec une grande affinité pour les récepteurs du TfR. L'ensemble a été évalué par nos collaborateurs afin de vérifier l'affinité de liaison vis-à-vis des récepteurs du TfR. Ce conjugué a montré une grande affinité précitées ($K_i = 27 \text{ nM}$), malgré de fortes altérations au niveau de sa structure causées par la présence du peptide. Pour le marquage radioactif, le peptide THRPPMWSPVWP bioconjugué ci-dessus a été dérivé avec le p-SCN-Bn-NOTA et radio-marqué au ^{68}Ga par nos collaborateurs. Les propriétés de ce peptide ont été étudiées en utilisant deux lignées cellulaires différentes présentant

les récepteurs TfR, soit des cellules de type tumoral glial (*U-87 MG*) et des *cellules* carcinomes de côlon humain (*HT-29*), afin d'étudier le potentiel du peptide à se comporter comme transporteur de molécules en utilisant les récepteurs TfR. L'affinité pour ces derniers a été de moins de 1% après une incubation de 60 minutes à 37°C. Ces résultats suggèrent que le concept utilisant le peptide THRPPMWSPVWP comme système de transport de molécules marquées à travers de la 'blood brain barrier' est peu efficace. Cependant, de nouvelles expériences devront être effectuées pour déterminer le potentiel de ce peptide à se comporter comme transporteur actif d'agents thérapeutiques en utilisant les récepteurs TfR.

D'autre part, du fait de l'optimisation des procédures de synthèse pour le radio marquage au ^{18}F de peptides biologiques actifs, dans le cadre d'études cliniques de 'PET', nous avons envisagé de synthétiser un nouveau synthon radio-marqué dérivant du glucose, le 2,3,4-tri-O-acetyl-1-azido-6- ^{18}F fluoro- β -D-glucopyranoside, pour radio-marquer des dérivés de type 'alkyne', par la technique de 'click chemistry'. Notre stratégie combine les avantages du processus de 'carbohydratation' des peptides et de la réaction de 'click chemistry'. Nous avons synthétisé le 2,3,4-tri-O-acetyl-1-azido-6-O- p-toluene sulfonyl β -D-glucopyranoside avec un rendement de 80%. Ce dernier possède un groupe p-toluène sulfonyl qui sert de précurseur pour radio-marquer la molécule au ^{18}F par une réaction de substitution nucléophile, et présente également un groupement 'azide', permettant le couplage rapide avec un peptide actif possédant une fonction 'alkyne'. Le 2eme composé préparé est le 2,3,4-tri-O-acetyl-1-azido-

6-fluoro- β -D-glucopyranoside, avec un rendement de 77%. Ce dernier servant de référence pour établir les conditions HPLC et par CCM nécessaires au radio-marquage. Les études préliminaires avec le ^{18}F comme nucléophile n'ont pas conduit au composé de type 'glucose' radio-marqué au ^{18}F . Nos efforts actuels se portent sur le radio-marquage du précurseur au ^{18}F en recristallisant ce dernier. Il a été montré par HPLC que ce dernier contient des impuretés, qui empêcheraient le radio-marquage au ^{18}F .

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CONTRIBUTIONS OF AUTHORS:

Our unpublished manuscript is a co operational work between different research institutions. The author of the thesis, Dina Nada, synthesized the peptide THRPPMWSPVWP and the peptide derivative NOTA-Ahx-THRPPMWSPVWP. Carmen Wängler synthesized the Fallypride-S-maleimido-THRPPMWSPVWP conjugate, and labeled NOTA-Ahx-THRPPMWSPVWP with ^{68}Ga . Georg Höfner and Klaus T. Wanner evaluated the binding affinity of Fallypride-S-maleimido-THRPPMWSPVWP to the D2 receptor using the D2 receptor expressing cell line HEK293. Simone Maschauer and Olaf Prante performed uptake experiments with the ^{68}Ga -labeled NOTA-Ahx-THRPPMWSPVWP using HT29 and U87MG cells expressing the transferrin receptors TfR1 and TfR2.

Note: Radio-labeling of the peptide THRPPMWSPVWP with ^{68}Ga was done by Dr. Carmen Waengler (McGill University, McConnell Brain Imaging Centre), by the group of Prof. Dr. Bartenstein (Munich University, Department of Nuclear Medicine, Germany) who possess a $^{68}\text{Ge}/^{68}\text{Ga}$ generator system and PD Dr Olaf Prante (University of Erlangen, Germany).

PREFACE

The main goal of our research was to investigate the potential of the THRPPMWSPVWP peptide to act as molecular taxi, having the potential to carry small molecules into the brain via receptor-mediated transport using the transferrin receptor TfR. This would be attained by performing uptake experiments and investigating its ability to accumulate in human TfR expressing cell lines. For the uptake experiments, it was necessary to find a method to radiolabel the peptide.

We therefore synthesized the peptide THRPPMWSPVWP and a derivative of the peptide NOTA-Ahx-THRPPMWSPVWP to facilitate its labeling with ^{68}Ga for cell uptake experiments. We also wanted to radiolabel the peptide with the radioisotope ^{18}F . In order to radiolabel the peptide with ^{18}F , a prosthetic group is needed. Hence, we investigated the possibility to synthesize a glucose based prosthetic group, to radiolabel an alkyne derivative of the peptide THRPPMWSPVWP with ^{18}F using click chemistry. The prosthetic group presented in our research might be used to radiolabel any biologically active alkyne-derivatized peptide. We designed the prosthetic group and succeeded in all steps towards its synthesis except for the last step which is the radio-labeling of the labeling precursor with ^{18}F .

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CHAPTER 1

Introduction

1.1 Positron Emission Tomography

Positron emission tomography (PET) is a high resolution, non-invasive, imaging technique which started in the early 1970s (1). Nowadays, it represents a unique tool for the in vivo study of biochemistry in living subjects (2). For doing PET, the patient is injected with a biologically active molecule which is radio-labeled with a suitable positron emitting radionuclide (tracer molecule), then, the patient is placed into a PET camera (fig 1). The detectors then detect the radioactivity distribution non-invasively finally revealing the actual area of accumulation of the tracer. 3D images are then reconstructed by computer analysis, giving a time-dependent resolved image of the tracer kinetics.

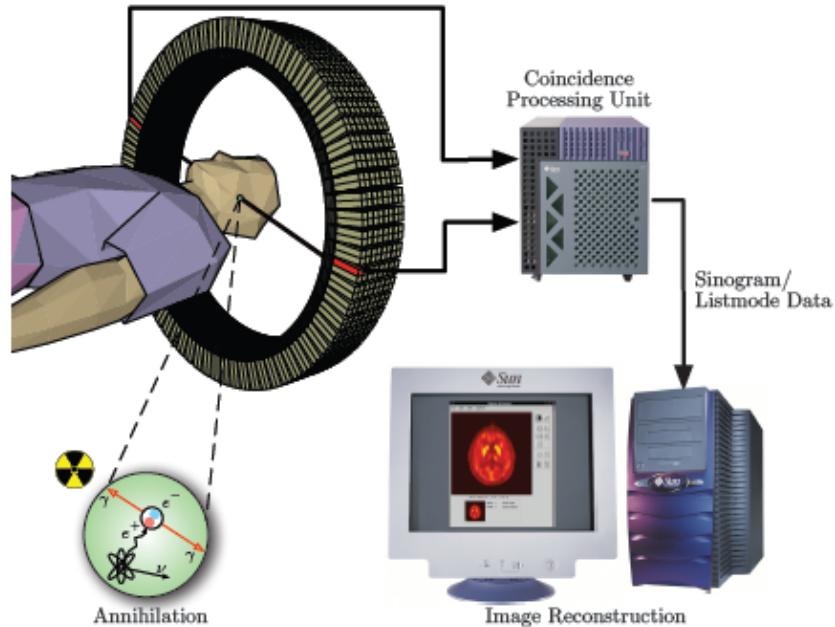


Fig 1: A patient, who was injected with a radioactive tracer probe, is placed into a PET camera. The radioactivity distribution of the PET tracer is determined in a time-dependent manner (3).

The radio-nuclides which are used for PET are positron emitters which decay by positron (β^+) emission. The positron is the antimatter particle of an electron and has a very short half life. This positron forms a positronium after traveling a few mm in tissue by combining with an electron (β^-) forming a hydrogen like system which exists only for a fraction of a second. In a subsequent annihilation reaction (fig 2), both masses, electron and positron, are converted into energy. This energy appears in the form of two 511 KeV gamma rays emitted in opposite directions (angle of 180°). These γ -rays are then detected simultaneously by PET detectors forming a line of response. If this is done with

thousands of detectors, this “coincidence imaging” which is the outstanding feature of a PET camera renders radioactivity quantification possible (1, 4). The detection principle of PET is thus not the positron detection, but the positronium annihilation leading to two simultaneously emitted gamma rays.

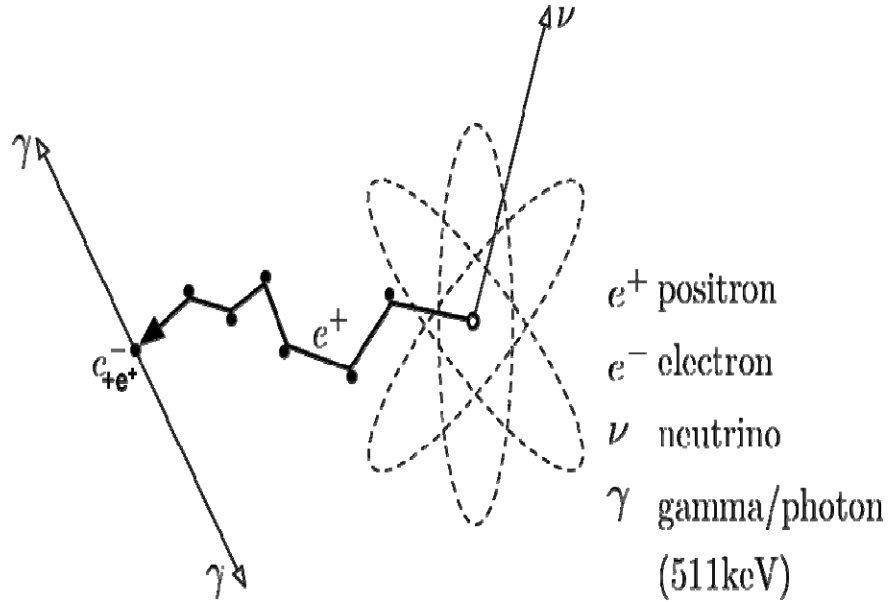


Fig 2: β^+ decay and subsequent positron-electron annihilation process resulting in two 511 KeV gamma rays (5).

PET provides unique information that is not provided by other imaging techniques (1). This imaging modality provides the advantage of quantifying biological distribution of known radioactively labeled molecules at a given point in time. With a series of images, changes in bio-distribution of these PET tracers can be studied and quantified in a time-dependent manner, in various living

systems (4). This quantitative analysis of PET images allows for the investigation of the mechanism of different diseases and their progress in detail (6).

PET has wide clinical applications especially in the fields of cardiology, oncology, and neurology (7). The most frequently applied radio-nuclides for PET are carbon-11 and fluorine-18 with physical half lives of 20 min and 110 min respectively. The most widely used radiopharmaceutical nowadays is 2-[^{18}F] fluoro-desoxyglucose (^{18}F]FDG) (4), a radio-labeled glucose analogue used for measuring energy consumption in cells (8).

In oncology, ^{18}F]FDG PET is widely used in diagnosis, staging, monitoring response to treatment and detecting recurrence of tumors (9). This is applicable in many types of tumors such as lung cancer, colorectal cancer, lymphoma, melanoma, esophageal cancer, head and neck cancer and breast cancer (10). After injection, ^{18}F]FDG which acts as a glucose analogue, accumulates in tumor cells which are metabolically very active (11, 4) allowing for the imaging of these cells by PET. It is expected that in the future PET will play a predominant role in the routine examination of malignant diseases (9).

1.2 Brain Imaging with PET

In neurology, PET is extremely useful, because it allows studying the different interactions between neuro-chemical pathways, understanding relationships between pharmacological changes and functional and behavioral responses, differential diagnosis of neurological disorders, evaluation and follow

up of treatment effects (6). These applications are possible with the availability of many PET tracers which are used in imaging and quantification of cerebral blood flow (H_2^{15}O , the PET nuclide is oxygen-15 with a half life of 2 min only), oxygen metabolism ($^{15}\text{O}_2$), glucose metabolism ($[^{18}\text{F}]\text{FDG}$), and receptor binding agents for example D2 receptors using $[^{11}\text{C}]$ raclopride (12) and $[^{18}\text{F}]$ fallypride (13, 14).

PET is very useful in helping in the detection and characterization of Alzheimer's disease (15) as well as in detecting abnormalities in brain biology and neurochemistry in mood and psychiatric disorders (16). In Parkinson's disease, there is a biochemical deficiency in the dopaminergic system which can be imaged by 6- $[^{18}\text{F}]$ fluorodopa ($[^{18}\text{F}]\text{FDOPA}$) or other dopaminergic ligands (6). Consequently, differential diagnosis as well as selection of the appropriate therapy can be achieved (6).

1.3 The human blood brain barrier is the ultimate obstacle for effective drug delivery to the brain

Unfortunately, only a small fraction of the brain PET tracer used for PET imaging enters the brain mostly by passive transport through the restrictive blood brain barrier (BBB). In fact, the delivery of drugs across the blood brain barrier (BBB) still represents a difficult problem in pharmaceutical drug design. This limits the application of promising substances which could be useful for therapeutic or diagnostic applications.

In this context, a conceivable concept to overcome this problem for diagnostic purposes would be the conjugation of radio-tracers, intended for brain

imaging with positron emission tomography (PET), to molecular taxis to accomplish carrier mediated transport across the highly restrictive BBB. Radiotracers for neurological imaging such as [^{18}F]DMFP (17), [^{18}F]FP (13, 14), [^{11}C] raclopride (18, 19), [^{18}F] flumazenil (20, 21), [^{18}F] altanserin (22, 23), [^{124}I]IBZM (24, 25) and [^{18}F]FP-CIT (26) would profit by this strategy to enhance their uptake into the brain for improved image quality and lower administered doses.

1.4 The transferrin receptor (TfR)

In principal, different approaches are available to overcome the BBB by receptor-mediated transport systems. The most frequently used transporter system is the transferrin receptor (TfR) which physiologically mediates the iron uptake into the brain by transferrin internalization (27, 28, 29, 30). Hence, substances binding to the transferrin receptor have been used as carriers for different classes of molecules into the brain. Furthermore, the TfR is present on many human cancer cell lines making it an attractive target for enhanced drug delivery of anti-cancer compounds (31).

Besides the endogenous transferrin which was used for the delivery of antibodies and nanoparticles into the brain (32, 33, 34), other ligands can be used for the transport via the transferrin receptor such as antibodies directed against the TfR. Examples include OX26, an antibody against the rat transferrin receptor used to transport nanoparticles over the blood brain barrier (34, 35, 36, 37), antibody fragments and scFvs targeting the TfR (37, 38). Interestingly, the extent

of the uptake of these conjugates has rarely been quantified and data about transport kinetics are still lacking. Especially for the development of new brain imaging agents on the basis of this proposed transporter mediated uptake concept, kinetic data taking the short half life of PET isotopes into consideration are of crucial importance. Thus, the concept of transporting small molecules over the blood brain barrier by targeting the transferrin receptor within the short time-frame dictated by the short half-life of PET isotopes used in brain imaging has to be determined.

1.5 Peptides binding to the transferrin receptor

Two small peptides – HAIYPRH and THRPPMWSPVWP – have been identified by phage display and have shown to target the human TfR and to internalize GFP in TfR-expressing cells (39). Recently, an artemisinin-conjugate of HAIYPRH has also been demonstrated to be a promising agent for cancer treatment of TfR-expressing cells (40). These two peptides have a binding affinity towards the TfR receptor with a K_d of approximately 10nM (40). Interestingly, there is not much data available quantifying the transport efficiency itself. In order to properly assess and judge a conceivable transporter system, kinetic data giving an insight into the uptake efficiency are needed. The transport of OX26 and derived conjugates into the rat brain for example has been controversially discussed in the literature. Although many OX26-drug conjugates have been proposed and it has been claimed that OX26 undergoes receptor-mediated transcytosis, the radio-labeling of OX26 with a radio-iodine isotopes revealed a

restricted uptake into the post-capillary compartment of the rat brain. This important data casts doubts on the usability of OX26 in general (41) and exemplifies the need to validate a potential carrier molecule appropriately.

From the two reported peptides, THRPPMWSPVWP showed a higher affinity to the TfR than HAIYPRH and was therefore the focus of our investigations. We sought to investigate the potential of THRPPMWSPVWP peptide to act as molecular taxi, which can carry small molecules to brain via receptor-mediated transport systems targeting TfR. This would be attained by performing uptake experiments and investigating its ability to accumulate in human TfR expressing cell lines. For uptake experiments to be done radio-labeling of the peptide is needed.

1.6 Radio-labeling of peptides for PET

As mentioned before, imaging with PET requires radio-labeling with a suitable positron emitting radionuclide. There are many positron emitters available e.g. ^{64}Cu , ^{11}C , ^{13}N , ^{15}O , ^{18}F , ^{66}Ga , ^{68}Ga , ^{76}Br , $^{94\text{m}}\text{Tc}$, ^{86}Y , ^{124}I and many others. The “organic radio-isotopes” are ^{11}C ($t_{1/2}=20.4$ min), ^{13}N ($t_{1/2}=10$ min), ^{15}O ($t_{1/2}=2$ min), and ^{18}F ($t_{1/2}=110$ min) (9). These radio-nuclides are produced in a cyclotron. Carbon, oxygen, nitrogen and hydrogen are the main components of biological molecules. Hydrogen has no positron emitting isotope, so ^{18}F is used to replace a hydrogen atom in molecules (7). The half-lives of ^{11}C , ^{15}O , and ^{13}N range from 2 to 20 minutes, restricting their use in terms of chemistry (42). On the

other hand very long half-lives might subject the patients to a higher dose of radiation. Thus, radio-nuclides whose half-lives are neither too short nor too long are needed.

^{18}F has an optimum half-life (110 min) for most investigations, which is long enough allowing some time for radio-synthesis and permits transportation from the cyclotron to outside clinics. Besides this, ^{18}F has many other advantages: it has a low positron emission energy (635 KeV) allowing for improved image quality, no additional γ -emissions and it can be produced with a high specific activity. In addition, its introduction into non-fluorine containing biological molecules does not lead to significant sterical changes, because the fluorine atom is small (isosteric to H) (42). However it has to be shown in every case that the fluorine-containing molecule still binds to the target (a receptor or an enzyme). For all these advantages and because of its excellent nuclear and chemical properties, ^{18}F is an almost ideal radionuclide for PET.

A good alternative for ^{18}F might be gallium-68 (^{68}Ga). ^{68}Ga is a positron emitter radionuclide. Its half-life is 68 minutes which is suitable to investigate the pharmacokinetics of most peptides (43). It decays by 89% positron emission and 11% electron capture. It is readily available through a $^{68}\text{Ge}/^{68}\text{Ga}$ generator. Its mother radionuclide germanium-68 (^{68}Ge) has a long half-life of 271 days (43). Generators have the advantage of the ease of transportation without the need of an on-site cyclotron.

1.7 Radio-labeling with ^{68}Ga

The Ga^{3+} ion is classified as a hard Lewis acid, which forms thermodynamically stable complexes with ligands that are hard Lewis bases (44). Hence, ligands with nitrogen and/or oxygen donor atoms (e.g. carboxylate and amine groups) are considered good chelating agents for Ga^{3+} . There are many suitable chelators for gallium available. The majority of these chelators are hexadentate ligands. Macrocyclic chelators, especially triaza ligands, have high conformational and size selectivity towards metal ions. Gallium forms high thermodynamically stable complexes with triazamacrocyclic ligands (44). This is because of the excellent fit of this small metal into the cavity of these ligands which have the ability to bind gallium with high efficiency. 1,4,7-Triazacyclononane-1,4,7-triacetic acid (NOTA) is such a suitable hexadentate chelator for gallium. The neutral Ga-(NOTA)-complex is a very stable complex which only undergoes dissociation under acidic conditions (44). So, coupling of NOTA or a derivative of NOTA to the peptide would allow for a rapid and efficient labeling of the peptide with ^{68}Ga .

1.8 Radio-labeling with ^{18}F

Radio-labeling of biologically active short peptides with ^{18}F for PET has been extensively studied in recent years. This has resulted in a large number of ^{18}F labeled peptides for PET, e.g. [^{18}F](D-Phe1)octreotide (45), [^{18}F] Galacto-

RGD (46), [^{18}F]FB-PEG-RGD (47), [^{18}F]NT (48), [^{18}F](NIe4,D-Phe7)- α -MSH (48). [^{18}F] Galacto-RGD showed promising results for the assessment of the expression of $\alpha_v\beta_3$ in tumor cells by PET (46).

So far, the synthetic procedures for labeling with ^{18}F are suboptimum, they are time consuming and multiple steps for their production are required reducing the overall radio-chemical yields (49). Automation is difficult to achieve. Consequently, there is a potential for optimization of the synthetic procedures for ^{18}F labeling of peptides.

1.8.1 Prosthetic groups for radio-labeling of peptides with ^{18}F

For the introduction of ^{18}F into peptides it is very important to synthesize a so called prosthetic group which is a small labeling agent already containing ^{18}F . The direct nucleophilic fluorination of bio-molecules with H-acidic functions is not possible as the nucleophilicity of ^{18}F is reduced by the formation of H-bonds present in the peptide (45).

Many attempts for the introduction of ^{18}F into peptides via prosthetic groups have been described in the literature. For example, N-succinimidyl 4- ^{18}F fluorobenzoate which was coupled to peptides through an acylation reaction, 2- ^{18}F fluoropropionic acid which was coupled to peptides by solid-phase synthesis, p- ^{18}F fluorophenacyl bromide which was coupled to peptides through an alkylation reaction, 4- ^{18}F fluorobenzyl halides which were coupled to peptides through a benzylation reaction, have been used (50). ^{18}F labeled thiols

were used for labeling N-terminal chloro acetylated peptides (51) and ^{18}F -labeled maleimide derivatives have been used as thiol-reactive reagents (52, 53). Toyokuni et al reported the synthesis of N-{4-[(4- ^{18}F) fluorobenzylidene) aminoxy] butyl} maleimide, which was coupled to a peptide containing cysteine in two steps (52). Bruin et al also described a maleimide derivative reagent which is pyridinyl-substituted maleimide reagent [^{18}F]FPyME, which was also used for coupling to peptides bearing a thiol functional group (53). Poethko et al. reported the labeling of aminoxy peptides with [^{18}F] fluorobenzaldehyde by oxime formation. This strategy was used for radiolabeling of RGD peptides and somatostatin analogues (54). One of the most commonly used ^{18}F prosthetic groups is 4-nitrophenyl 2- ^{18}F fluoro propionate which is conjugated to the amino group of the target peptide through an acylation reaction (45).

Unfortunately, most of these prosthetic groups increased the lipophilicity of the peptides leading to a reduction in their bioavailability causing a high hepatic and intestinal uptake (49). Consequently, a chemical modification is needed, introducing a more hydrophilic prosthetic labeling precursor to overcome the unfavorable pharmacokinetics of commonly applied labeling synthons.

1.8.2 Carbohydration

Carbohydration seems to be a promising chemical tool. Sugar compounds are hydrophilic compounds, reducing the overall lipophilicity when attached to biologically active peptides, improving their properties such as bioavailability

(55), solubility under physiological conditions (56), resistance against proteases (57), and blood-brain barrier penetration (58).

Glycopeptide derivatives of an oligopeptide rennin inhibitor exhibited excellent intravenous and oral hypotensive activity. Clearance of these peptides was shifted from the fast liver biliary pathway to the slow renal pathway, leading to an increased concentration in the serum, which consequently led to prolongation of the hypotensive activity (59, 60). Glycosylated derivatives of the tetrapeptide Gly-Gly-Tyr-Arg were studied and proved to have increased resistance to aminopeptidases. Moreover, the Na⁺ dependent glucose transporter SGLT-1 helped in the intestinal absorption of α and β -glucopyranoside derivatives (61). Schottelius et al (62) attempted to modify the pharmacokinetics of somatostatin receptor-agonists using different sugar compounds. The results showed a reduction in liver uptake and an elevation in tumor uptake, leading to high tumor-to-organ ratios. Wester and his group made several studies to the optimization of octreotide analogues (63, 64, 65) and integrin antagonists (66, 67), using the strategy of carbohydration. They proved that these modifications improved the pharmacokinetic properties of peptides efficiently without affecting their affinities to their binding sites.

Consequently it would be an obvious advantage to use a radio-labeled glucose derivative as a prosthetic group for the introduction of ¹⁸F into biologically active peptides to get less lipophilic derivatives. Few studies reported the use of an ¹⁸F labeled glucosyl donor for glycoconjugation of peptides (68, 69, 70). Tetra-O-acetylated 2-[¹⁸F] fluoro-2-deoxy-glucopyranose was studied, but

has the disadvantage of lacking chemoselectivity when coupling to the peptide (68, 69). 3,4,6-Tri-O-acetyl-2-deoxy-2-[^{18}F] fluroglucopyranosyl phenylthiosulfonate was also investigated as an ^{18}F labeled thiol-reactive glycosyl donor for peptides (70). This approach was actually successful, succeeding in introducing ^{18}F to the cyclic peptide c (RGDfc) without affecting its $\alpha_v\beta_3$ binding affinity. However, this approach is limited to cysteine-containing peptides only. Recently three groups reported the use of [^{18}F]FDG for ^{18}F labeling and glycosylation of peptides (71, 72, 73). Wuest et al reported the conversion of [^{18}F]FDG into [^{18}F]FDG-maleimidehexyloxime which was then coupled to a peptide containing a thiol group chemoselectively (71). FDG was also coupled to aminooxy-derivatized peptides by oxime formation (72, 73). This method has the disadvantage of the tedious separation of [^{18}F]FDG from excess glucose.

1.8.3 Click reaction in radio-chemistry

As mentioned above, the other problem which is encountered with the currently used prosthetic groups for radio-labeling peptides with ^{18}F is the time and the multiple steps needed for the radio-synthesis. This is reducing the overall radio-chemical yields of the final labeled peptide (49). Consequently, a rapid and chemo-selective method for labeling peptides with ^{18}F is needed.

A promising way which might fulfill this requirement is the so called “click reaction”. The CuI catalyzed Huisgen 1,3-dipolar cycloaddition of azides and alkynes forming 1,2,3-triazole was named by Sharpless et al (74, 75). This

reaction is extremely versatile, works well in many different solvents using a wide range of temperatures and pH values within reasonable reaction times (76). Moreover, the cycloaddition of the alkyne and azide proceeds in the presence of many unprotected functional groups like alcohols, carboxylic acids, and amines and it is not affected by steric factors. Coupling of tertiary azides e.g. proceeds with high yields (76). Furthermore, the alkynes and azides are relatively stable under different conditions and can easily be introduced into different organic molecules (76). In addition, the 1,2,3-triazole formed has similar polarity and size as an amide bond (76).

There are already some successful reports on radio-labeling of peptides with ^{18}F using this click reaction (77, 78, 79). Marik et al reported the synthesis of ω -[^{18}F]-fluoroalkynes which were then coupled to several peptides bearing azide groups. The products were obtained in 10 min, with RCYs (radio-chemical yields) of 54-99% and a radiochemical purity of 81-99% (77). Glaser et al reported the synthesis of 2-[^{18}F] fluoroethyl azide, which was then coupled to a model peptide bearing an alkyne group by click reaction. The product was obtained in $\approx 90\%$ RCY after purification by HPLC (78). Another study used the same strategy for the introduction of ^{18}F into a RGD peptide where the product was obtained with high specific activity (79). An in vivo study of the behavior of the synthesized peptide revealed good pharmacokinetic properties and metabolic stability (79).

These above mentioned advantages point towards the combination of glucosidation and click chemistry. The rationale of using an ^{18}F labeled glucose derivative is that it not only acts as a prosthetic group for the introduction of ^{18}F to

biologically active peptides, but also helps improve their pharmacokinetic properties without affecting their binding affinities to the target receptor, and it can be introduced in a fast and efficient way into peptides such as our before mentioned TfR-targeting peptide using “click chemistry”.

1.9 Aim of the project

1. Synthesize the peptide THRPPMWSPVWP :

This peptide which according to the literature binds to the TfR-receptor, would first be conjugated by Carmen Waengler (McGill University) with the D2 receptor-ligand fallypride (which was chosen as an example of radiotracers for neurological imaging that could in theory benefit from an increased transport across the BBB), to test whether this modification would affect the binding affinity of fallypride towards the D2 receptor. This is crucial because the whole project of finding a method to carry imaging agents across the BBB by conjugating them to carrier peptides depends on the prerequisite that the conjugate (comprising the radio-tracer molecule and the carrier molecule) still binds to the receptor. If this is not the case the finding of a transport mechanism would be useless even if the BBB penetration would be successful.

2. Synthesize the peptide derivative NOTA-Ahx-THRPPMWSPVWP:

This is a conjugate of the peptide with the NOTA chelator in order to facilitate the radio-labeling of the peptide with ^{68}Ga which will be done by Dr. Carmen Waengler (McGill University, McConnell Brain Imaging Centre), by the group of Prof. Dr. Bartenstein (Munich University, Department of Nuclear Medicine, Germany) who possess a $^{68}\text{Ge}/^{68}\text{Ga}$ generator system and PD Dr Olaf Prante (University of Erlangen, Germany). This will allow our collaborators in Germany to perform uptake experiments of this TfR-targeting peptide into human TfR expressing cells. These data should provide evidence whether this peptide is a suitable carrier molecule for the transport of small molecules over the BBB enabling a more efficient neurological imaging by means of PET.

3. Design of a novel glucose based prosthetic group to radiolabel peptides with ^{18}F :

Due to the current need for optimization of the synthetic procedures for ^{18}F labeling of peptides, we investigated the possibility to synthesize a glucose based prosthetic group 2,3,4-tri-O-acetyl-1-azido-6- ^{18}F -fluoro- β -D-glucopyranoside (fig 3), to radio-label alkyne derivatized peptides with ^{18}F using click chemistry. Our strategy would combine the advantages of carbonylation of peptides with the fast and efficient click reaction for radio-labeling. This glucose derivative bears ^{18}F at one position (at C6) and an azide moiety (at C1) rendering the coupling to alkyne-derivatized peptides possible.

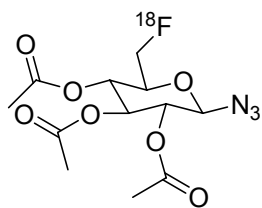


Fig 3: structure of 2,3,4-tri-O-acetyl-1-azido-6-[^{18}F]-fluoro- β -D-glucopyranoside labeled at C6 with ^{18}F and bearing an azide group at C1.

Chapter 2

Results and Discussion

2.1 Synthesis of the peptide THRPPMWSPVWP (1)

As the transferrin receptor has been shown to be a target for efficient transport over the blood brain barrier (BBB) (27, 28, 29, 30), this carrier system was investigated for the delivery of small organic molecules into transferrin receptor expressing cells located at the BBB. This concept of enhanced transport could find application in radiopharmaceutical chemistry to increase the low brain uptake of radiotracers already used for neurological PET imaging in patients. Radiotracers like [^{18}F] fallypride, a receptor-ligand for imaging striatal as well as extrastriatal D2 receptors, could be attached to suitable carrier molecules improving their brain uptake. This should enable a higher accumulation of these compounds in the brain and therefore be useful to improve the overall image quality by concurrently using lower injected doses. For transferrin receptor targeting, the recently identified peptide THRPPMWSPVWP (**1**) (fig 4), with a high affinity to the transferrin receptor and the ability to transport GFP into transferrin receptor expressing cells (39), was investigated. Most important, this peptide has been reported to have a distinct binding position from endogenous transferrin. This would reduce the competition in uptake with transferrin itself hopefully leading to a higher uptake in the brain.

In order to prove the validity of the concept of using this peptide as a molecular taxi, we decided to synthesize the peptide THRPPMWSPVWP (**1**) and synthesize a conjugate between the peptide and the D2 receptor-ligand fallypride (which was chosen as an example of radiotracers for neurological imaging that could in theory benefit from an increased transport across the BBB). To test

whether this modification would affect the binding affinity of fallypride towards its D2 receptor, the synthesis of the fallypride-THRPPMWSPVWP conjugate was done by Dr. Carmen Waengler (McGill University, McConnell Brain Imaging Centre). The radio-labeling with ^{68}Ga was performed by Dr. Carmen Waengler, by the group of Prof. Dr. Bartenstein (Munich University, Department of Nuclear Medicine, Germany) who possess a $^{68}\text{Ge}/^{68}\text{Ga}$ generator system and PD Dr Olaf Prante (University of Erlangen, Germany).

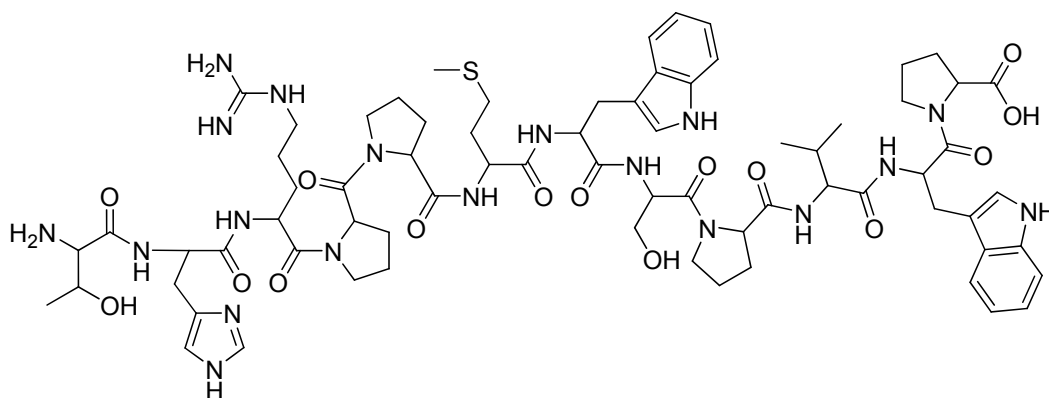
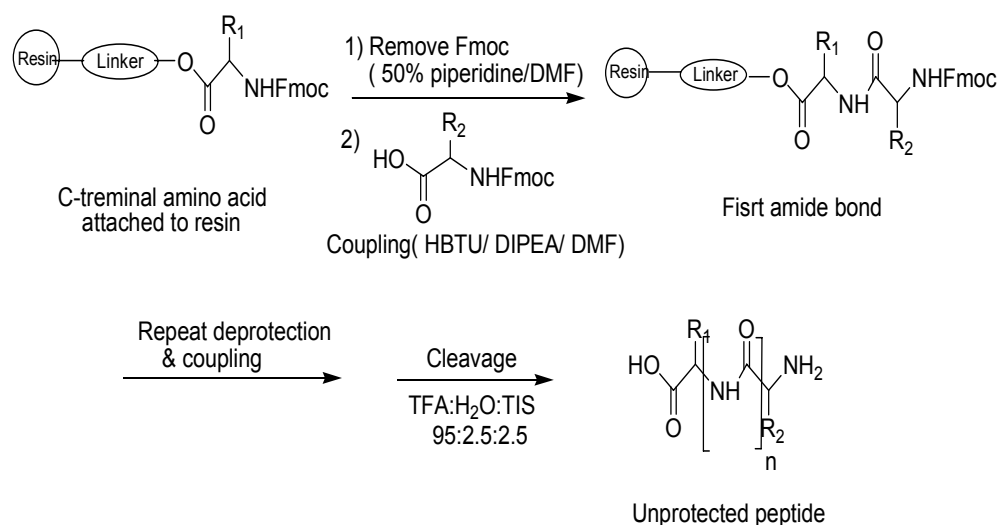


Fig 4: structure of TfR targeting peptide THRPPMWSPVWP **1**

[These letters are symbols of the amino acids constituting the peptide where T is threonine, H is histidine, R is arginine, P is proline, M is methionine, W is tryptophan, S is serine and V is valine]

The synthesis of all peptides was carried out using standard Fmoc-solid-phase-peptide synthesis (80), (Scheme 1).



Scheme 1: general scheme for standard Fmoc-solid-phase-peptide synthesis

A conjugate of maleimido-derivatized THRPPMWSPVWP with a derivative of D2 receptor ligand fallypride was synthesized (fig 5) by Dr. Carmen Waengler and evaluated at the University of Erlangen (Germany, group PD Dr. Olaf Prante) as to its binding affinity to the D2 receptor using the D2 receptor expressing cell line HEK293 (81). Despite the major structural alterations of the small compound fallypride now conjugated to the TfR targeting peptide, the binding affinity of this non-optimized model-conjugate to the D2 receptor was still high with a K_i of 27 nM even though less than for fallypride ($K_i = 0.1$ nM) itself. These findings demonstrate that even a structural alteration of this magnitude can lead to compounds still binding to their target, legitimizing the

proposed concept of facilitated transport of structurally small radiopharmaceuticals by conjugating them to much larger molecular transporters.

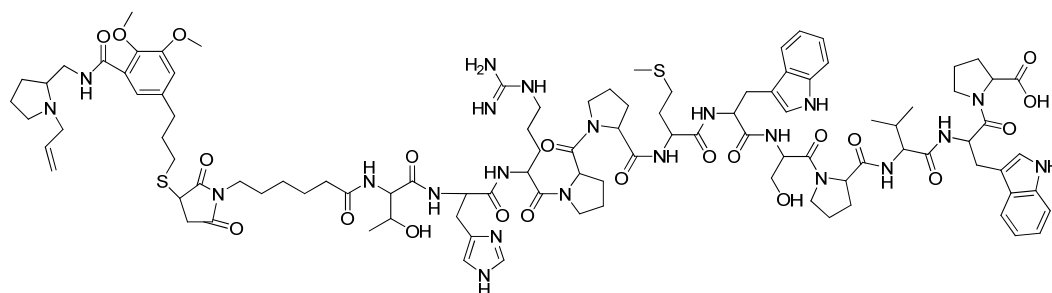


Fig 5: Structure of the conjugate of fallypride-S-maleimido-THRPPMWSPVWP

2.2 Synthesis of the peptide derivative NOTA-Ahx-THRPPMWSPVWP (2):

In order to evaluate the potential of the peptide THRPPMWSPVWP (**1**) to transport small organic molecules conjugated to its N-terminal end over the blood brain barrier into the brain, we synthesized a derivative of the peptide THRPPMWSPVWP containing the macrocyclic chelator 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA) (fig 6) to enable labeling with ^{68}Ga .

We used the NOTA derivative, S-2-(4-Isothiocyanatobenzyl)-1,4,7-triazacyclononane-1,4,7-triacetic acid (p-SCN-Bn-NOTA) (fig 7).

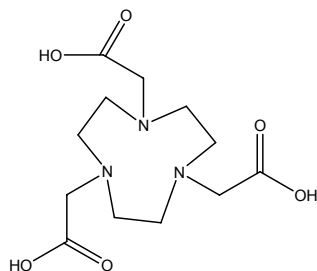


Fig 6: structure of 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA)

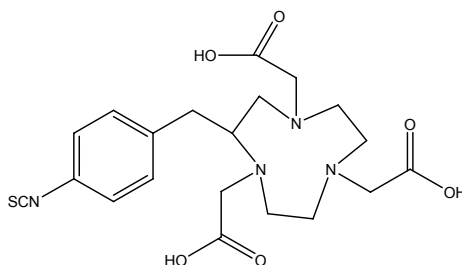
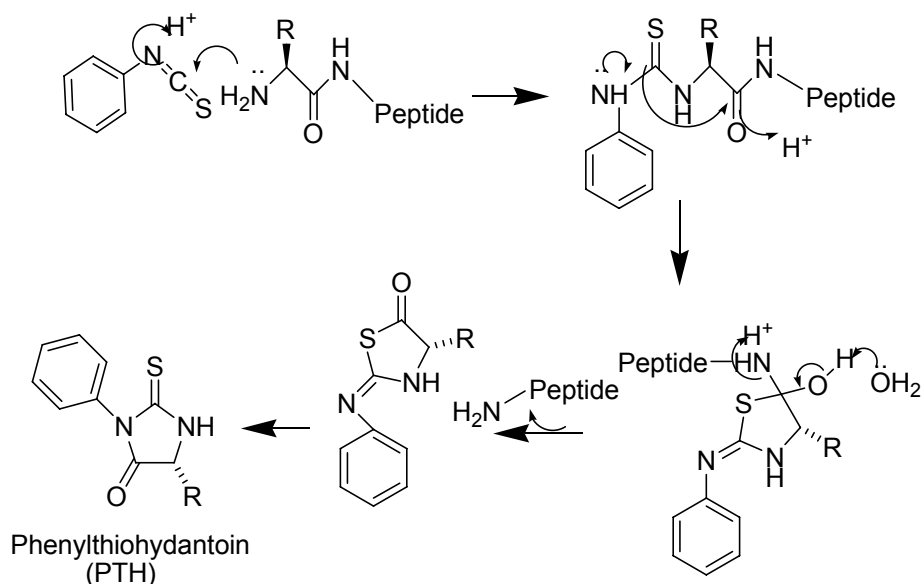


Fig 7: structure of S-2-(4-Isothiocyanatobenzyl)-1,4,7-triazacyclononane-1,4,7-triacetic acid (p-SCN-Bn-NOTA)

As a first trial, we tried to directly couple this NOTA derivative to the peptide on resin by solid phase synthesis. After cleavage from the resin, we observed several peaks by HPLC, and by collection of the different fractions and checking their masses we found only smaller masses than the mass of the desired peptide. This was probably due to the thiocyanate (SCN) group present in the NOTA derivative. This group is responsible for the well known reaction “Edman dergradation” (Scheme 2) which is cleavage of the amide bond between amino acids.



Scheme 2: mechanism of Edman degradation.

We thus decided to add a linker between the peptide and the NOTA derivative. We chose 6-amino hexanoic acid (Ahx) which resembles amino acids in having both the amino group and the carboxylic group, making it easy to be coupled to the peptide (by Fmoc solid phase method). The five carbon atoms of the amino hexanoic acid compound constitute a long chain which would protect the amide bond from Edman degradation caused by the SCN group of the NOTA derivative. This approach was successful, the amino group of the amino hexanoic acid was reacted with the SCN group of the NOTA derivative resulting in a thio-urea formation and the coupling of NOTA to the N-terminal end of the peptide yielding NOTA-Ahx-THRPPMWSPVWP (**2**) (fig 8).

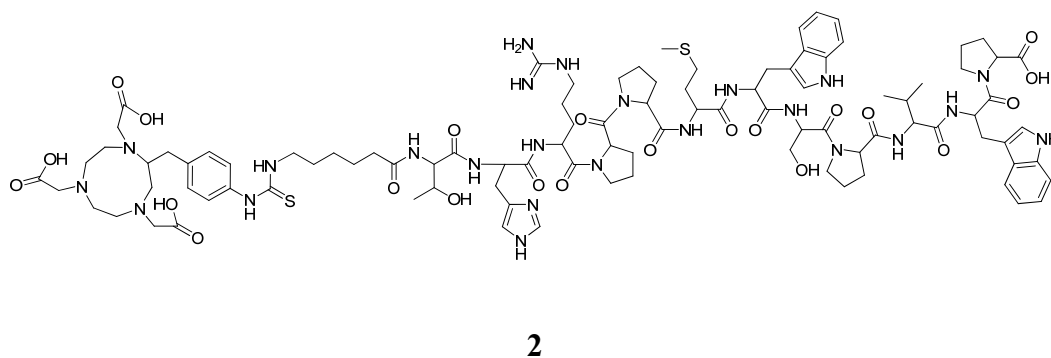


Fig 8: structure of the conjugate NOTA-Ahx-THRPPMWSPVWP (**2**) for the radio- labeling with ^{68}Ga

The NOTA-Ahx-THRPPMWSPVWP (**2**) was radiolabeled with ^{68}Ga (fig 9) by Dr. Carmen Waengler, by the group of Prof. Dr. Bartenstein and PD Dr Olaf Prante (University of Erlangen, Germany).

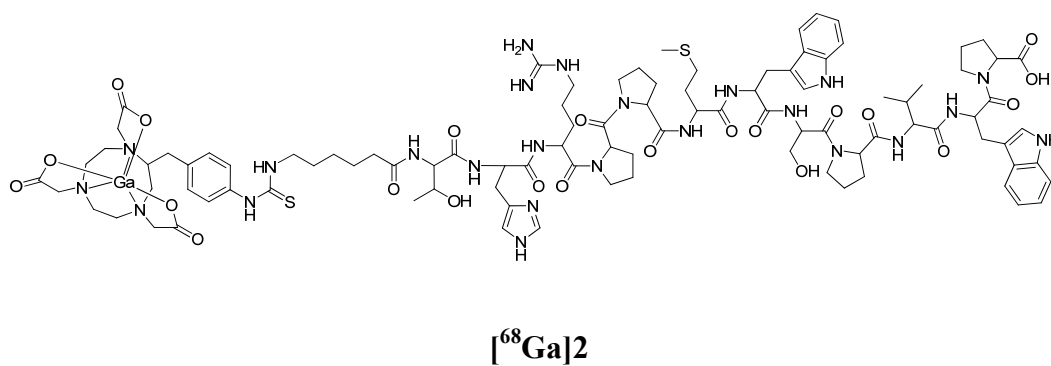


Fig 9: structure of ^{68}Ga -NOTA-Ahx-THRPPMWSPVWP (**[^{68}Ga]2**)

Uptake experiments with the ^{68}Ga -labeled NOTA-Ahx-THRPPMWSPVWP (**[^{68}Ga]2**) using HT29 (human colon adenocarcinoma cell

line) and U87MG (human glioblastoma cell line) cells expressing the transferrin receptors TfR1 and TfR2 were performed by PD Dr. Olaf Prante (University of Erlangen, Germany). As can be seen from fig 10 the labeled peptide-NOTA derivative showed only low uptake rates of less than 1% ID/mg protein after 60 minutes of incubation at 37°C to the used cell lines.

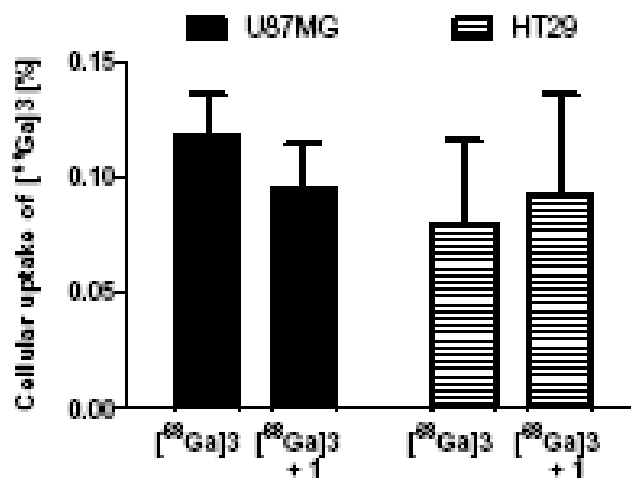


Fig 10: Uptake of ⁶⁸Ga-NOTA-Ahx-THRPPMWSPVWP on U87MG and HT29 cells at 37°C after 60 min of incubation in the presence and absence of underivatized peptide (1, 50 µM).

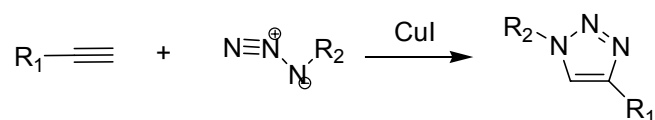
This small uptake could be due to the conjugation although even bigger conjugates were synthesized which still showed internalization into transferrin receptor expressing cells (13, 14). Oh et al (14) investigated the influence of their TfR binding peptide artemisinin-conjugate on cancer cells but did not quantify the uptake of the substance. Another critical point is the fact that the cells that were initially used for the identification of the TfR-

binding peptides by phage display were not derived from a human cell line but from a chimeric chicken embryo fibroblasts cell line not comparable to human cell lines expressing the naïve TfR.

2.3 Designing a novel glucose based prosthetic group to radiolabel peptides with ^{18}F :

As mentioned before there is a current need for optimization of the synthetic procedures for ^{18}F labeling of peptides, we thus investigated the possibility to synthesize a glucose based prosthetic group which could be coupled to peptides with the fast and efficient click chemistry. It was our intention to use this method to label our TfR peptide (**1**) with ^{18}F to yield a differently labeled derivative for the cell uptake experiments.

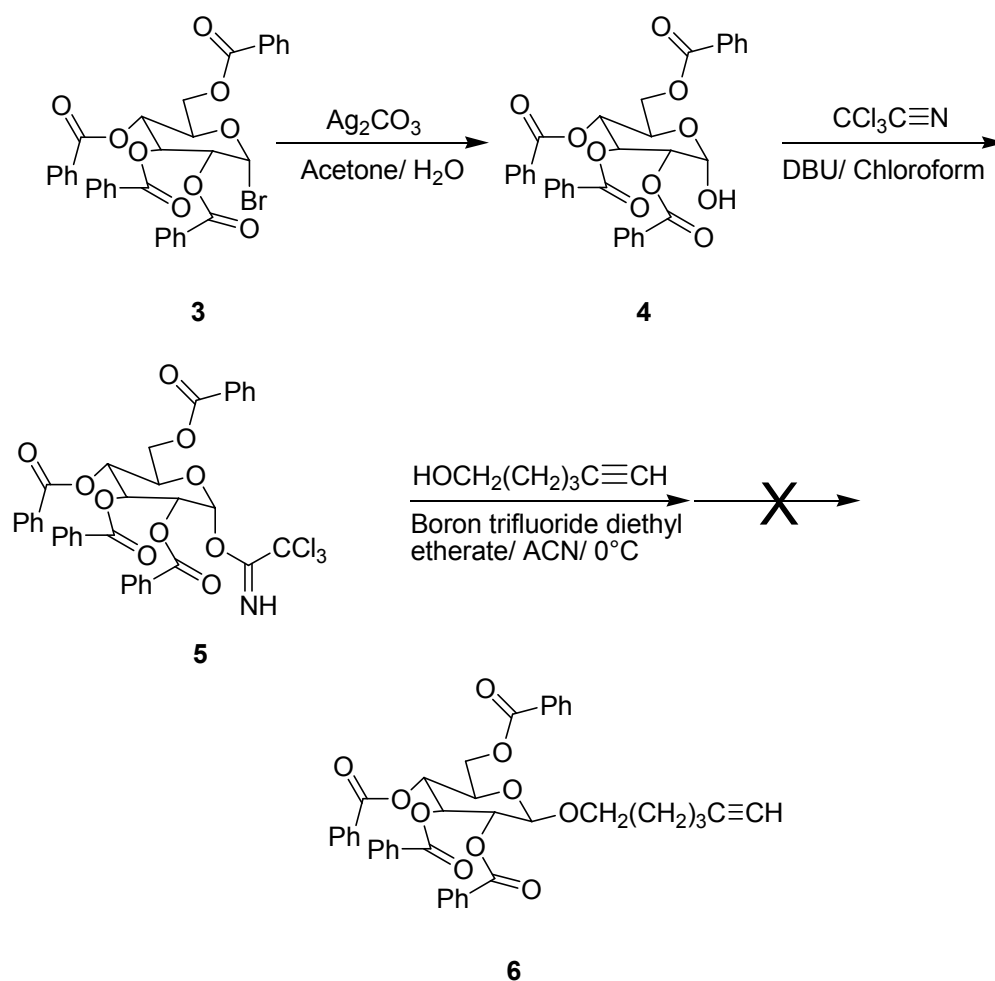
In order for a prosthetic group to be coupled to peptides by click chemistry, it should either have an alkyne moiety or an azide group. These two functional groups involved in the click reaction, as shown in scheme 3, where R1 and R2 may be either the peptide or the prosthetic group forming the triazole.



Scheme 3: reaction scheme of the click reaction

We first decided to design our prosthetic group such that it contains the alkyne moiety, which would then be coupled to peptides bearing an azide moiety.

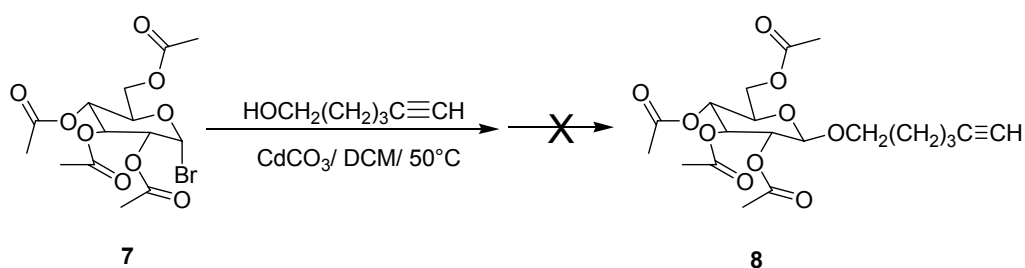
The first step to synthesize this prosthetic group (bearing an alkyne moiety) was to synthesize the compound 2,3,4,6-tetra-O-benzoyl-1-O-hex(6)yn- α,β -D-glucopyranoside (**6**). The proposed scheme for its synthesis is shown in scheme 4.



Scheme 4: synthetic scheme for the synthesis of 2,3,4,6-tetra-O-benzoyl-1-O-hex-6-yn- α,β -D-glucopyranoside (**6**) bearing the clickable alkyne moiety.

Compound **4** 2,3,4,6-tetra-O-benzoyl- α -D-glucopyranose and **5** 2,3,4,6-tetra-O-benzoyl-1-(2,2,2-trichloroacetamido)- α -D-glucopyranoside were successfully synthesized with yields of 74% and 70% respectively. The trichloroacetamido group was chosen because it is a good leaving group which would facilitate the coupling of the alcohol 5-hexyl-1-ol. Unfortunately, the coupling step of the alcohol 5-hexyl-1-ol did not work. A good leaving group like trifluoromethane sulfonate (triflate, OTf) or p-toluene sulfonate (tosylate, OTos) was intended to be introduced at position 6 (C6) in order to facilitate the nucleophilic substitution of $^{18}\text{F}^-$.

We also tried to couple the alcohol 5-hexyl-1-ol to 2,3,4,6-tetra-O-acetyl-1-bromo- α -D-glucopyranoside (**7**) (Koenig's knorr reaction) to form 2,3,4,6-tetra-O-acetyl-1-O-hex(6)yn- α - β -D-glucopyranoside (**8**) as shown in scheme 5, but this trial also did not yield the desired compound.



Scheme 5: synthetic scheme for the synthesis of 2,3,4,6-tetra-O-acetyl-1-O-hex-6-yn- α - β -D-glucopyranoside (**8**) bearing the clickable alkyne moiety.

As a back-up strategy we decided to couple an azide moiety to the sugar derivative at the anomeric position (at C1) instead of an alkyne moiety which would then be coupled to alkyne derivatized peptides.

The newly designed prosthetic group for labeling peptides with ^{18}F is 2,3,4-tri-O-acetyl-1-azido-6- ^{18}F -fluoro- β -D-glucopyranoside (^{18}F **19**) as shown in fig 3.

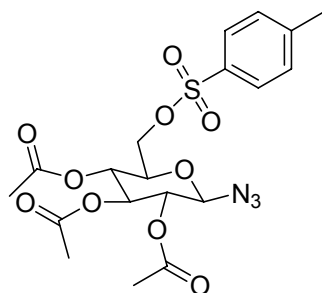
In order to synthesize this prosthetic group there are two main steps to be considered:

Step 1: Synthesis of a labeling precursor (compound **15**, scheme 6) based on glucose bearing an azide group as well as a suitable leaving group such as triflate or tosylate to introduce $^{18}\text{F}^-$ by nucleophilic substitution as well as synthesis of the non-radioactive fluorinated standard compound 2,3,4-tri-O-acetyl-1-azido-6-fluoro- β -D-glucopyranoside (compound **19**, scheme 7).

Step 2: Radio-labeling of the labeling precursor (compound **15**, scheme 6) with ^{18}F to yield the ^{18}F -prosthetic group (^{18}F **19**) for peptide labeling.

• **Step 1:** *Two glucose-derivatives were synthesized:*

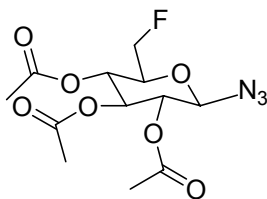
- 1) The labeling precursor 2,3,4-tri-O-acetyl-1-azido-6-O-p-toluene sulfonyl- β -D-glucopyranoside (compound **15**, fig 11): This is a glucose derivative which has the two required chemical moieties to introduce the ^{18}F and to couple the compound to an alkyne derivatized peptide.



15

Fig 11: structure of the labeling precursor 2,3,4-tri-O-acetyl-1-azido-6-O-p-toluene sulfonyl- β -D-glucopyranoside (**15**)

- 2) The non-radioactive reference compound 2,3,4-tri-O-acetyl-1-azido-6-fluoro- β -D-glucopyranoside (compound **19**, fig 12): This compound is exactly like the ^{18}F -labeled prosthetic group, but with ^{19}F instead of ^{18}F to be used as a cold reference i.e. to establish HPLC and TLC conditions for identification.



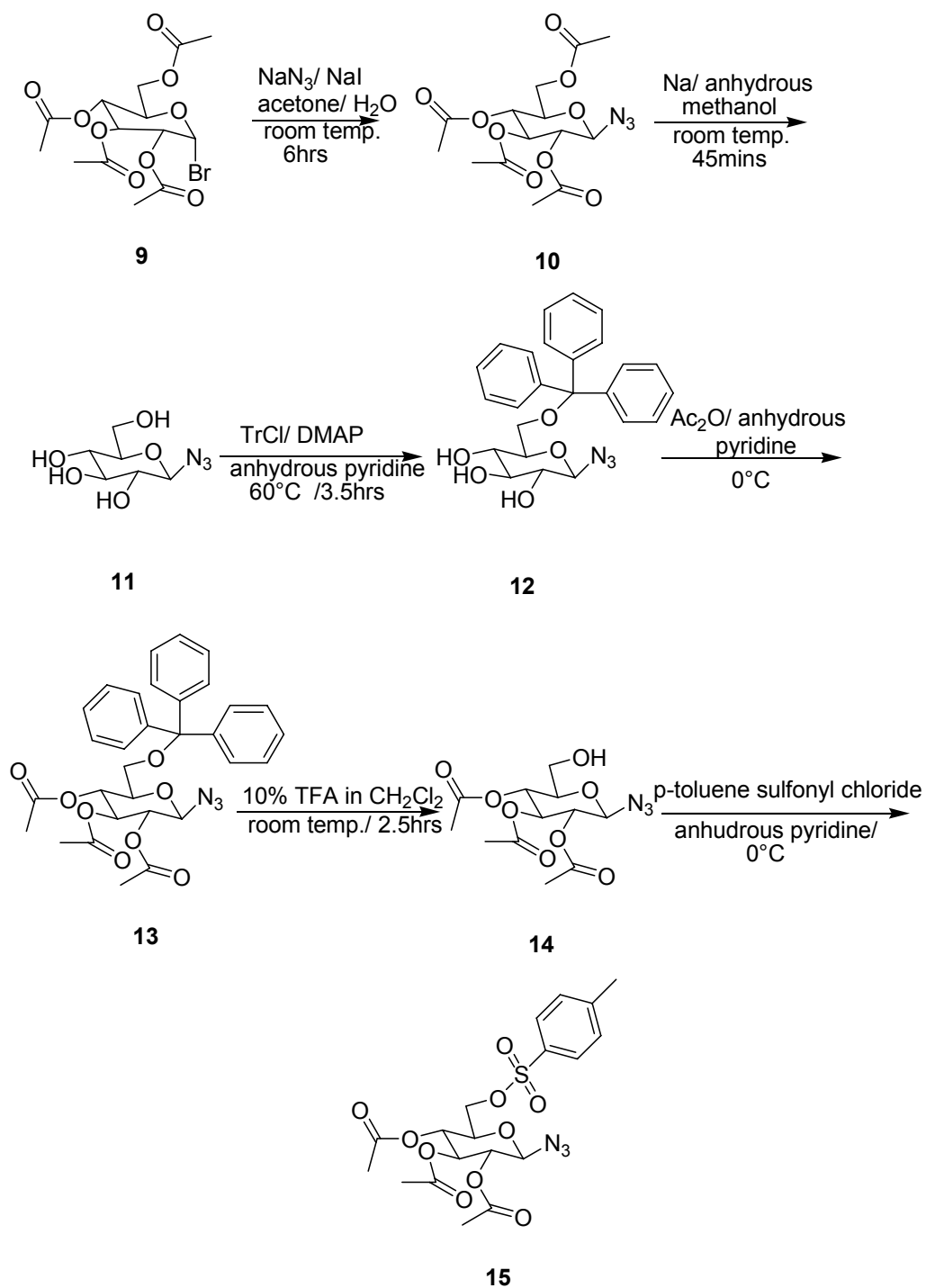
19

Fig 12: structure of the non-radioactive reference compound 2,3,4-tri-O-acetyl-1-azido-6-fluoro- β -D-glucopyranoside (**19**)

1) Synthesis of the labeling precursor:

The labeling precursor 2,3,4-tri-O-acetyl-1-azido-6-O-p-toluene sulfonyl- β -D-glucopyranoside (**15**) was synthesized in six steps as shown in scheme 6. In the first step the azide group was introduced at the anomeric position (at C1). 2,3,4,6-tetra-O-acetyl-1-bromo- α -D-glucopyranoside (**9**) was used as the starting material. It was dissolved in a mixture of acetone and water and was then reacted with sodium azide, using sodium iodide as a catalyst. This solvent system was chosen to bring the starting material (**9**) and sodium azide in solution. 2,3,4,6-tetra-O-acetyl-1-azido- β -D-glucopyranoside (**10**) was obtained in good yield (73.5%) in spite of the formation of 2,3,4,6-tetra-O-acetyl β -D-glucopyranose as a by product which was expected due to the presence of water in the solvent system. The azide group was introduced at the anomeric position (C1) with β configuration. This was expected due to the neighboring group (acetyl group) participation mechanism which was confirmed by measuring the coupling constant ($J=8.85$) between H1 (anomeric position) and H2. Step two involved the removal of the acetyl protecting groups from **10** by using sodium methoxide which was added to **10**, using methanol as a solvent to give 1-azido- β -D-glucopyranoside (**11**) (yield=86%). In step three a tri-phenyl methyl (trityl) group was introduced by reacting **11** with trityl chloride in anhydrous pyridine using dimethyl amino pyridine (DMAP) as a catalyst. The trityl group is a bulky group that reacts with a primary alcohol rather than a secondary one, so it was chosen for protection of the primary alcoholic group at position C6 to give 1-azido-6-O-trityl- β -D-glucopyranoside (**12**) (yield=75%). In step four the other alcoholic groups of **12** at positions C2, C3, C4 were re-protected with acetyl groups by reacting **12** with acetic anhydride in anhydrous pyridine to give 2,3,4-

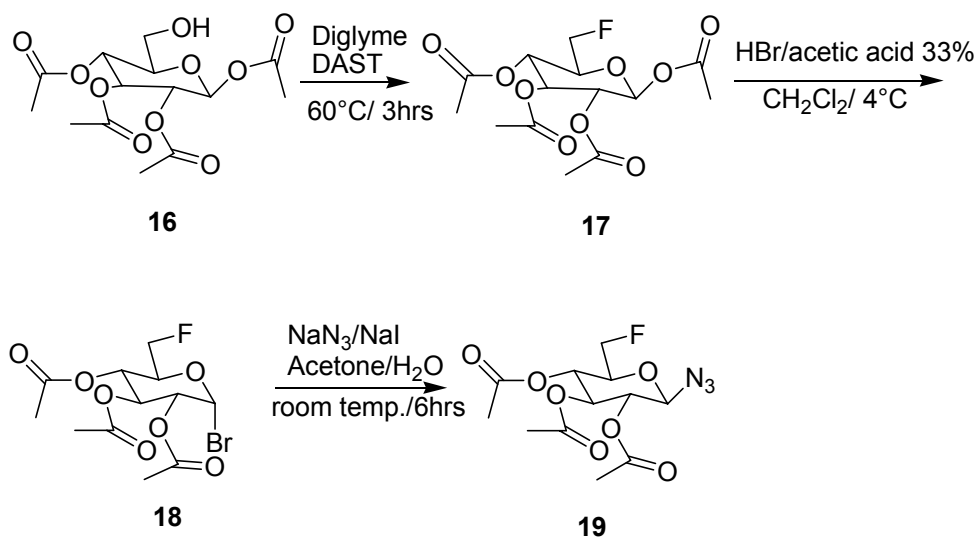
tri-O-acetyl-1-azido-6-O-trityl- β -D-glucopyranoside (**13**) (yield=85%). In step five the trityl group at C6 was removed under acidic conditions using trifluoro acetic acid (TFA) in dichloromethane to give 2,3,4-tri-O-acetyl-1-azido- β -D-glucopyranoside (**14**) (yield=79%). In step six we intended to introduce a good leaving group at position C6. We first tried to introduce a triflate group (OTf) by reacting **14** with 2,2,2-trifluoroacetic anhydride. We chose the triflate group as it is a better leaving group than the tosylate group. We did two trials for the introduction of the triflate group into **14**. In the first attempt we used (**14**, 2,2,2-trifluoroacetic anhydride, 1:1) in anhydrous pyridine. In this trial no reaction happened after the reaction was left overnight. By comparing the reaction mixture with the starting material (**14**) by TLC it was obvious that no reaction took place. In the second attempt we used (**14**, triflic anhydride, 1:1.2) in dichloromethane using triethyl amine as a catalyst. By performing a TLC we observed multiple spots, the main one was identified as being the starting material. As a back up strategy we managed to introduce the tosylate instead of the triflate at position C6. This was done by reacting **14** with p-toluene sulfonyl chloride in anhydrous pyridine to give the labeling precursor 2,3,4-tri-O-acetyl-1-azido-6-O-p-toluene sulfonyl- β -D-glucopyranoside (**15**) (yield=80%).



Scheme 6: synthetic scheme for the synthesis of the labeling precursor 2,3,4-tri-O-acetyl-1-azido-6-O-p-toluene sulfonyl-β-D-glucopyranoside (**15**)

2) Synthesis of the non-radioactive reference compound 2,3,4-tri-O-acetyl-1-azido-6-fluoro- β -D-glucopyranoside (19):

As a first trial to synthesize the non-radioactive reference compound 2,3,4-tri-O-acetyl-1-azido-6-fluoro- β -D-glucopyranoside (**19**), we tried to introduce the fluorine atom by reacting **14** with diethyl amino sulfur trifluoride (DAST). This reaction was performed in several solvents such as dichloromethane and diglyme but none of them yielded the desired product. To obtain the required compound 2,3,4-tri-O-acetyl-1-azido-6-fluoro- β -D-glucopyranoside **19**, compound 1,2,3,4-tetra-O-acetyl β -D-glucopyranoside (**16**) was used as the starting material. The fluorine atom was introduced by reacting **16** with DAST in diglyme to give 1,2,3,4-tetra-O-acetyl-6-fluoro- β -D-glucopyranoside (**17**) (yield=83%). In the next step the bromine atom was introduced at the anomeric position (C1), this would facilitate the nucleophilic substitution by the azide group at that position (C1). In this step **17** was reacted with HBr/acetic acid mixture in dichloromethane to give 2,3,4-tri-O-acetyl-1-bromo-6-fluoro- α -D-glucopyranoside (**18**) (yield=46%). In the last step the azide group was introduced by the reaction of **18** with sodium azide in a mixture of acetone and water using sodium iodide as a catalyst to give the reference compound 2,3,4-tri-O-acetyl-1-azido-6-fluoro- β -D-glucopyranoside (**19**) (scheme 7) (yield=77%).



Scheme 7: synthetic scheme for the synthesis of the non-radioactive reference compound 2,3,4-tri-O-acetyl-1-azido-6-fluoro-β-D-glucopyranoside (**19**)

Step 2: *Radio-labeling of the labeling precursor **15** with ^{18}F :*

1) Production of ^{18}F :

No-carrier added aqueous $^{18}\text{F}^-$ was produced in the cyclotron by irradiation of [^{18}O]H₂O via the ^{18}O (p,n) ^{18}F nuclear reaction.

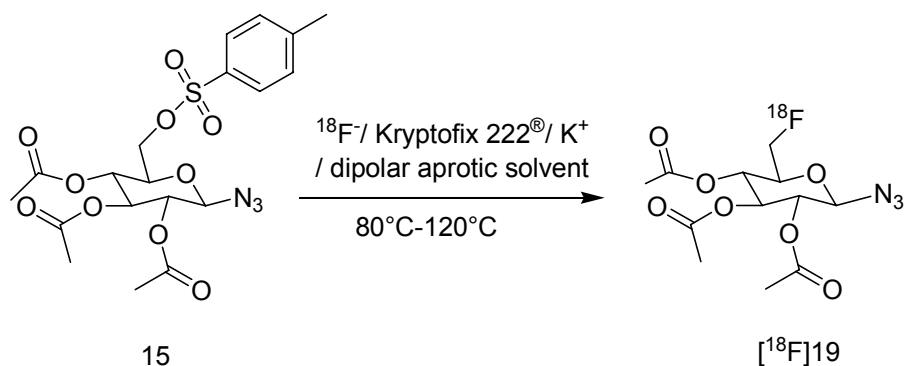
2) Drying of anionic $^{18}\text{F}^-$:

Since $^{18}\text{F}^-$ is a very weak nucleophile in water, the drying of $^{18}\text{F}^-$ had to be done before starting the radio-labeling experiments. $^{18}\text{F}^-$ was extracted with an anion exchange resin cartridge (QMA, Waters). Kryptofix 222[®] (10-15 mg)/K₂CO₃ (0.5N, 20-30 μL) solution in acetonitrile (400-800 μL) with addition of a small amount of water (50-100 μL) was used to elute the cartridge, and the resulting mixture was dried

in a glass reactor by applying a stream of nitrogen and gentle vacuum at elevated temperature of 90°C. After the first drying step, acetonitrile (1 mL) was added again and the drying procedure was repeated to ensure total dryness of the $^{18}\text{F}^-$ /Kryptofix 2.2.2[®]/K⁺ complex.

3) Radio-labeling experiments:

As mentioned before the labeling precursor was designed such that it bears a good leaving group like tosylate, this group should theoretically be easily substituted by anionic $^{18}\text{F}^-$ in a dipolar aprotic solvent such as acetonitrile, DMF and DMSO at elevated temperature as shown in scheme 8.



Scheme 8: replacing the tosylate group in **15** by $^{18}\text{F}^-$ through a nucleophilic substitution reaction to yield **[^{18}F]19**.

Several experiments have been done to radiolabel precursor **15** with $^{18}\text{F}^-$. Different dipolar aprotic solvents including acetonitrile, DMF and DMSO were used for the $^{18}\text{F}^-$ labeling reactions as the nucleophilicity of anions is enhanced in the presence of aprotic solvents whereas it is retarded in the presence of protic

solvents. We also tried tertiary amyl alcohol which in spite of being a protic solvent has been reported by Kim et al to increase the nucleophilicity of fluoride ions significantly without the need of any catalyst. At the same time the formation of by-products is decreased (82). The labeling experiments were carried out at elevated temperatures which ranged from 80°C to 120°C (table 1).

Table 1: conditions used in the different radio-labeling experiments

Radio-labeling experiment	Amount of labeling precursor (15)	Solvent	Amount of ^{18}F from stock solution (10 mCi/mL)	Reaction time	Temp.	Result
Exp1	5mg	250 μl DMSO	50 μl	2h	90°C	Unidentified radio-active product
Exp2	5mg	300 μl DMSO	100 μl	3h	110°C	Unidentified radio-active product
Exp3	10mg	500 μl Tert-amyl alcohol	50 μl	3h	80°C	No reaction
Exp4	6.2mg	450 μl Tert-amyl alcohol	50 μl	4h	110°C	No reaction
Exp5	6mg	450 μl ACN	20 μl	4h	80°C	Unidentified radio-active product
Exp6	9mg	450 μl ACN	50 μl	5h	110°C	Unidentified radio-active product
Exp7	7.4mg	450 μl DMF	50 μl	5h	90°C	Unidentified radio-active product
Exp8	12mg	400 μl Tert-amyl alcohol	70 μl	5h	120°C	Unidentified radio-active product
Exp9	6mg	450 μl Tert-amyl alcohol	50 μl	4h	120°C	Unidentified radio-active product

Note: 1mg Sodium Iodide was used as a catalyst in experiment 9

The labeling reactions were monitored every 20 minutes by radio-TLC using a radio-TLC scanner, using **19** as the cold reference standard to identify the radio-labeled compound by its retention factor on the TLC plate. Unfortunately we did not manage to radiolabel compound **15** with $^{18}\text{F}^-$ no matter of what conditions were used. What was observed, using the radio-TLC scanner, was the formation of an unidentified radioactive product which travelled with an Rf of 0.77 (fig 13), where as the Rf of the reference compound **19** is 0.38. These results might be explainable by the presence of a small amount of impurity in **15** which was labeled instead of the labeling precursor. This is because the amount of used $^{18}\text{F}^-$ is very minute (in the nano-molar range) compared to the amount of the labeling precursor (in the milli-molar range), hence any minute amount of impurity (which could be favorably labeled than our compound **15**) could negatively affect our radio-labeling yields.

Although we purified **15** by column chromatography and confirmed its structure by ^1H -NMR, ^{13}C -NMR and mass spectroscopy, an impurity was finally qualitatively confirmed by HPLC. Thus, further purification such as re-crystallisation of **15** was tried. The re-crystallisation was done using anhydrous ethanol where compound **15** was totally soluble at 50°C. After cooling in the fridge overnight, the obtained crystals were dried under vacuum, and the radio-labeling experiments were performed again in acetonitrile at 80°C. Unfortunately we did not manage to radio-label our labeling precursor with $^{18}\text{F}^-$ in spite of the disappearance of the impurity.

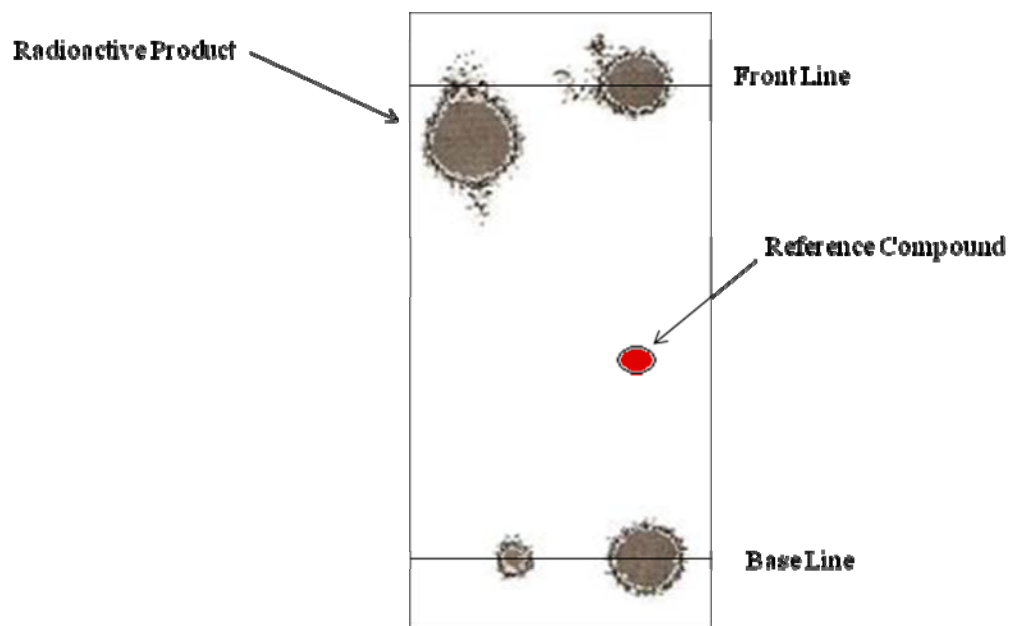


Fig 13: results of radio-labeling experiments using the labeling precursor **15** TLC obtained from the radio-TLC scanner 1 hour after performing the radio-labeling experiment (where 12 mg of **15**, 400 μ l tert-amyl alcohol, 70 μ l of ^{18}F from stock solution (10 mCi/mL) were used at 120°C): the radio-active product (an unidentified impurity) with Rf of 0.77 does not match the Rf of the reference compound **19** (red spot) (0.38).

2.4 Summary and conclusion:

The peptide THRPPMWSPVWP was successfully synthesized and conjugated by Dr. Carmen Waengler with the D2 receptor ligand fallypride. The conjugate was evaluated as to its binding affinity to the D2 receptor at the University of Munich (Germany, group Prof. Dr. Wanner). The binding affinity of this conjugate towards D2-receptor was still high ($K_i = 27\text{nM}$) even though less than fallypride ($K_i = 0.1\text{nM}$) itself.

We have also successfully coupled NOTA (1,4,7-triazacyclononane-1,4,7-triacetic acid) to the peptide THRPPMWSPVWP which was then radiolabeled with ^{68}Ga by Dr. Carmen Waengler (McGill University), by the group of Prof. Dr. Bartenstein (Munich University, Department of Nuclear Medicine, Germany) who possess a $^{68}\text{Ge}/^{68}\text{Ga}$ generator system and PD Dr Olaf Prante (University of Erlangen, Germany) . Uptake experiments with the ^{68}Ga -labeled peptide were performed using human transferrin receptor (TfR) expressing cancer cell lines HT29 and U87MG. The ^{68}Ga -radiolabeled derivative of THRPPMWSPVWP (^{68}Ga -labeled NOTA-Ahx-THRPPMWSPVWP) showed only a low uptake of less than 1% after 60 minutes of incubation at 37°C into the used cell lines. These results suggest that the accumulation of the ^{68}Ga -labeled conjugates of the TfR-binding peptides casts doubts on the applicability of the peptide THRPPMWSPVWP as a vehicle for a drug delivery of PET tracers into the brain. However, further experiments have to be carried out to determine the potential of this peptide as transferrin receptor targeting carrier molecule.

We have also investigated the possibility to synthesize the glucose based labeling synthon (prosthetic group) 2,3,4-tri-O-acetyl-1-azido-6-[^{18}F]-fluoro- β -D-glucopyranoside, to radiolabel alkyne derivatized peptides with ^{18}F using click chemistry. This new method was intended to be used for an ^{18}F -labeling of the forementioned TfR-targeting peptide THRPPMWSPVWP to get another, differently labeled peptide exhibiting a different lipophilicity. The lipophilicity is an important factor influencing the accumulation of the labeled compound in vitro as well as in vivo. Therefore, a ^{18}F -labeled glucose derivative capable of being conjugated to the TfR-targeting peptide by click chemistry would be a reasonable way of obtaining a ^{18}F -labeled derivative of the peptide. The first glucose derivative synthesized was 2,3,4-tri-O-acetyl-1-azido-6-O-p-toluene sulfonyl- β -D-glucopyranoside which was supposed to serve as a labeling precursor bearing an azide group ready for coupling to an alkyne derivative of a peptide by click chemistry and a p-toluene sulfonyl group (tosyl-group) amenable to a radio-labeling with $^{18}\text{F}^-$ by a nucleophilic substitution reaction. The second compound was 2,3,4-tri-O-acetyl-1-azido-6-fluoro- β -D-glucopyranoside which served as a reference compound to establish HPLC and TLC conditions for the radio-labeling experiments. Radio-labeling of the labeling precursor 2,3,4-tri-O-acetyl-1-azido-6-O-p-toluene sulfonyl- β -D-glucopyranoside with $^{18}\text{F}^-$ did not work probably due to the presence of an unidentified impurity and even a second re-crystallisation did not lead to the desired radio-labeled product.

CHAPTER 3

In vitro and initial in vivo evaluation of ^{68}Ga -labeled transferrin receptor (TfR) binding peptides as potential carriers for enhanced drug transport into TfR expressing cells

“SUBMITTED”

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Abstract

Introduction: An often proposed mechanism to overcome the system of efflux transporters and tight junctions present at the blood brain barrier (BBB) is the active, receptor-mediated transport via endocytosis. Among the proposed transport vehicles, the transferrin receptor (TfR) has been considered to be one of the most attractive targets. Besides different proteins, it has recently been shown that two peptides are also able to bind to the TfR and are subsequently internalized into TfR-expressing cells. **Methods:** In this study we evaluated the ability of one of these peptides – THRPPMWSPVWP, labeled with ^{68}Ga – to become internalized into human TfR-expressing cells via endocytosis to determine its potential to act as a carrier system for the transport of small molecules such as brain imaging agents for Positron Emission Tomography (PET) across the BBB utilizing the TfR receptor. To prove the validity of this concept, we first synthesized a conjugate consisting of the D2 receptor ligand fallypride and THRPPMWSPVWP. The conjugate retained a high D2 receptor affinity ($K_i = 27 \text{ nM}$, determined using HEK293 cells) despite the severe structural alteration exerted by the peptide. **Results:** Two derivatives of THRPPMWSPVWP, containing a NOTA or DOTA moiety introduced via different strategies were labeled with ^{68}Ga in high radiochemical yields ($> 96\%$) and a radiochemical purity of 96-98%. The uptake of the ^{68}Ga -labeled peptides into TfR-bearing cells was investigated using two human TfR-expressing cell lines (U87MG and HT-29 cells) to assess the capability of the peptide to act as a carrier molecule targeting the TfR. *In vitro* binding studies revealed negligible

uptake of the tested ^{68}Ga -labeled conjugates ranging from 0.08 to 0.66% after 60 min incubation at 37°C. The nonspecific uptake was assessed using ^{68}Ga -labeled DOTA (2,2',2'',2'''-(1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrayl)tetraacetic acid) and NOTA (2,2',2''-(1,4,7-triazonane-1,4,7-triyl)triacetic acid), showing uptake values within the same range as the tested ^{68}Ga -labeled peptide conjugates. Initial *in vivo* experiments with ^{68}Ga -DOTA-S-maleimido-THRPPMWSPVWP in two healthy rats showed a mean brain uptake of 0.037% ID/g, confirming the results obtained *in vitro*. **Conclusion:** These results suggest that the accumulation of the ^{68}Ga radio-labeled conjugates of the TfR-binding peptide THRPPMWSPVWP into TFR expressing human cell lines is nonspecific and too low to render this peptide suitable as a possible carrier molecule for a receptor mediated transport of compounds across the BBB within the limited timeframe allowed for short-lived positron emitters.

Key-words: blood brain barrier (BBB), active drug transport, transferrin receptor (TfR)

1. Introduction

The delivery of drugs across the blood brain barrier (BBB) still represents a difficult problem in pharmaceutical drug design. This limits the application of promising substances which could be useful for therapeutic or diagnostic applications. In this context, a conceivable concept to overcome this problem for diagnostic purposes could be the conjugation of radiotracers, intended for brain

imaging with positron emission tomography (PET), to molecular taxis to accomplish carrier mediated transport across the highly restrictive BBB. Radiotracers for neurological imaging such as ^{18}F -DMFP [1], ^{18}F -FP [2,3], ^{11}C -raclopride [4,5], ^{18}F - or ^{11}C -labeled flumazenil [6,7], ^{18}F -altanserin [8,9], ^{123}I -IBZM [10,11] and ^{123}I -FP-CIT [12] would profit by this strategy to enhance their uptake into the brain for improved image quality and lower administered doses. In principal, different approaches are available to overcome the BBB by receptor-mediated transport systems. The most frequently used transporter system is the TfR which physiologically mediates the iron uptake into the brain by transferrin internalization [13-16]. Hence, substances binding to the TfR have been used as carriers for different classes of molecules into the brain. Furthermore, the TfR is present on many human cancer cell lines making it an attractive target for enhanced drug delivery of anti-cancer compounds [17]. Besides the endogenous transferrin which was used for the delivery of antibodies and nanoparticles into the brain [18-20], other ligands can be used for the transport via the TfR such as antibodies directed against the TfR. Examples include OX26, an antibody against the rat TfR used to transport nanoparticles over the blood brain barrier [20-23], antibody fragments and scFvs targeting the TfR [23,24]. Interestingly, the extent of uptake of these conjugates has hardly been quantified and data about transport kinetics are still lacking. Especially for the development of new brain imaging agents on the basis of this proposed transporter mediated uptake concept, kinetic data taking the short half life of PET isotopes into consideration are of crucial importance. Thus, the concept of transporting small molecules over the blood

brain barrier by targeting the TfR within the time-frame dictated by the short half-life of PET isotopes used in brain imaging has to be evaluated. For exact quantification, well defined conjugates with a known stoichiometry are needed. This means that small carrier molecules, which can be site-specifically modified with the radiopharmaceutical in an accurately defined way, are preferential. Two small peptides – HAIYPRH and THRPPMWSPVWP – have been identified by phage display and have shown to target the human TfR and to internalize GFP in TfR-expressing cells [25]. Recently, an artemisinin-conjugate of HAIYPRH has also been demonstrated to be a promising agent for cancer treatment of TfR-expressing cells [26]. However, data quantifying the transport efficiency itself are rare. In order to properly assess and judge a conceivable transporter system, kinetic data giving an insight into the uptake efficiency are needed. The transport of OX26 and derived conjugates into the rat brain for example has been controversially discussed in the literature. Although many OX26-drug conjugates have been proposed and it has been claimed that OX26 undergoes receptor-mediated transcytosis, the radio-labeling of OX26 with radioiodine isotopes revealed a restricted uptake into the post-capillary compartment of the rat brain. This important data casts doubts on the usability of OX26 in general [27] and exemplifies the need to validate a potential carrier molecule appropriately. Among the two previously reported peptides [25], THRPPMWSPVWP showed a higher affinity to the TfR than HAIYPRH and was therefore the focus of our investigations. The peptide was derivatized at its *N*-terminus with the D2 receptor-ligand fallypride. Despite this substantial modification with the

structurally large peptide, the conjugate maintained a relatively high D2 receptor affinity. On the basis of these positive results, the peptide was radiolabeled with ^{68}Ga and investigated with regard to its specific uptake in human TfR expressing cell lines. These data should provide evidence whether this peptide is a suitable carrier molecule for the transport of small molecules over the BBB enabling a more efficient neurological imaging by means of PET.

2. Materials and methods

2.1. General

All commercially available chemicals were of analytical grade and used without further purification. The resin for peptide synthesis (Fmoc-Pro-NovaSyn® TGT resin, Fmocprotected amino acids and p-SCN-Bn-NOTA were purchased from NovaBiochem (Nottingham, UK) and Macrocyclics (Dallas, USA), respectively. Thiol-DOTA (2,2',2''-(10- (2-(2-mercaptoethylamino)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid) and SiFA-SH (4-(di-tert-butylfluorosilyl)benzenethiol) were synthesised according to published procedures [28,29]. The analytical and semipreparative HPLC system used was an Agilent 1200 system equipped with a raytest Gabi Star radioactivity detector together with a Chromolith Performance (RP- 18e, 100-4.6 mm, Merck, Germany) and a Chromolith (RP-18e, 100-10 mm, Merck, Germany) column, respectively. ESI and MALDI mass spectra were obtained by using a Finnigan MAT95Q and a Bruker Daltonics Microflex LT mass spectrometer. The human glioblastoma cell line U87MG was purchased from the American Type Culture Collection (ATCC, HTB-14); the human colon adenocarcinoma cell line HT-29

was obtained from European Collection of Cell Cultures (ECACC, 91072201). Fetal calf serum (FCS), Dulbecco's modified Eagle Medium (DMEM) and nonessential amino acids (NAA) were obtained from Invitrogen (Karlsruhe, Germany). Human transferrin and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (Taufkirchen, Germany). ¹²⁵I-transferrin was obtained from Perkin Elmer (NEX 212, 1.8 GBq/μmol, Mechelen, BE). The synthesis of fallypride-S-maleimido-THRPPMWSPVWP will be described elsewhere.

2.2. Synthesis of THRPPMWSPVWP (1)

The peptide was synthesized on solid support applying standard Fmoc-solid-phase-peptide synthesis as described by Wellings et al. [30]. The peptide was cleaved from the resin by incubating with a mixture of TFA (trifluoroacetic acid) : TIS (triisopropyl silane) : H₂O (95:2.5:2.5, 2mL) for 45 minutes and was suspended in diethylether. After washing three times with diethylether, the product was purified via semi-preparative HPLC using a gradient of 0-100% MeCN + 0.1% TFA over 30 minutes and was isolated as white solid after lyophilisation (13.4 mg, 8.9 μmol, 30 % yield).

MALDI-MS (m/z) for [M] (calculated): 1489.1 (1489.73).

2.3. Synthesis of maleimido-THRPPMWSPVWP (2)

Maleimido hexanoic acid (84.5 mg, 400 μmol) was coupled to side chain protected THRPPMWSPVWP on resin (100 μmol) and the product was cleaved from the solid support as described before. Purification was carried out via semi-preparative HPLC using a gradient of 20-45% MeCN + 0.1% TFA over 6 minutes

and was isolated as off-white solid after lyophilisation (65 mg, 39 μ mol, 39 % yield). ESI-MS (m/z) for [M + H]⁺ (calculated): 1683.56 (1683.80).

2.4. Synthesis of *NOTA-THRPPMWSPVWP* (**3**)

A solution of Fmoc-protected amino hexanoic acid (21 mg, 60 μ mol) and HBTU (O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate) (22 mg, 58.5 μ mol) in DMF (1 mL) was incubated for 2 minutes with DIPEA (diisopropylethylamine) (10.3 μ L, 60 μ mol). The reaction mixture was added to side chain protected THRPPMWSPVWP (**1**, 15 μ mol) on resin and reacted for 3 hours. The Fmoc group was removed from the *N*-terminus by incubating the resin with a solution of 50% piperidine in DMF (2 mL) for 5 minutes and subsequently, a solution of *p*-SCN-Bn-NOTA (*S*-2-(4-Isothiocyanatobenzyl)-1,4,7-triazacyclononane-1,4,7-triacetic acid) (17mg, 30 μ mol) in DMF was added and reacted for 24 hours. The product was cleaved from the solid support as described for **1** and purified via semi-preparative HPLC using a gradient of 0-100% MeCN + 0.1% TFA over 30 minutes and was isolated as white solid after lyophilisation (9.85 mg, 4.79 μ mol, 32% yield).

MALDI-MS (m/z) for [M] (calculated): 2052.2 (2052.97).

2.5. Synthesis of *DOTA-S-maleimido-THRPPMWSPVWP* (**4**)

To a freshly prepared solution of maleimido-THRPPMWSPVWP (**2**, 10 mg, 5.9 μ mol) in phosphate buffer (0.1M, pH6) : MeCN 1:1 (500 μ L) was added a solution of thiol-DOTA (5.5 mg, 11.9 μ mol) in water (100 μ L). The pH of the solution was adjusted to 7.2 using phosphate buffer (0.5M, pH 7.2, 200 μ L) and the mixture was reacted for 5 minutes. The product was purified via semi-

preparative HPLC using a gradient of 0-100% MeCN + 0.1% TFA over 10 minutes and was isolated as white solid after lyophilisation (21.1 mg, 5.6 μ mol, 95% yield). ESI-MS (m/z) for [M]⁺ (calculated): 2146.04 (2146.01).

2.6. Synthesis of SiFA-S-maleimido-THRPPMWSPVWP (**5**)

To a freshly prepared solution of maleimido-THRPPMWSPVWP (**2**, 7.5 mg, 4.45 μ mol) in phosphate buffer (0.1M, pH6) : MeCN 1:1 (500 μ L) was added a solution of SiFA-SH (2.4 mg, 8.9 μ mol) in MeCN (100 μ L). The pH of the solution was adjusted to 7.2 using phosphate buffer (0.5M, pH 7.2, 200 μ L) and the mixture was reacted for 5 minutes. The product was purified via semi-preparative HPLC using a gradient of 0-45% MeCN + 0.1% TFA over 5 minutes and was isolated as white solid after lyophilisation (5.9 mg, 3.0 μ mol, 67% yield). ESI-MS (m/z) for [M]⁺ (calculated): 1952.95 (1952.93).

2.7. Radiochemistry

2.7.1. ⁶⁸Ga solution

A commercially available ⁶⁸Ge/⁶⁸Ga generator (obtained from Eckert&Ziegler, Bonn, Germany and originating from Obninsk, Russia) was eluted with 2*1 mL 0.1 M HCl and the first milliliter was discarded. To the second milliliter were added 100 μ L of a 1.25 M sodium acetate solution in water yielding a pH of 3.5 – 4.0. This buffered solution was directly used for labeling.

2.7.2. Radiolabeling of the NOTA- and DOTA-conjugated peptides (**3**) & (**4**) with ⁶⁸Ga

A solution of the respective peptide **3** or **4** (10 nmol) in HEPES buffer (0.025M, pH 4.0, 250 μ L) was added to 240-340 MBq of ⁶⁸Ga in sodium acetate

solution (1.1 mL; 0.13 M; pH 3.5 – 4.0) and incubated for 10 minutes at room temperature (**3**) or 99°C (**4**). The reaction mixtures were analyzed by analytical HPLC. The radiolabeled peptides ^{68}Ga -**3** and ^{68}Ga -**4** were found to be 96-98% pure.

2.7.3. Radiolabeling of NOTA and DOTA with ^{68}Ga

A solution of NOTA or DOTA (10 nmol) in sodium acetate buffer (0.025 M, 150 μL) was added to 140 MBq of ^{68}Ga in sodium acetate solution (1.1 mL; 0.13 M; pH 3.5 – 4.0) and incubated for 10 minutes at room temperature (NOTA) or 99°C (DOTA) at a final pH of 4.5. The reaction mixtures were analyzed by radio-thin layer chromatography. The radiolabeled products ^{68}Ga -NOTA or ^{68}Ga -DOTA were found to be 96-98% pure.

*2.7.4. Radiolabeling of SiFA-S-maleimido-THRPPMWSPVWP (**5**) with ^{18}F*

*2.7.4.1. Direct labeling of SiFA-S-maleimido-THRPPMWSPVWP (**5**)*

A solution of SiFA-S-maleimido-THRPPMWSPVWP (**5**) (10-150 nmol, 20-300 μg) in dry DMSO (500 μL) was added to the dried $[\text{nBu}_4\text{N}]^{18}\text{F}$ complex (2.6-4.5 MBq) and reacted for 5-10 minutes at room temperature. As determined by analytical HPLC, up to 12% product was formed. Final purification has not been performed.

*2.7.4.2. Two-step labeling of maleimido-THRPPMWSPVWP (**2**) with ^{18}F -SiFA-thiol*

A solution of SiFA-thiol (25-150 nmol, 49-300 μg) in dry DMSO (100 μL) was added to the dried $[\text{nBu}_4\text{N}]^{18}\text{F}$ complex (1.9-4.2 MBq) and reacted for 5-10 minutes at room temperature. As determined by analytical HPLC,

radiochemical yields of up to 31% ^{18}F -SiFA-thiol were achieved. A solution of maleimido-THRPPMWSPVWP (**2**) (45-200 nmol, 76-337 μg) in phosphate buffer (0.5M pH 4.0, 500 μL) was added and the pH of the mixture was adjusted to pH 7.2 by addition of phosphate buffer (0.5M, pH 7.2, 200 μL) and reacted for 10 minutes at room temperature. Conjugation yields of ^{18}F -SiFA-thiol to (**2**) were up to 11% as determined by analytical HPLC. Final purification has not been performed.

2.8. Dopamine D2 receptor binding

2.8.1. Stable transfection and membrane preparation

The mammalian expression vector pcDNA3.1+ containing the cDNA for the human dopamine D2 receptor (NM_000795) was obtained from the cDNA resource center of the university of Missouri-Rolla. HEK293 cells were cultured in Dulbecco's modified Eagles medium (DMEM) supplemented with 10 % fetal calf serum and 100 U/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin in a humidified atmosphere (92% air, 8% CO_2) at 37°C. All media, sera, additives and antibiotics for cell culture were purchased from PAA (Cölbe, Germany). Transfections were performed using FuGENE6 (Roche, Mannheim, Germany) according to manufacturer's protocol: 3.5×10^6 HEK cells (per 145 cm^2 flask) were plated in DMEM before transfection. On the next day cells were treated with a mixture of 60 μL FuGENE6 and 5 μg cDNA. Two days after transfection the cells were split in DMEM supplemented with 10% fetal calf serum and 100 U/ml penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin and 500 $\mu\text{g}/\text{mL}$ geneticin (selection medium). Having reached a confluence of about 30% stably transfected cells were plated 1:10000 in

selection medium. About two weeks later single colonies of stably transfected cells were isolated and further cultivated in selection medium. Cells from dishes (145 cm²) with a confluence > 85 % were isolated, washed two times with PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 1.75 mM KH₂PO₄, pH 7.4) and homogenized in a buffer containing 50mM, 1 mM EDTA, 5 mM MgCl₂, 0.1 mM DTT, 100µg/ml bacitracin, 5 µg/mL soybean trypsin inhibitor adjusted with HCl to pH 7.4 using a Polytron PT A7 (Kinematica Polytron, Littau-Lucern, Switzerland). Aliquots of this suspension, corresponding to approximately 2 mg/mL protein were stored at -80 °C. Aliquots of about 2 mL were rapidly defrosted, diluted with 20 mL binding buffer (50 mM Tris, 120 mM NaCl, 5 mM KCl, 1 mM EDTA, 4 mM MgCl₂ adjusted to pH 7.6 with HCl) and centrifuged (Kendro Sorvall, SS34 rotor, 20000 rpm, 4 °C, 20 min). The pellets were resuspended in binding buffer to yield a final protein content of about 80 µg/mL.

2.8.2. [³H] Spiperone binding

Binding assays were performed according to the protocol described by Malmberg et al. with minor modifications [32]. In competition experiments aliquots of about 2 µg protein (determined as described above) were incubated in the presence of 36 pM [³H]siperone (3.55 TBq/mmol, Amersham/GE Healthcare), test compounds in varying concentrations in a total volume of 7.5 mL (8 mL polystyrene tubes, Sarstedt AG & Co., Nümbrecht, Germany) at 30°C in a shaking water bath for 6 h. Afterwards the samples were filtrated over Whatman GF/B filters presoaked in 0.3 % polyethylenimine with a Brandel M-24R harvester. The filters were rinsed with cold 50 mM Tris HCl buffer pH 7.4 (4 x 3

mL) and counted in 3 mL Rotiszint Eco Plus using a Packard TriCarb 1600 liquid scintillation counter. Nonspecific binding was determined in presence of 1 μ M (+)-butaclamol. The concentration of a competing test compound that inhibited 50 % of specific binding (IC₅₀) was calculated using the respective function of Prism 4.02 (GraphPad Software, San Diego, CA, USA) for competition experiments. Top and bottom of the competition curve were constrained to values obtained for the controls without competitor (total binding, top) or with 1 μ M (+)-butaclamol (nonspecific binding, bottom). *K_i*-values were calculated based on a *K_d*-value of 41 pM for [³H] siperone obtained in saturation experiments and transformed to *pK_i*-values. If not stated otherwise, the data are expressed as means $\pm$ S.E.M. of three separate experiments, each carried out in triplicate.

2.9. In vitro Competition Binding Assays using ¹²⁵I-transferrin

Competition binding experiments were performed following the method described previously [31]. U87MG cells and HT-29 cells were routinely cultured in DMEM medium supplemented with sodium pyruvate (1 mM), nonessential amino acids (0.1 mM) and 10% FCS in a humidified atmosphere at 37°C. Cells were harvested, suspended in medium supplemented with 10% DMSO and samples with a concentration of 500,000 cells (U87MG) and 106 cells (HT-29) in 0.3 mL per Eppendorf tube were prepared. Each tube was centrifuged at 210 g for 2 min. The cell pellet was washed once before blocking buffer (DMEM containing 15 mM HEPES, 0.1 mM PMSF and 1% BSA; 250 μ L) was added, and incubation was continued for 15 min at room temperature under shaking. After centrifugation and aspiration of the blocking buffer, the washing procedure was

repeated once using 250 μ L of binding buffer (DMEM containing 15 mM HEPES, 0.1 mM PMSF and 0.1% BSA). Subsequently, cells were incubated on a shaker in the presence of varied amounts of competing ligand (50 pmol – 50 μ mol peptide competitor, compounds **3**, **4** and **5**) with 370 Bq/tube (2.06 nmol) ^{125} I-transferrin in a total volume of 100 μ L binding buffer at 37°C for 60 min. After the incubation time, tubes were centrifuged, the supernatants were aspirated and the cell pellets were washed twice 12 with binding buffer (2 $\times$ 250 μ L). The cell pellets with bound ^{125} I-transferrin were transferred in counting tubes for measuring the radioactivity with a γ -counter (Wallac Wizard).

Nonspecific binding of ^{125} I-transferrin was measured in the presence of a more than 104-fold molar excess of transferrin and was subtracted from total binding to yield specific binding. Each data point is an average of at least three independent experiments, each performed in triplicate. IC₅₀ values were calculated by use of the software program GraphPad Prism. For *in vitro* binding experiments with ^{68} Ga-labeled NOTA-THRPPMWSPVWP (^{68} Ga-**3**), ^{68} Ga-labeled DOTA-S-maleimido-THRPPMWSPVWP (^{68} Ga-**4**), ^{68} Ga-NOTA or ^{68} Ga-DOTA, the incubation step was performed with 0.5 - 1.0 MBq of the respective tracer in the absence or presence of 50 μ M THRPPMWSPVWP (**1**) in a total volume of 100 μ L binding buffer at 37°C for 60 min. The determination of cellular uptake was performed as described above for the binding experiments with ^{125} I-transferrin.

2.10. Animal experiments

The initial animal experiment was conducted according to protocols approved by the local Animal Protection Authorities (Regierung Mittelfranken,

Germany, No. 621-2531.31-28/06). Female Sprague–Dawley rats (170-180 g) were housed in a temperature- and air-controlled room with free access to food and water. Animals were maintained on a 12-h light/dark cycle. The biodistribution of ^{68}Ga -labeled DOTA-S-maleimido-THRPPMWSPVWP ^{68}Ga -**4** was determined by i.v. injection of two animals with 11-15 MBq (200 μL) through the tail vein under isoflurane anesthesia. Under anesthesia, rats were sacrificed by decapitation 10 min and 20 min post injection. Blood and tissue samples from liver, kidney, muscle and brain were rapidly removed, measured for radioactivity and weighed. The results were expressed as the % of injected dose per gram of wet tissue (%ID/g).

3. Results and discussion

As the TfR has been shown to be a target for efficient transport over the blood brain barrier [13-16], this carrier system was investigated for the delivery of small organic molecules into TfR expressing cells. This concept of enhanced transport could find application in radiopharmaceutical chemistry to increase the low brain uptake of radiotracers already used for neurological PET imaging in patients. Radiotracers such as ^{18}F -fallypride (Fig. 1), a receptor-ligand for imaging striatal as well as extrastriatal D2 receptors, normally enter the brain passively and reach very low final concentrations. Their conjugation to suitable carrier molecules should provide a higher accumulation of these ligands in the brain and therefore be useful to improve the overall image quality. In addition, this would also allow a reduction of the administered dose of the radiotracer to the patient. For TfR targeting, the recently identified peptide THRPPMWSPVWP

with a high affinity to the TfR and the ability to transport GFP into TfR expressing cells was investigated. A conjugate of maleimidoderivatized THRPPMWSPVWP (2) and *N*-((1-allylpyrrolidin-2-yl) methyl)-5-(3-mercaptopropyl)-2,3-dimethoxybenzamide (thio-fallypride, Fig. 1), a derivative of the D2 receptor ligand fallypride was synthesized and evaluated as to its binding affinity to the D2 receptor on HEK293 cells [32] (Fig. 1). Despite these major structural alterations, the D2 receptor binding affinity of this first, non-optimized model-conjugate was still adequate with a K_i of 27 nM, even though considerably less than for fallypride (K_i = 0.1 nM) itself. These preliminary and by all means conceptual findings demonstrate that even a structural alteration of this magnitude can lead to compounds still binding to their target, legitimizing the proposed concept of facilitated transport of low molecular weight radiopharmaceuticals by conjugating them to much larger molecular transporters.

3.1 Synthesis of the DOTA-, NOTA- and SiFA-peptide derivatives (3, 4, 5) and their radiolabeling with ^{18}F and ^{68}Ga

In order to evaluate the potential of the peptide THRPPMWSPVWP to actively transport small organic molecules conjugated to its *N*-terminal end over the BBB into the brain, we synthesized three derivatives of this lead peptide namely a DOTA, NOTA and SiFA derivative. All derivatives differed substantially in their *N*-terminus and were radiolabeled via different strategies with ^{68}Ga (for the DOTA and NOTA derivative) and ^{18}F (for the SiFA derivative). The ^{68}Ga -labeled conjugates were subsequently evaluated in vitro as to their binding and uptake into the two human TfR expressing cell lines HT29 and

U87MG which are known to express the human TfR subtypes 1 and 2 (hTfR1 and hTfR2). The two synthesized derivatives containing the macrocyclic chelators NOTA (**3**) and DOTA (**4**) were labeled with ^{68}Ga and one peptide derivative bearing a SiFA (silicon fluoride acceptor) moiety (**5**) was labeled with ^{18}F using a one- and a two step procedure. It was lately shown that SiFAs represent a favorable strategy for simple and convenient introduction of ^{18}F into various biomolecules by simple isotopic exchange [29, 33, 34] (Fig. 2). The peptide synthesis of THRPPMWSPVWP was carried out using standard Fmoc-solidphase- peptide synthesis [30] and the derivatization reactions were performed by reacting the protected on-resin peptide with hexanoic acid followed by p-SCN-Bn-NOTA and maleimido hexanoic acid, respectively. The hexanoic acid linker was introduced to avoid Edman degradation by direct coupling of p-SCN-Bn-NOTA. The maleimide-conjugated peptide (**2**) was derivatized with thiol-DOTA and SiFA-SH after cleavage and purification of the peptide from the resin in aqueous solution (phosphate buffer, 0.1M, pH 7.2 : MeCN 1:1) at pH 7.2 for 5 minutes. The purified peptides were subsequently labeled with ^{68}Ga and ^{18}F . For ^{68}Ga -labeling, the corresponding peptide derivatized with DOTA (**4**) or NOTA (**3**) was labeled with 250-300 MBq ^{68}Ga at pH 3.7 and 99°C (for **4**) or room temperature (for **3**) for 10 minutes and the labeled products were obtained in radiochemical yields of >96% and radiochemical purities of 96-98%. For ^{18}F -labeling, two different approaches were pursued: Direct labeling of SiFA-maleimido- THRPPMWSPVWP (**5**) by isotopic exchange and a two-step labeling of maleimido-THRPPMWSPVWP (**2**) with ^{18}F -SiFA-thiol which gave

12 and 11% of the labeled peptide, respectively, as determined by analytical HPLC. However, due to the relatively low radiochemical yields and the formation of several side-products which complicated the isolation of the labeled peptide, the cell binding experiments were carried out using only the ^{68}Ga -labeled peptides.

3.2 Competitive binding studies of 3, 4 and 5 on HT29 and U87MG cells pre-incubated with ^{125}I -transferrin

Although it has already been shown in the literature [25,26] that THRPPMWSPVWP does not bind to the same cavity of the human TfR as apo-transferrin and thus does not compete with endogenous apo-transferrin, we first aimed at verifying that our derivatized peptides also do not bind to the apo-transferrin binding site. Therefore, we carried out a competitive binding study using ^{125}I -transferrin on the colon carcinoma cell line HT29 and the glioblastoma cell line U87MG expressing both subtypes TfR1 and TfR2 and TfR1 alone, respectively [17]. In these experiments, displacement of receptor-bound ^{125}I -transferrin was challenged by NOTATHRPPMWSPVWP (**3**), DOTA-S-maleimido-THRPPMWSPVWP (**4**), SiFA-S-maleimido-THRPPMWSPVWP (**5**), underivatized THRPPMWSPVWP (**1**) and apo-transferrin in a concentration-dependent manner ranging from 10^{-4} to 10^{-12} -molar (Fig. 3). As shown in Fig. 3, specific binding of ^{125}I -transferrin to the TfR present on the two cell lines (HT29 and U87MG) was not inhibited by either the peptide (**1**) or the peptide derivatives (**3**, **4** and **5**), indicating that the peptides do not bind to the same cavity on the TfR. In contrast, apotransferrin itself was highly potent to replace bound ^{125}I -

transferrin with an IC₅₀ of 13±2 nM on HT29 cells that express hTfR1 and hTfR2, and an IC₅₀ of 71±24 nM on U87MG cells expressing only hTfR2.

3.3 Cell uptake experiments of ⁶⁸Ga-3 and ⁶⁸Ga-4 on HT29 and U87MG cells

To be applicable as drug carriers across the BBB, specific binding and uptake of the peptide derivatives in TfR-expressing cells are prerequisites. Therefore, uptake experiments with the ⁶⁸Ga-labeled peptides (⁶⁸Ga-3) and (⁶⁸Ga-4) were performed using HT29 and U87MG cells. As can be seen from Fig. 4, the ⁶⁸Ga-labeled peptide derivatives revealed negligible cellular uptake of far less than 1% after 60 minutes of incubation at 37°C. Incubation of U87MG and HT29 cells with the NOTA derivative ⁶⁸Ga-3 showed uptake values of 0.118±0.018% (U87MG, n=5) and 0.080±0.036% (HT29, n=7). In the presence of 50 µM of **1**, the cellular uptake of ⁶⁸Ga-3 was not affected significantly (0.095±0.020% (U87MG) and 0.092±0.044% (HT29)), thereby reflecting low if not absent specific binding of ⁶⁸Ga-3 in both TfR-expressing cell lines (Fig. 4). A similar situation was observed for the DOTA derivative ⁶⁸Ga-4 that notably showed slightly higher uptake values when compared with the NOTA peptide ⁶⁸Ga-3. However, the cellular uptake did not differ significantly in the presence and absence of 50 µM of **1** (0.656±0.184% vs. 0.603±0.068% (U87MG, n=8-12); 0.245±0.110% vs. 0.173±0.081% (HT29, n = 8-12)) (Fig. 4). In addition, we assessed the nonspecific tracer uptake in the cell lines by comparison with uptake studies using the ⁶⁸Ga-labeled unmodified metal chelators ⁶⁸Ga-NOTA and ⁶⁸Ga-DOTA (Table 1), observing uptake values within the same range as the tested ⁶⁸Ga-labeled conjugates ⁶⁸Ga-3 and ⁶⁸Ga-4.

Table 1: Cellular uptake values of ^{68}Ga -NOTA and ^{68}Ga -DOTA in U87MG and HT29 cells, respectively.

Uptake values in the absence (control) and presence of 50 μM of underivatized peptide (**1**) are expressed as mean $\pm$ standard deviation (S.D.), $n = 6-10$ (* p values of < 0.05 were considered to be significant. Statistical analysis was determined by the non-parametric Mann-Whitney test).

	Uptake (^{68}Ga -NOTA, %)			Uptake (^{68}Ga -DOTA, %)		
	Control	+1	$p^{\#}$	Control	+1	$p^{\#}$
U87MG	0.038 ± 0.021	0.049 ± 0.017	0.428	0.128 ± 0.099	0.110 ± 0.050	0.792
HT29	0.027 ± 0.028	0.024 ± 0.016	0.713	0.044 ± 0.037	0.035 ± 0.013	0.875

The observed negligible uptake of the radiolabeled peptides ^{68}Ga -**3** and ^{68}Ga -**4** could be due to the conjugation with the chelator, although even larger peptide conjugates were synthesized that still showed biological activity [25, 26]. However, Oh et al. investigated the influence of a TfR-binding peptide artemisinin-conjugate on cancer cells [26], but did not quantify the uptake of the substance into TfR-expressing cells. Another critical point is the fact that the cells that were initially used for the identification of the TfR-binding peptides by phage display were not derived from a human cell line but from a chimeric chicken embryo fibroblasts cell line not comparable to human cell lines expressing the native TfR [25]. To sum up our *in vitro* binding experiments, the ^{68}Ga -labeled

derivatives of THRPPMWSPVWP show only a nonspecific uptake in human TfR-expressing carcinoma cell lines. In addition, we performed an initial *in vivo* experiment in two healthy rats using ^{68}Ga -labeled DOTA-S-maleimido-THRPPMWSPVWP (^{68}Ga -4) to determine the capability of the labeled peptide to penetrate the BBB. Besides predominant kidney uptake and apparently slow blood clearance, the mean brain uptake of ^{68}Ga -4 was determined to be extremely low

(Table 2), confirming our *in vitro* results of negligible binding to the TfR.

Table 2: Preliminary biodistribution data with ^{68}Ga -4

Organ	% ID/g tissue	
	10 min pi	20 min pi
Blood	1.52	1.05
Liver	0.35	0.24
Kidney	2.78	1.89
Muscle	0.17	0.12
Brain	0.046	0.028

Data are expressed as mean values from three tissue samples determined for one animal at each time point.

4. Conclusion

In conclusion, our results suggest that the accumulation of conjugates of the TfR-binding peptide is too low and nonspecific to render the peptide THRPPMWSPVWP suitable as a carrier system for enhanced BBB transport of conjugated compounds. This also casts doubts on the applicability of this concept to use the peptide THRPPMWSPVWP as a vehicle for a drug delivery into the brain. However, further experiments with more conjugates have to be carried out to determine the potential of this peptide as a transferrin receptor targeting carrier molecule. The nature of the compound conjugated to the peptide might have an actual impact on the binding to the TfR and hence influence the uptake kinetics.

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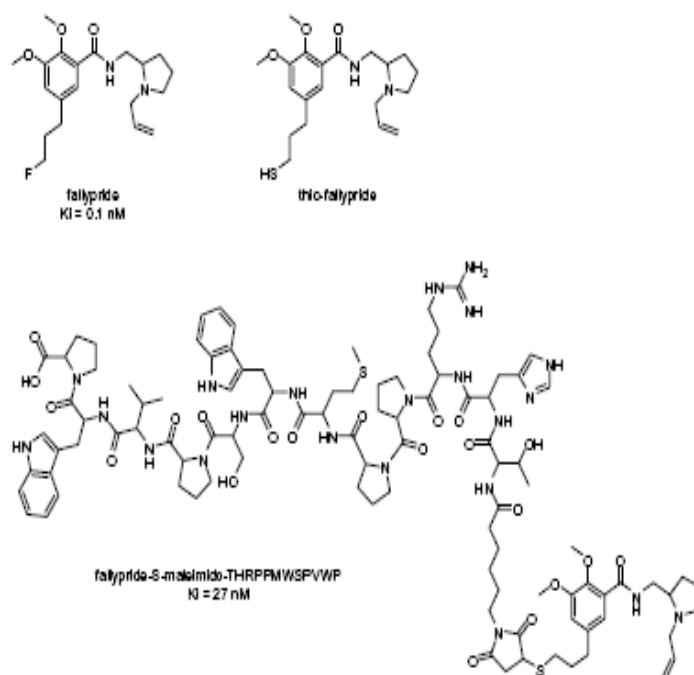


Fig. 1: Structures of the D2 receptor ligands fallypride and thio-fallypride as well as the corresponding conjugate fallypride-S-maleimido-THRPPMWSPVWP.

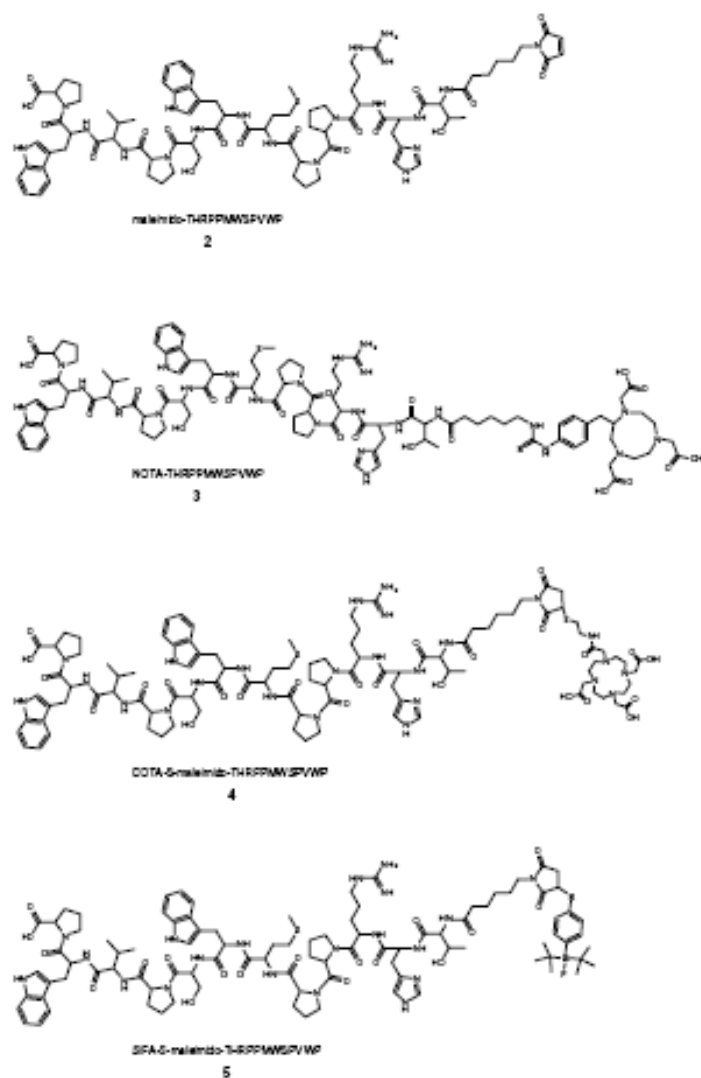


Fig. 2: Structures of the peptide derivatives maleimido-THRPPMWSPVWP (2), NOTA-THRPPMWSPVWP (3), DOTA-S-maleimido-THRPPMWSPVWP (4) and SIFA-S-maleimido-THRPPMWSPVWP (5).

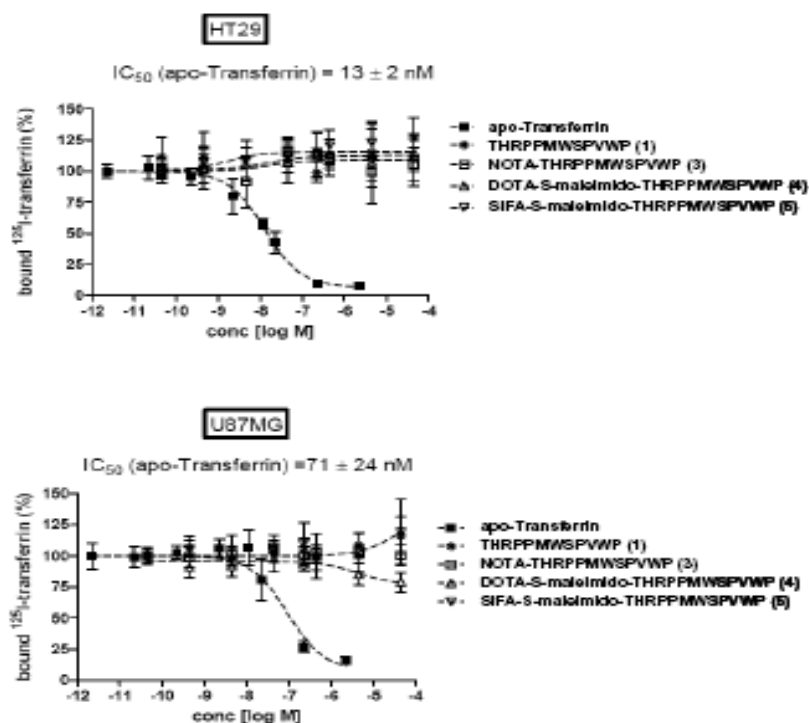


Fig. 3: Competitive binding study on HT29 and U87MG cells using apo-transferrin, THRPPMWSPVWP (1), NOTA-THRPPMWSPVWP (3), DOTA-S-maleimido-THRPPMWSPVWP (4) and SIFA-S-maleimido-THRPPMWSPVWP (5) as competitors for receptor-bound ^{125}I -transferrin. Values are expressed as mean $\pm$ standard deviation (S.D.), as determined from 2-3 independent experiments each performed in triplicate.

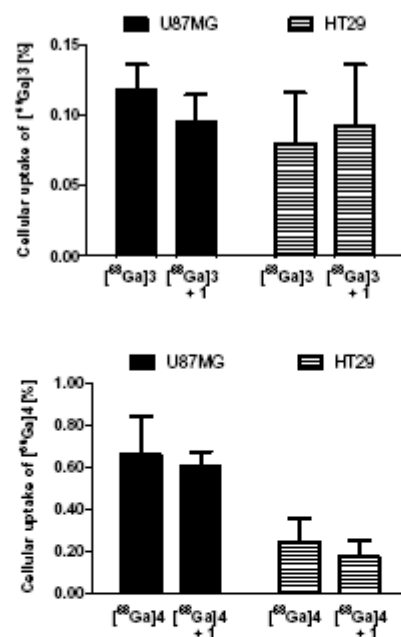


Fig. 4: Cellular uptake of ⁶⁸Ga-labeled NOTA-THRPPMWSPVWP (⁶⁸Ga-3) and DOTA-S-maleimido-THRPPMWSPVWP (⁶⁸Ga-4) on U87MG and HT29 cells at 37°C after 60 min of incubation in the presence and absence of underivatized peptide (1, 50 μM). Uptake values are expressed as mean ± standard deviation (S.D.), as determined from two to four independent experiments each performed in triplicate.

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CHAPTER 4

Experimental Part

Methods and Materials:

Resins for peptide synthesis, Fmoc-protected amino acids were purchased from NovaBiochem (Nottingham, UK) and p-SCN-Bn-NOTA was purchased from Macrocyclics (Dallas, USA). Solvents and reagents were purchased from Sigma-Aldrich (Ontario, Canada) and were used directly without further purification. 2,3,4,6-tetra-O-acetyl-1-bromo- α -D-glucopyranoside was purchased from Alfa Aesar (U.S.A). 1,2,3,4,-tetra-O-acetyl- β -D-glucopyranoside was purchased from AMRI (U.S.A).

MALDI mass spectra were obtained by using a Bruker Daltonics Microflex LT mass spectrometer.

Accurate mass measurements were performed on a LC-MSD-TOF instrument from Agilent technologies in positive electrospray mode. Either protonated molecular ions $(M+H)^+$ or sodium adducts $(M+Na)^+$ were used for empirical formula confirmation.

NMR spectra were obtained using Varian instrument 300MHz.

An Instant Imager radio-TLC scanner (Packard, Canberra) was used for determining the radio-chemical yields of the ^{18}F -labeling reactions.

Peptide THRPPMWSPVWP (1):

The peptide was synthesized on solid support applying standard Fmoc-solid-phase-peptide synthesis (80). The resin “NovaSyn® resin” (0.03 mmol), pre-linked to the C-terminal amino acid proline was first swollen in dichloromethane (DCM) (3 mL) and then washed with dimethyl formamide (DMF) (3 mL) 10 times. The Fmoc group was removed from the N-terminus by incubating the resin with a solution of 50% piperidine in DMF (2 mL) for 1 minute, then the same step was repeated for another 5 minutes. Each side-chain protected amino acid (0.12 mmol), together with O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU) (44 mg, 0.12 mmol), were dissolved in DMF (0.5 mL), then diisopropylethylamine (DIPEA) (20.7 μ L, 0.12 mmol) was added and mixed. The mixture was then added to the resin and reacted at different length of times as shown in table 2. The peptide chain was thus assembled consecutively where the resin was washed after each coupling step 10 times with DMF (3 mL). Each coupling step was preceded by removal of the Fmoc group from the N-terminus of the peptide chain. The peptide was then cleaved from the resin by incubating the resin material with a mixture of trifluoroacetic acid (TFA) / triisopropyl silane (TIS) / H₂O (95/2.5/2.5, 2 ml) for 45 minutes and the crude peptide was precipitated with cold diethyl ether (20 mL). After washing the crude peptide three times with diethyl ether (5 mL), the peptide was purified via semi-preparative HPLC using a gradient of 0-100% MeCN + 0.1% TFA over 30 minutes and was isolated as a white solid after lyophilisation (13.4 mg, 8.9 μ mol, 30 % yield).

MALDI-MS (m/z) for [M] (calculated): 1489.1 (1489.73).

Table 2: List of side-chain protected amino-acids constituting the TfR targeting peptide THRPPMWSPVWP **1** (proline was attached to the resin) and their coupling times

Amino Acid	Coupling time
Trp(Boc)	40 min
Val	20 min
Pro	20 min
Ser(tBu)	40 min
Trp(Boc)	20 min
Met	35 min
Pro	35 min
Pro	55 min
Arg(Pbf)	55 min
His(Trt)	100 min
Thr(tBu)	35 min

NOTA-Ahx- THRPPMWSPVWP (2):

A solution of Fmoc-protected amino hexanoic acid (21 mg, 0.06 mmol) and O-(benzotriazol-1-yl) -N,N,N',N'- tetra methyl uronium hexafluorophosphate (HBTU) (22 mg, 0.06mmol) in DMF (1 mL) was mixed with diisopropylethylamine (DIPEA) (10.3 μ L, 0.06 mmol). The reaction mixture was added to side chain protected THRPPMWSPVWP (0.015 mmol) on resin and reacted for 3 h. The Fmoc group was removed from the N-terminus by incubating the resin with a solution of 50% piperidine in DMF (2 mL) for 5 minutes and subsequently, a solution of S-2-(4-isothiocyanatobenzyl)-1,4,7-triazacyclononane-1,4,7-triacetic acid (p-SCN-Bn-NOTA) (17 mg, 0.03 mmol) in

DMF (2 mL), and diisopropylethylamine (DIPEA) (20.7 μ L, 0.12 mmol) was added and reacted for 24 h. The product was cleaved from the resin as described above and purified via semi-preparative HPLC using a gradient of 0-100% MeCN + 0.1% TFA over 30 minutes and was isolated as a white solid after lyophilisation (9.9 mg, 4.8 μ mol, 32% yield).

MALDI-MS (m/z) for $[M^+]$ (calculated): 2052.2 (2052.97).

2,3,4,6-Tetra-O-benzoyl- α -D-glucopyranose (4) (83):

To a solution of 2,3,4,6-tetra-O-acetyl-1-bromo- α -D-glucopyranoside (**3**) (1.17 g, 1.78 mmol) in a mixture of acetone (9.5 mL) and water (0.5 mL), Ag_2CO_3 (808 mg, 1.65 mmol) was added under argon. The reaction was left to stir overnight at room temperature. The reaction was filtered and the filtrate was concentrated under vacuum. The residue was purified using column chromatography (chloroform-ethyl acetate, 9.3:0.7) to give white crystals of **4** (781 mg, 1.31 mmol, yield=74%).

^1H -NMR $[(\delta, CDCl_3), [8 (m, 8H), 7.34 (m, 13H), \text{protons of benzene rings together with the solvent chloroform}], [6.22 (t, J=9.9, 1H), 5.7 (m, 1H), 5.2 (dd, J=3.45, 1H), 4.6 (m, 1H), 4.4 (m, 1H), 4 (m, 1H), 3.3 (d, J=3.6, 1H), \text{protons of sugar}], [2 (s, 1H), 1.6 (s, 1H), 1.2 (m, 3H), \text{unidentified peaks}].$

Esi (m/z) for $[M+Na]$ (calculated): 619.3 (619.17).

2,3,4,6-Tetra-O-benzoyl-1-(2,2,2-trichloroacetamdate)- α -D-glucopyranoside (5) (84):

4 (343 mg, 0.575 mmol) was dissolved in anhydrous dichloromethane (4 mL) under argon. The solution was cooled to 0°C and trichloroacetonitrile (173 µL, 1.73 mmol) and DBU (8.6 µL, 0.05 mmol) were added. The reaction mixture was left to stir overnight at room temperature. The solvent was removed under vacuum and the residue was purified using column chromatography (petroleum ether-ethyl acetate, 3:1) to give **5** as a yellowish solid (296 mg, 0.4 mmol, yield=70%).

¹H-NMR [(δ, CDCl₃), [8.5 (s,1H), proton of NH], [7.9 (m,8H), 7.38 (m,13H), protons of benzene rings together with the solvent chloroform], [6.8 (d,J=3.7,1H), 6.2 (t,J=9.9,1H), 5.7 (t,J=9.8,1H), 5.58 (dd,J=3.7,1H), 4.58 (m,2H), 4.47 (m,1H), protons of sugar], [1.53 (s,5H), 1.1 (s,3H), unidentified peaks]

Esi (m/z) for [M+Na] (calculated): 762.25 (762.08)

2,3,4,6-Tetra-O-acetyl-1-azido-β-D-glucopyranoside (10) :

A solution of 2,3,4,6-tetra-O-acetyl-1-bromo-α-D-glucopyranoside (**9**) (7.91 g, 0.0192 mol) in a mixture of acetone (100 mL) and water (100 mL) (1:1) was treated with sodium azide (2.5 g, 0.038 mol) and sodium iodide (catalytic amount) under argon. After 6 h the solvents were evaporated under vacuum and the residue was extracted with chloroform (500 mL), washed with water (500 mL), brine solution (1 x 10 mL) and the organic phase was dried with sodium sulphate and concentrated under vacuum. The residue was purified by column chromatography (chloroform-ethyl acetate, 9:1) to give **10** as a white powder (5.27 g, 0.014 mol, yield=73.5%).

¹H-NMR [(δ , CDCl₃), 5.2 (t, J=9.43 Hz, 1H), 5.1 (t, J=9.81, 1H), 4.9 (t, J=8.92, 1H), 4.6 (d, J=8.84, 1H, proton at anomeric position), 4.2 (dd, J=4.76, 4.75, 1H, one proton of c6), 4.1 (dd, J=2.32, 2.33, 1H, the other proton of c6), 3.8 (m, 1H), protons of the sugar], [2.1 (s, 3H), 2.07 (s, 3H), 2.02 (s, 3H), 2 (s, 3H), protons of acetyl].

¹³C-NMR [(δ , CDCl₃), (20.74 carbons of acetyl), (61.82, 68.04, 70.79, 72.76, 74.2, 88.09 carbons of the sugar), (169.38, 170.29, 170.78 carbonyls)].

Esi (m/z) for [M+Na] (calculated): 396.3 (396.11).

1-Azido- β -D-glucopyranoside (11) (85):

Compound **10** (300 mg, 0.8 mmol) was added to a stirred solution prepared from (4.5 mg, 0.19 mmol) Na in 3 ml methanol. After 45 minutes all of the starting material was dissolved and TLC (chloroform-methanol, 9.7:0.3) showed only the presence of non-migrating material. The pale yellow solution was neutralized with Dowex[®] (50WX2-200 (H)) ion-exchange resin (10 mg) for 5 min and filtered. The filtrate was evaporated under vacuum and the residue was purified using column chromatography (chloroform-methanol, 8:2) to give white crystals of **11** (140 mg, 0.68 mmol, yield=86 %).

¹H-NMR [(δ , D₂O), 4.7 (d, J=8.71, 1H, anomeric), 3.9 (dd, J=2.04, 2.18, 1H), 3.7 (dd, J=5.65, 5.42, 1H), 3.5 (m, 2H), 3.35 (s, 1H), 3.2 (t, J=8.97, 1H), protons of sugar].

¹³C-NMR [(δ , D₂O), (60.67, 69.29, 72.94, 75.85, 78, 90.22, carbons of sugar)].

Esi (m/z) for [M+Na] (calculated): 228.05 (228.07).

1-Azido-6-O-trityl- β -D-glucopyranoside (12) (86):

A solution of **11** (362 mg, 1.76 mmol) in anhydrous pyridine (4 mL) was treated with tri-phenyl methyl chloride (trityl chloride) (738 mg, 2.6 mmol) and DMAP (43.2 mg, 0.354 mmol) at 60°C under Argon. After 3.5 h, the reaction mixture was cooled to room temperature, methanol (4 mL) was added, and the solvent was evaporated under vacuum. The residue was purified by column chromatography (petroleum ether-ethyl acetate, 1:1) to give white crystals of **12** (587 mg, 1.3 mmol, yield=75%).

¹H-NMR [(δ , CDCl₃) [7.4 (m,6H), 7.25 (m,9H), protons of trityl], [4.46 (d,J=8.5,1H,anomeric), 3.44 (m,7H), protons of the sugar + OH], [4.35 (s, 1H, OH), 3.87 (s, 1H, OH)]

¹³C-NMR [(δ , CDCl₃), (127.45, 128.18, 128.8, 143.64, carbons of trityl), (63.87, 71.35, 73.36, 76.4, 76.7, 87.32, carbons of the sugar)].

Esi (m/z) for [M+Na] (calculated): 470.17 (470.18).

2,3,4-Tri-O-acetyl-1-azido-6-O-trityl- β -D-glucopyranoside (13) (87):

Acetic anhydride (535 mg, 5.2 mmol) was added drop-wise to a stirred solution of **12** (306 mg, 0.68 mmol) in anhydrous pyridine (3 mL) at 0°C. The reaction mixture was left overnight at room temperature and the solvents were evaporated under vacuum at 40°C. The residue was extracted with chloroform (100 mL), washed with water (100 mL) and brine solution (1x10 mL). The organic phase was dried with sodium sulphate and concentrated under vacuum. The residue was purified by column

chromatography (chloroform-petroleum ether, 8.5:1.5) to give white crystals of **13** (332 mg, 0.58 mmol, yield=85%).

¹H-NMR [(δ, CDCl₃) [7.2 (m,9H), 7.35 (m,6H), protons of trityl], [5.2 (t,J=9.76,1H), 5.1 (t,J=9.35,1H), 4.9 (t,J=8.77,1H), 4.6 (d,J=8.68,1H,anomeric), 3.6 (m,1H), 3.2 (dd,J=2.11,1H), 3 (dd,J=4.25,1H), protons of the sugar], [2 (s,3H), 1.9 (s,3H), 1.7 (s,3H) protons of acetyl].

¹³C-NMR [(δ, CDCl₃), (20.81 carbons of acetyl), (61.81, 68.34, 71.16, 73.12, 75.76 carbons of the sugar), (127.3, 128, 128.8, 143.61, carbons of trityl), (168.99, 169.51, 170.47 carbonyls)]

Esi (m/z) for [M+Na] (calculated): 596.2 (596.21).

2,3,4-Tri-O-acetyl-1-azido-β-D-glucopyranoside (14) (88):

Compound **13** (300 mg, 0.52 mmol) was treated with 10% TFA (300 μL, 4 mmol) in chloroform (3 mL) at room temp for 2.5 h. The reaction mixture was concentrated under vacuum and the residue was purified by column chromatography (chloroform-ethyl acetate, 1:1) to give **14** as a yellow oil (136 mg, 0.41 mmol, yield=79%).

¹H-NMR [(δ, CDCl₃), [5 (t,J=9,1H), 4.88 (t,J=9,1H), 4.6 (d,J=8.7,1H,anomeric), 4.5 (dd,J=3.79,1H), 4.3 (dd,J=1.4,1.8,1H), 3.6 (m,2H), protons of the sugar] , [2.1 (s,3H), 2.09 (s,6H), protons of acetyl].

¹³C-NMR [(δ, CDCl₃), (171.97, 171.37, 169.66 carbonyls), (88.12, 76.8, 76.42, 70.83, 68.65, 62.79, carbons of the sugar), (20.8 carbons of acetyl)].

Esi (m/z) for [M+Na] (calculated): 354.09 (354.1).

2,3,4-Tri-O-acetyl-1-azido-6-O-p-toluene sulfonyl-β-D-glucopyranoside (15) (89):

To a stirred solution of **14** (500 mg, 1.5 mmol) in anhydrous pyridine (5.5 mL) at 0°C p-toluene sulfonyl chloride was added (863 mg, 4.5 mmol) under argon. The mixture was gradually warmed up to room temperature and stirred overnight. The solvent was removed under vacuum. The residue was extracted with chloroform (100 mL), washed with water (100 mL) and brine solution (1x10 mL). The organic phase was dried with sodium sulphate and concentrated under vacuum. The residue was purified by column chromatography (chloroform-ethylacetate, 9.1:0.9) to give white crystals of **15** (584 mg, 1.2 mmol, yield=80%).

¹H-NMR [(δ, CDCl₃), [7.6 (d,J=7.84, 2H,2protons of benzene ring), 7.2 (d,J=7.9,2H other 2 protons of benzene ring)], [5.14 (t,J=9.14,1H), 4.78 (t,J=9.14,1H), 4.73 (t,J=9.47,1H), 4.5 (d,J=8.87,1H), 4.14 (d,J=12.47,1H), 3.97 (dd,J=4.21, 4.18,1H), 3.6 (d,J=9.87,1H), protons of the sugar], [2.29 (s,3H), protons of terminal methyl attached to benzene ring], [1.98 (s,3H), 1.92 (s,3H), 1.8 (s,3H), protons of acetyl].
¹³C-NMR [(δ, CDCl₃), (20.58, 20.78 carbons of acetyl), (21.73 carbon attached to benzene ring), (61.57, 70.77, 71.70, 73.82, 76.9, 87.86 carbons of sugar), (127.82, 130, 133.26, 145.62 carbons of benzene ring), (169.2, 170, 170.5 carbonyls)].

Esi (m/z) for [M+Na] (calculated): 508.1 (508.11).

1,2,3,4-Tetra-O-acetyl-6-fluoro-β-D-glucopyranoside (17) (90):

A solution of 1,2,3,4,-tetra-O-acetyl β-D-glucopyranoside (**16**) (2.63 g, 0.0075 mol) in diglyme (10 mL) was added to a cooled (-10°C) and stirred solution of diethylaminosulfur trifluoride (4 mL, 0.03 mol) in diglyme (10 mL). The temperature

was slowly raised to 60°C and maintained there for 3 h. The solvent was removed under vacuum. The residue was extracted with chloroform (200 mL), washed with water (200 mL) and brine solution (1x10 mL). The organic phase was dried with sodium sulphate and concentrated under vacuum. The residue was purified by column chromatography (chloroform-ethyl acetate, 9:1) to give **17** as a white powder (2.2 g, 0.0062 mol, yield=83%).

¹H-NMR [(δ, CDCl₃), [5.7 (d, J=8.21, 1H, anomeric proton), 5.22 (t, J=9.31, 1H), 5 (m, 2H), 4.4 (2 X ddd, J=2.4, 4, 10.5, 47 coupling with the F, 2H, the two protons of carbon 6), 3.8 (m, 1H), protons of sugar], [2.08 (s, 3H), 2.02 (s, 3H), 2 (s, 3H), 1.9 (s, 3H), protons of acetyl groups].

¹³C-NMR [(δ, CDCl₃), (20.71, 20.73 carbons of acetyl), (67.63, 67.72, 70.32, 79.66, 81.99, 91.76 carbons of sugar), (169.1, 169.3, 169.4, 170.3 carbonyls)].

¹⁹F-NMR [(δ, CDCl₃), -56.458 (dt, J=22.6)].

Esi (m/z) for [M+Na] (calculated): 373.09 (373.1).

2,3,4-Tri-O-acetyl-1-bromo-6-fluoro-α-D-glucopyranoside (18) (84):

To a solution of **17** (3.15 g, 0.0089 mol), in dichloromethane (25 mL), HBr/acetic acid (33%) (3.1 mL) was added drop wise at 4°C. The reaction mixture was left to stir overnight at room temperature. The solvent was evaporated under vacuum and the residue was neutralized with sodium bicarbonate solution, extracted with chloroform (200 mL) and washed with water (200 mL) and brine solution (1x 10 mL), dried with sodium sulphate, concentrated under vacuum and purified with column

chromatography (chloroform-ethyl acetate, 9.9:0.1) to give **18** as a white powder (1.52 g, 0.004 mol, yield=46%).

¹H-NMR [(δ , CDCl₃) [6.3 (d, J=3.99, 1H, anomeric proton), 5.5 (t, J=9.8, 1H), 5.1 (t, J=9.5, 1H), 4.79 (q, J=4, 1H), 4.5 (m, 1H), 4.4 (m, 1H), 4.2 (m, 1H), protons of the sugar], [2.07 (s, 3H), 2.04 (s, 3H), 2.01 (s, 3H), protons of the acetyl groups].

¹³C-NMR [(δ , CDCl₃), (20.79 carbons of acetyl), (66.89, 70.66, 72.9, 79.74, 80.91, 86.66, carbons of sugar), (169.47, 169.89, 170 carbonyls)].

¹⁹F-NMR [(δ , CDCl₃), -56.89 (dt, J= 24.83)].

Esi (m/z) for [M+Na] (calculated): 392.99 (393.01).

2,3,4-Tri-O-acetyl-1-azido-6-fluoro- β -D-glucopyranoside (19):

A solution of **18** (1.49 g, 0.004 mol) in a mixture of acetone (25 mL) and water (10 mL) was treated with sodium azide (0.53 g, 0.008 mol) and sodium iodide (catalytic amount) under argon. After 6 h, the solvents were evaporated under vacuum. The residue was extracted with chloroform (100 mL), washed with water (100 mL) and brine solution (1x10 mL). The organic phase was dried with sodium sulphate and concentrated under vacuum. The residue was purified by column chromatography (chloroform-ethyl acetate, 9.5:0.5) to give **19** as a white powder (1.03 g, 0.003 mol, yield=77%).

¹H-NMR [(δ , CDCl₃) [5.2 (t, J=9.49, 1H), 5 (t, J=9.88, 1H), 4.9 (t, J=8.89, 1H), 4.62 (d, J=8.85, 1H, anomeric carbon), 4.54 (m, 1H), 4.38 (m, 1H), 3.7 (m, 1H), protons of sugar], [2.03 (s, 3H), 2.01 (s, 3H), 1.97 (s, 3H), protons of acetyl groups].

^{13}C -NMR $[(\delta, \text{CDCl}_3), (20.75 \text{ carbons of acetyl}), (67.76, 70.71, 74.79, 80.54, 81.71, 88, \text{carbons of sugar}), (169.36, 169.48, 170.32 \text{ carbonyls})]$.

^{19}F -NMR $[(\delta, \text{CDCl}_3), -62.183 (\text{dt}, J= 24.89)]$.

Esi (m/z) for $[\text{M}+\text{Na}]$ (calculated): 356.08 (356.1).

Radiochemistry:

No-carrier added aqueous $^{18}\text{F}^-$ was produced in the cyclotron by irradiation of $[^{18}\text{O}]\text{H}_2\text{O}$ via the $^{18}\text{O} (\text{p}, \text{n}) ^{18}\text{F}$ nuclear reaction.

Drying of $^{18}\text{F}^-$: $^{18}\text{F}^-$ was extracted with an anion exchange resin cartridge (QMA, Waters). Kryptofix 222/ K_2CO_3 solution (10-15 mg Kryptofix, 20-30 μL 0.5M K_2CO_3 , 400-800 μL acetonitrile, 50 μL water) was used to elute the cartridge, and the resulting mixture was dried in a glass reactor by applying a stream of nitrogen and vacuum at 90°C . The drying step was repeated with 1 mL of acetonitrile.

15 (5 mg, 0.01 mmol) in 250 μL of either acetonitrile, DMF, DMSO or tertiary amyl alcohol was mixed with 50 μL taken from a 1 mL stock solution of $^{18}\text{F}^-$ in the same solvent (10 mCi/mL). The temperature was adjusted between 80 - 120°C . The reaction was monitored by radio-TLC using **19** as the cold reference.

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6.0 Appendices

Characterization of the compounds

