Modular Approaches in PET-tracer Development

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Chapter 7

Discussion, Future Perspective and Conclusion

7.1 Discussion

The aim of this thesis is to present different modular approaches within the field of positron emission tomography (PET) imaging, particularly within the design of imaging agents and PET-tracers, chemical reactions suitable for radiosynthesis and automation. To this end, modularity was introduced to achieve these different aims, such as a stepwise design approach, a modular medical imaging platform targeting the prostate-specific membrane antigen which enables the coupling of different imaging tags and optimization of the binding motif of the ligand to modulate binding affinity by introducing a modular strategy to create multivalency and its automation on a continuous-flow radiosynthesis module.

In **chapter 2**, we discussed the concept of multivalency and its relation to binding of ligands to targets, which appeared to be a very useful concept for medical imaging applications as it improves the binding avidity of compounds. As the binding avidity influences the binding potential of imaging agents, multivalency would be able to improve the image properties of multivalent imaging agents, however until now the clinical use is limited. This is caused, among other reasons, by the challenging synthesis of those multivalent constructs and several *in vivo* obstacles that are faced by these molecular multivalent imaging agents when it comes to their pharmacokinetic behavior [1,2]. This is reflected by the fact that there is no multivalent molecular imaging agent used in clinics yet. As elaborated in this chapter, knowledge regarding multivalent interactions is still lacking. One reason why multivalent compounds are very interesting in medical imaging are their underlying thermodynamics, which provide well-grounded equations that represent the beneficial effects of multivalency on binding avidity in the form of a lowered K_d [3]. The lowered K_d is caused by the higher Gibbs free energy as a consequence of the entropy increase due to the higher valency of multivalent imaging agent compared to the monovalent equivalents and the additional intermolecular interactions based on the degeneracy coefficient Ω_i and possible cooperativity effects [3-5] supported by the high concentration of ligands of the imaging agent at the binding site [6]. It is even so significant, that many groups, including ourselves as presented in chapter 6, tend to decorate a core molecule with multiple ligands that show good monovalent binding properties, and appropriate linker length and expect to obtain increased binding affinity [4]. The here presented design approach shows, how important it is to define the different requirements of such a molecule, e.g. ligand concentration and linker length, and to be able to determine the different building blocks that are necessary to obtain a multivalent imaging agent. Since the different building blocks can be changed, we can here also talk about a modular approach to selectively change properties of the imaging agent to improve its *in vitro* and *in vivo* behavior. The here presented stepwise approach might not yet be the best solution to create a multivalent molecular imaging agent, but it is intended as starting-point to combine the information that we already have obtain within the field of multivalency and further develop and adjust this approach we might be able to have MMIAs in the clinics.

In **chapter 3**, we introduced a modular molecular platform that relies on copper(I)-catalyzed azide-alkyne [3+2]-cycloaddition (CuAAC) and is aimed at targeting the prostate-specific membrane antigen (PSMA). PSMA PET imaging is an attractive target for nuclear medicine

and serves here as a showcase to present that our modular imaging agent approach can be realized without loss of binding potential. This can be of importance for other binding motifs than PSMA, as common approaches to introduce other imaging tags, such as paramagnetic-metal chelators for magnetic resonance imaging or fluorescent- groups, end up in structural changes of the binding motif [7,8] or can have influence on the binding affinity, especially when used in multimodal imaging approaches [9]. In the specific case of PSMA targeting, it is known that these large imaging agents are still very well tolerated when they are located outside the entrance lid [10], which was discovered to be a tunnel-like region with a length of 20 Å [11]. The alkyne-functionality of the modular molecular platform targeting PSMA enables the coupling of every desired signaling moiety given it has the azide-functionality, which provides a universal molecular platform for different imaging modalities without altering its pharmacophore. However, we also faced some unexpected, yet interesting, challenges. Despite the successful competitive binding assay using the reference compound F-PSMA-MIC01 against [^{68}Ga]PSMA-11, we were not able to obtain *in vitro* results of tracer uptake, internalization and efflux rate of [^{18}F]PSMA-MIC01 in cell cultures. FACS analysis revealed sufficient PSMA expression of the used LNCaP cells. Additionally, cell culture media with and without serum were used to see whether the inability so detect tracer uptake is caused by protein binding effects – however, no tracer uptake could be detected in all cases.

In **chapter 4,** we presented the new FlowSafe radiosynthesis module that combines in-batch with continuous-flow microfluidics. The synthesizer can be adjusted to the required conditions for several different radiotracer syntheses and shows the modularity on the level of radiotracer production, which forms the third pillar of the radiotracer accessibility [12]. As all parts are connected by tubes, connectors and needles, 4 different radiotracers could be synthesized with only minor changes in the setup of the radiosynthesis module. However, all parts can be changed, such as a microreactor with more inlets to include a third reactant instead of 2, as used in this study. This shows that even the technology of radiosynthesis productions can be modular. In the end, we were able to fully automize the production of [^{18}F]PSMA-MIC01 using the prototype of the FlowSafe to obtain injectable sterilized product for preclinical *in vivo* studies, as presented in chapter 3.

In **chapter 5**, we continued our work on the modular PSMA-targeting platform by targeting the arene binding site. After the discovery of the arene-binding site, located at the entrance lid of PSMA, it was confirmed that the introduction of an aryl-group improves the binding affinity of PSMA-ligands [13,14] by the π-π stacking of the phenyl -ring and Trp541 [15]. However, until now it was expected that the linker length was the main determinant whether the arene binding site is targeted by an phenyl-ring or not [14,16]. This is supported by the design of [^{68}Ga]PSMA-11 ([^{68}Ga]PSMA-11), in which it was supposed that the aromatic part of the chelator improves binding [17]. Interestingly, all reported tracers used an electron-deficient phenyl-ring without mentioning the reasons behind it, while we used an electron-rich phenyl-ring. This shows that despite the well-studied characteristics of PSMA, there are still aspects that need further investigation. This study was performed with the same modular molecular platform as used for [^{18}F]PSMA-MIC01 and our library on PSMA-targeting modular platforms got extended with the azide-functionalized modular platform, which had an additional methylene group at

modular platform, while the fluorinated alkyne-synthon is one methyl-group shorter. This produced F-PSMA-MIC03 with the same molecular weight, chemical formula and reaction conditions, but enables us to investigate the effect of the different triazole-orientation caused by regioselective CuAAC and the flexible methyl-group between aromatic and triazole ring. Additionally, we could quite easily modify the linker that is coupled to the moiety to target the arene-binding site without changing the pharmacophore.

Despite the promising properties obtained by our PSMA-targeting modular platform, we shifted to another modular platform in **chapter 6**, which is aimed in targeting the cardiac β-adrenergic receptors. The work described in this chapter was intended to show the effect of multivalency on the binding avidity of ligands targeting the β-adrenergic receptors. A collaboration, started a few years ago, inspired us to continue with this project, as it has several advantages compared to PSMA targeting. One advantage is that β-adrenergic receptors only reduces the size of the binding pocket a bit when a ligand is bound to the pharmacophore [18], while PSMA is able to rearrange itself to open the arene binding site which alters the positioning of the key residues that are responsible for the ligand-target interaction and opens the entrance lid [15]. As this study was intended as a proof-of-concept, the choice of the β-adrenergic receptors ligand simplifies the ligand design and the main information obtained in this project is, if multivalency will increase the binding affinity of the monomeric ligand and will be independent on the structural changes. Targeting PSMA in this context already involves more properties to incorporate into the design, namely the remote arene binding [15] site as well as tunnel-like entrance lid of around 20 Å [11]. Therefore, we targeted cardiac β-adrenergic receptors (βARs) in chapter 6 by designing a new ligand and using the modular CuAAC click reaction to achieve a trimeric ligand which was directed to improve the binding affinity. However, targeting βAR PET imaging is often restricted to poor binding affinity *in vivo* associated with high non-specific uptake profile. It was assumed that multivalency can increase the binding affinity and improve its *in vivo* behavior. Our multivalency approach included the synthesis of a novel propranolol-derivative that is able to undergo CuAAC for multimerization purposes. We succeeded in synthesizing a monovalent βAR ligand which showed to have affinity to the β-adrenergic receptors on rat C6 glioma cells. The first step was made in preparing a new β-antagonist for PET imaging purposes, which showed interaction with the desired target. However, this study was performed with a race mate of the monomer and consequently a mixture for the trimer. In order to improve the binding affinity, the synthesis needs to be optimized to obtain solely the active (*S)*-enantiomer. The easiest way to synthesize the (*S*)-enantiomer would be by replacing epibromohydrin with (*S*)-glycidyl-nosylate as previously reported by our group [19]. After showing that our approaches for synthesizing a trimeric βAR showed improved binding avidity, this is another potential platform also for the PSMA-platform used in chapter 3 and 5.

7.2 Future Perspective

This thesis provides an overview on modular aspects of PET tracer development, which involves the design of modular molecular platforms that can easily be synthesized with click reactions and automated. At the same time, a lot of work presented in this thesis is still in progress. Therefore, an outlook is provided on the next steps that are envisioned in future.

For **multivalent molecular imaging agents**, we are still in the fundamental research phase in determining and clarifying how multivalency actually improves binding avidity *in vivo*. Antibodies, in form of immunoglobulin M, are natural examples of multivalent agents found throughout the body proofing that there are advantages of multimers compared to monomers [20–23]. However, its function is still not completely understood, as it was discovered that it is involved in the early pathogen detection stage which is then replaced by IgA and IgG [22]. IgM is a good example, in which multiple antibodies with low binding affinities strengthen its interaction with the target by having repeating units of the same antibody [22]. In order to achieve the same effect of increasing binding affinity, we need to gain more information about its behavior *in vivo* to be able to apply multivalency in medical imaging by figuring out the main hurdles in the translational phase from preclinical to clinical trials, which is true not only for multivalent agents, but imaging agents in general. *In silico* studies can help to understand the correlations between the multivalent imaging agent and its surrounding such as protein binding with blood plasma proteins, e.g. human serum albumin and α-1-acid glycoproteins [1,24], but do not explain the fundamental gaps of the correlation between binding affinity, composition, protein binding and *in vivo* behavior that hinders researchers to synthesize a successful multivalent imaging agent that shows the desired properties in human. Another factor might be, that the blood plasma proteins of rodents show small structural differences compared to human proteins, which results in different protein binding profiles between preclinical and clinical phases and different non-specific uptake *in vivo*. This difference could be easily assessed beforehand by structure- activity-relationship determinations and surface-plasmon-resonance measurements [1,24] of imaging agent - protein complexes.

However, with the large amount of research groups all over the world involved in research areas such as pharmacology, medicine, medicinal chemistry, drug delivery and medical imaging, this problem is being tackled from different point of views. If we combine the expertise of every single discipline, we might be already further than we think. As we detected in the process of preparations of **chapter 2**, we could find several publications in the field of drug delivery which can easily be translated into the field of multivalent medical imaging agents, in which we want to mention the reviews of Tjandra & Thodarsson [4] or Blanco et al. [25], which were also used in chapter 2 for describing the thermodynamics and binding modes and pharmacokinetics caused by biological obstacles, respectively. Furthermore, there are already several nanoparticle-drug complexes that obtained FDA approval or the European CE certification [26]. However, medical imaging needs to consider more properties than drug delivery systems, as they need to provide the required tissue accumulation. Although, when we combine drug delivery with medical imaging, we are able to study and understand the pharmacokinetics of the drug delivery system and can optimize it for the required needs, but we also obtain a highly specific drug complex, as we optimized the tissue uptake for medical imaging purposes. Additionally, we found that we are already able to pre-define the characteristics of the linker by biophysical and mathematical approaches, which includes the stiffness and length of the liner, and the influence of the cell membrane stiffness [27–29]. However, usually the translation into the synthetic approaches requires experts collaborating from all fields.

The application of the **copper-catalyzed click reaction** in radiopharmaceutical sciences is established [30]. However, especially after the development of its copper-free variant, the strain promoted azide-alkyne click reaction, [31] and other bioorthogonal click reactions [32–34], CuAAC is widely avoided due to the toxicity of copper(I) [35]. In this thesis we aimed to show the large opportunity offered by click chemistry in radiopharmaceutical sciences. Without changing the binding motif of PSMA, we improved the binding affinity and obtained 4 different PSMA-targeting compounds (F-PSMA0MIC01 – F-PSMA-MIC04) with different binding properties.

However, for **[^{18}F]PSMA-MIC01** *in vitro* experiments to determine tracer uptake in PSMA positive cell lines and assessment of internalization and efflux rates were problematic. After excluding the problem on the cellular level by confirming the PSMA expression, a careful step-by-step analysis should be made to determine the problem, such as the possibility of a too low molar activity. Further, the introduced electron-rich aromatic in **[^{18}F]PSMA-MIC02** showed sub-optimal face-to-edge π-π stacking. For improving binding affinity of PSMA-tracers, a set of different electron-deficient aromatic phenyl rings should be studied to find the best match with the arene binding site. It is difficult to discriminate small structurally changes by computational approaches, as we presented in this study with the triazole arrangement. Since our study was not based on the crystal structure of our compounds with PSMA, but a purely computational approach, the triazole moiety of all compounds was fitted into the same position during the molecular docking and dynamics studies. Therefore, it is advised to test *in vitro* several PSMA-tracers using both modular platforms with alkyne-and azide-functionality, including different linker lengths, as it was shown in the study by Zhang *et al.* that this can improve or hinder binding to PSMA [15]. Nevertheless, after successful radiolabeling and automation of [^{18}F]PSMA-MIC02, it would be interesting to investigate the effect of increased binding affinity of [^{18}F]PSMA-MIC02, compared to [^{18}F]PSMA-MIC01, in terms of organ distribution and tumor accumulation. Since the log*D* is slightly lower, a higher liver uptake is expected, as described earlier [33]. On the other hand, a higher tumor accumulation, due to a higher binding affinity, is expected as well. The here obtained RCY of 9 % is still quite low, although it would be sufficient to perform *in vivo* studies, it would be appreciated to optimize the radiosynthesis.

The **FlowSafe** radiosynthesis module used in this study is a prototype and showed typical technical issues that needed to be solved during the development of a radiosynthetic procedure. Several valves had to be replaced because of a too high pressure observed when using DMSO, which might have been earlier detected when the pressure meter did not had to be by-passed during the radiolabeling. Another drawback is that the pressures created by the syringe pumps exceed the limits of the valves, which will cause long-term problems when the valves are ageing. Several issues should be carefully examined: It might be useful to adjust the pressure limits of the valves, either by replacing the valves with those of higher resistance, or by determining the maximal flow-speed limits per solvent and include them in the manual. Another recommended adjustment concerns the user interface of Absynth: since it is important to follow the status of the FlowSafe by temperature of the different reactors, it would be useful to follow the script and the report in the same window. Unfortunately, in the current version of Absynth,

this is not possible. Therefore, errors such as a stuck pump can only be back-tracked post-factum and might lead to follow-up problems. But in the end, the FlowSafe provided quite reliable results in terms of conversion and radiochemical purity. The molar activities obtained where still quite low, which is a matter of upscaling the radioactivity, since the used radioactivity was usually below 15 GBq.

The **mono- and trivalent β-adrenergic receptor ligands** introduced here need further investigation. Molecular docking studies suggested that 2,6-dihydroxynaphthalene does not provide the optimal positioning of the compound in the binding pocket, as presented in chapter 6. To obtain a higher binding affinity of the monomeric βAR ligand, different attachment-points for the triethylene-glycol linker and the β-hydroxypropanolamine were docked in AutodockVina [36]. This shows that the fit between our proposed monomer using 1,6-dihydroxynaphthalene and the parent cyanopindolol shows a good fit within the binding pocket (Figure 2). But as shown for the case of the clinically used metoprolol, docking studies and real life do not always match. In order to obtain the best ligand, a set of different naphthalenes should be made, modified at the ortho-, meta- and para-positions to determine the best position for the monomer as well as for the trimer. Additionally, the effect should be tested on the influence of the flexible polyethylene glycol ligand, as the consensus docking study suggests some kind of interactions. Therefore, it might be a good idea to stabilize the ligand within the pharmacophore with a short hydrophobic linker such as 1,3-propanediol or 1,4,butanediol, which is then coupled to the naphthalene and the triethylene glycol linker. This reduces the interaction of oxygens of the PEG linker within the binding pocket, but still introduced the flexibility of the ligand. A similar approach was used for a hydrophobic linker, in which the introduction of a flexible linker improved the binding affinity [37].

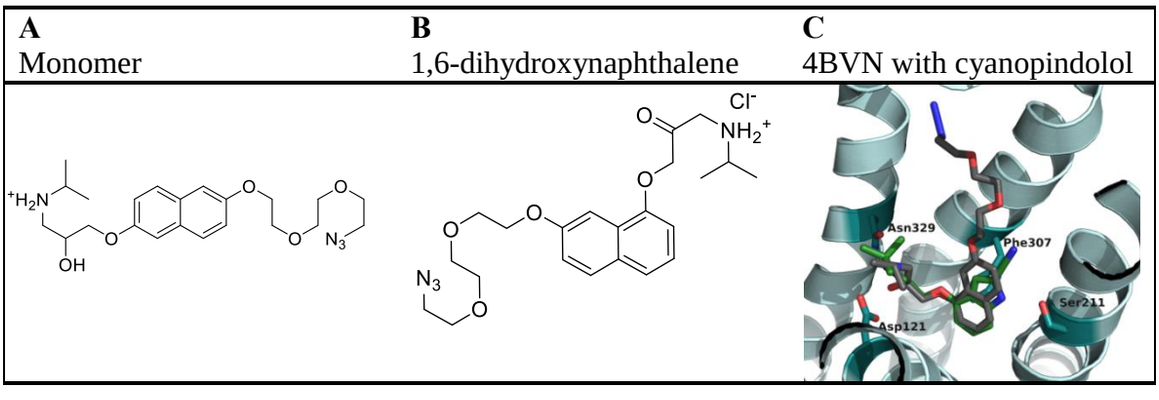

Figure 2. A possible outlook on an improved version of the monomer used in this study. (A) The monomer used in this study, which provides a suboptimal attachment point for the linker at the naphthalene. (B) The better choice of the monomer, using 1,6-dihydroxynaphthalene instead of the here presented 2,7-dihydroxynaphthalene. (C) Docking pose of the suggested improved monomer using 1,6-hydroxynaphthalene.

We successfully applied the versatile and modular CuAAC click reaction in order to synthesize a trivalent β-adrenergic receptors binding ligand, which also showed similar binding affinity than the monomer. The effect of multivalency is usually referred to as the binding affinity determined by the inhibitory concentration at half maximum (IC_{50}) [3], just as in this thesis presented as $logIC_{50}$ data. However, as described in chapter 2, only monovalent ligands have

binding affinity, multivalent ligands have avidity, therefore the $\log IC_{50}$ obtained by using the concentration of a multivalent compound is not completely comparable with the $\log IC_{50}$ of a monomer, as the multimer has several copies of one ligand and the monomer only one while the same molar concentration range is used. A more precise comparison would be the determination of the dissociation constant K_d and should become mandatory for multivalency studies. However, as a first indicative study, as performed in chapter 6, the IC_{50} is a valid parameter, which should then include the ratio IC_{50}^{multi} $(IC_{50}^{monomer})^{-1}$, as this ratio expresses the relative potency (rp), which is related to the enhancement factor [5]. In order to evaluate the effect of multimerization, this ratio can be divided by the number of ligands n of the multimers and one can say that a multivalency effect occurs when rp/n is >1, and does not occur when rp/n is <1 [5]. As we obtained a rp of 3.29 and thus a rp/n of 1.09, we can say that we could detect a small multivalency effect of the enantiomer-mixture and thus it is worth to further improve this trimeric β_1AR by synthesizing the enantiomer-pure variant.

Another point for improvement regarding the trimer was the solubility of the final compound: although it is composed of three hydrophilic triethylene-glycol linkers, it showed a reduced solubility after freeze-drying. Solubility might also be one reason why the yield is so low, as it was purified by prep-HPLC using acetonitrile-H_2O as eluent which may have caused losses of compound. However, LCMS showed quantitative conversion of the core triethynylbenzene. The later fractions of the trimer were contaminated with the monomer and could not be used for *in vitro* studies. By adjusting the eluens, the peak obtained on prep-HPLC might get sharper and could result in reduction of the contamination with unreacted monomer. Solubility issues might have influenced the *in vitro* binding at the target site, in case some aggregation occurs. Altogether, a more hydrophilic core or a longer linker length should be considered to improve the solubility, as even colloidal structures found to be effective in drug delivery systems given they have linker that is not too long [38]. Another possible option might be the use of a short linear polymer instead of a trimeric core, as the reduced receptor density might result in larger distances between the receptors on the cell surface. The linear polymeric backbone can then be prepared fully out of a hydrophilic polymer which solves the problematic use of the hydrophobic core. However, the conclusion of this multivalency experience is that it needs definitely more than a multivalent core and a binding ligand to improve binding affinity.

All in all, this thesis showed the different types of **modularity** in the field of PET tracer development. We have shown that the design of a multivalent imaging agent is dependent on many different factors, such as the receptor density, linker length, and multivalent scaffold but also the type of synthesis required to obtain the desired ligand concentration. We further showed that a modular synthesis approach enables the development of a molecular platform, which in this thesis addressed the field of prostate cancer and heart failure. The multivalent approach used for the synthesis of the trimer that is targeting the β-adrenergic receptors, can be adapted for prostate cancer by changing the linker properties for the requirements for PSMA. The important consideration here would be to target the arene-binding site and having the multivalent core outside the entrance funnel with a linker length that enables the simultaneous binding to two or more antigens. We presented also a new modular radiosynthesis module which can be adapted to several set-ups and connections of in-batch reactors and microreactors.

However, this thesis also comprises a broad range of molecular design, synthesis, *in vitro* and *in vivo* studies but it also shows inside into the technical side of radiochemistry. Therefore, the different chapters of this thesis provide different perspectives of modularity in the field of PET tracer development, which, referring to the definition of modular, formed a complete whole of 4 years of work with new ideas how to improve future experiments.

7.3 Conclusion

This thesis, entitled *'Modular Approaches in PET tracer Development – Radiotracer Design, Synthesis and Automation for Prostate Cancer and Heart Failure'*, gave an overview of different approached to include modularity into the design, synthesis and automation. The modular design was exemplified by the stepwise design approach for multivalent molecular imaging probes, in which different building blocks, such as the ligand, its linker and the molecular construction, need to be combined into one final multivalent imaging agent. The modular synthesis was shown in the successful synthesis of in total 4 prostate-specific membrane antigen ligands (F-PSMA-MIC01 – F-PSMA-MIC04) and one trimeric β-adrenergic receptor ligands synthesized using the modular copper(I)-catalyzed [3+2]-cycloaddition (CuAAC). Finally, the automation of 4 different fluorine-18 based radiotracers could provide an insight into the modularity of the FlowSafe radiosynthesis module, which could be adjusted for the synthesis just by rearrangement of the different parts.

However, this was just an initial step of showing how much more we can prepare with the tools we already have, which can be seen in the form of the stepwise synthesis approach, but also the application of CuAAC to show that we can synthesize modular imaging agents in short time. This knowledge can be used for future studies demonstrating that this modular approach can lead to many more medical imaging agents with similar binding affinities.

7.4 References

[1] E. Fabini, U. H. Danielson, *J. Pharm. Biomed. Anal.* **2017**, *144*, 188–194.
[2] T. Uehara, A. Sensui, S. Ishioka, Y. Mizuno, S. Takahashi, H. Takemori, H. Suzuki, Y. Arano, *Mol. Pharmarmaceutics* **2020**, *17*, 5, 1621 - 1628.
[3] P. I. Kitov, D. R. Bundle, *J. Am. Chem. Soc.* **2003**, *125*, 52, 16271–16284.
[4] K. C. Tjandra, P. Thordarson, *Bioconjug. Chem.* **2019**, *30*, 3, 503–514.
[5] Y. H. W. Derks, D. W. P. M. Löwik, J. P. M. Sedelaar, M. Gotthardt, O. C. Boerman, M. Rijpkema, S. Lütje, S. Heskamp, *Theranostics* **2019**, *9*, 23, 6824–6839.
[6] G. Y. Mitronova, G. Lukinavičius, A. N. Butkevich, T. Kohl, V. N. Belov, S. E. Lehnart, S. W. Hell, *Sci. Rep.* **2017**, *7*, 12319.
[7] R. Hübner, X. Cheng, B. Wängler, C. Waengler, *Chem. – A Eur. J.* **2020**, doi: 10.1002/chem.202002386 [ahead of print].
[8] J. Tykvart, J. Schimer, A. Jančařík, J. Bařinková, V. Navrátil, J. Starková, K. Šrámková, J. Konvalinka, P. Majer, P. Šácha, *J. Med. Chem.* **2015**, *58*, 10, 4357–4363.
[9] S. Lütje, S. Heskamp, A. S. Cornelissen, T. D. Poeppel, S. A. M. W. van den Broek, S. Rosenbaum-Krumme, A. Bockisch, M. Gotthardt, M. Rijpkema, O. C. Boerman, *Theranostics* **2015**, *5*, 12, 1388–1401.
[10] V. Pichler, N. Berroterán-Infante, C. Philippe, C. Vraka, E.-M. Klebermass, T. Balber, S. Pfaff, L. Nics, M. Mitterhausr, W. Wadsak, *J. Nucl. Med.* **2018**, *59*, 9, 1350–1354.
[11] K. Kopka, M. Benešová, C. Bařinka, U. Haberkorn, J. Babich, *J. Nucl. Med.* **2017**, 58, 17S–26S.
[12] M. Benešová, U. Bauder-Wüst, M. Schäfer, K. D. Klika, W. Mier, U. Haberkorn, K. Kopka,

M. Eder, *J. Med. Chem.* **2016**, *59*, 5, 1761–1775.
[13] A. X. Zhang, R. P. Murelli, C. Barinka, J. Michel, A. Cocleaza, W. L. Jorgensen, J. Lubkowski, D. A. Spiegel, *J. Am. Chem. Soc.* **2010**, *132*, 36, 12711–12716.
[14] T. Liu, J. R. Nedrow-Byers, M. R. Hopkins, C. E. Berkman, *Bioorg. Med. Chem. Lett.* **2011**, *21*, 23, 7013–7016.
[15] M. Eder, O. Neels, M. Müller, U. Bauder-Wüst, Y. Remde, M. Schäfer, U. Hennrich, M. Eisenhut, A. Afshar-Oromieh, U. Haberkorn, K. Kopka, *Pharmaceuticals* **2014**, *7*, 7, 779–796.
[16] T. Warne, P. C. Edwards, A. S. Doré, A. G. W. Leslie, C. G. Tate, *Science.* **2019**, *364*, 6442, 775 – 778.
[17] A. van Waarde, J. Doorduin, J. R. de Jong, R. A. Dierckx, P. H. Elsinga, *Neurochem. Int.* **2008**, *52*, 4-5, 729–733.
[18] N. E. Holodick, N. Rodríguez-Zhurbenko, A. M. Hernández, *Front. Immunol.* **2017**, *8*, 872.
[19] A. C. Davis, K. H. Roux, M. J. Shulman, *Eur. J. Immunol.* **1988**, *18*, 7, 1001–1008.
[20] V. B. Klimovich, *Biochemistry. (Mosc).* **2011**, *76*, 534–549.
[21] C. Grönwall, G. J. Silverman, *J. Clin. Immunol.* **2014**, *34*,12-21.
[22] K. W. Lexa, E. Dolghih, M. P. Jacobson, *PLoS One* **2014**, *9*, 4, e93323.
[23] M. McKenzie, S. M. Ha, A. Rammohan, R. Radhakrishnan, N. Ramakrishnan, *Biophys. J.* **2018**, *114*, 8, 1830–1846.
[24] N. Ramakrishnan, R. W. Tourdot, D. M. Eckmann, P. S. Ayyaswamy, V. R. Muzykantov, R. Radhakrishnan, *R. Soc. Open Sci.* **2016**, *3*, 160260.
[25] T. A. Shewmake, F. J. Solis, R. J. Gillies, M. R. Caplan, *Biomacromolecules* **2008**, *9*, 11, 3057–3064.
[26] J. Marik, J. L. Sutcliffe, *Tetrahedron Lett.* **2006**, *47*, 37, 6681–6684.
[27] L. S. Campbell-Verduyn, L. Mirfeizi, A. K. Schoonen, R. A. Dierckx, P. H. Elsinga, B. L. Feringa, *Angew. Chem. - Int. Ed.* **2011**, *50*, 47, 11117–11120.
[28] J.-P. Meyer, P. Adumeau, J. S. Lewis, B. M. Zeglis, *Bioconjug. Chem.* **2016**, *27*, 12, 2791–2807.
[29] E. Saxon, J. I. Armstrong, C. R. Bertozzi, *Org. Lett.* **2000**, *2*, 14, 2141–2143.
[30] T. Reiner, B. M. Zeglis, *J. Labelled Comp. Radiopharm.* **2014**, *57*, 285–290.
[31] J. Li, P. R. Chen, *Nat. Chem. Biol.* **2016**, *12*, 129–137.
[32] O. Trott, A. J. Olson, *J. Comput. Chem.* **2010**, *31*, 2, 455–461.
[33] J. Wang, M. Dzuricky, A. Chilkoti, *Nano Lett.* **2017**, *17*, 10, 5995–6005.
[34] K. Abstiens, M. Gregoritza, A. M. Goepferich, *ACS Appl. Mater. Interfaces* **2019**, *11*, 1, 1311–1320.