Pretargeted Imaging Beyond the Blood-Brain-Barrier

Vladimir Shalgunov1,*, Sara Lopes van den Broek1,*, Ida Vang Andersen3, Rocío García Vázquez2, Nakul R. Raval2, Mikael Palner2, Yuki Mori3, Gabriela Schäfer4, Hannes Mikula5, Natalie Beschörner3, Maiken Nedergaard3, Stina Svyänen6, Matthias Barz2, Gitte M. Knudsen7, Umberto M. Battisti3,*8, Matthias M. Herth1,7*

1 Department of Drug Design and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen, Universitetsparken 2, 2100 Copenhagen, Denmark; 2 Center for Integrated Molecular Brain Imaging, Rigshospitalet Copenhagen University Hospital, Blegdamsvej 9, DK-2100 Copenhagen, Denmark; 3 Center for Translational Neumedicine, University of Copenhagen, Blegdamsvej 3B, DK-2200 Copenhagen, Denmark; 4 Leiden Academic Centre for Drug Research, Leiden University, Einsteinweg 55, 2333CC Leiden, the Netherlands; 5 Institute of Applied Synthetic Chemistry, Technische Universität Wien (TU Wien), Getreidemarkt 9, 1060 Vienna, Austria; 6 Rudbeck Laboratory, Department of Public Health and Caring Sciences, University of Uppsala, Dag Hammarskjölds väg 20, 75185 Uppsala, Sweden; 7 Department of Clinical Physiology, Nuclear Medicine & PET, Rigshospitalet Copenhagen University Hospital, Blegdamsvej 9, 2100 Copenhagen, Denmark

*Authors contributed equally, # Corresponding authors (matthias.herth@sund.ku.dk and umberto.battisti@sund.ku.dk)

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Abstract

Pretargeting is a powerful nuclear imaging strategy to achieve enhanced imaging contrast for nanomedicines. It reduces the radiation burden to healthy tissue. Pretargeting is based on bioorthogonal chemistry. The most attractive reaction for this purpose is currently the tetrazine ligation, which occurs between trans-cyclooctene (TCO) tags and tetrazines (Tzs). Pretargeted imaging beyond the blood-brain-barrier (BBB) has not been reported thus far. In this study, we developed Tz imaging agents that are capable to ligate in vivo to targets beyond the BBB. We chose to develop 18F-labeled Tzs as they can be applied to positron emission tomography (PET) - the most powerful molecular imaging technology. Fluorine-18 is an ideal radionuclide for PET due to its almost ideal decay properties. Fluorine-18 also allows - as a non-metal radionuclide - to develop Tzs with physicochemical properties enabling passive brain diffusion. In order to develop these imaging agents, we applied a rational drug design approach. This approach was based on estimated and experimental determined parameters such as the BBB score, pretargeted autoradiography contrast, in vivo input and washout curves as well as on metabolism studies. From initially 18 developed structures, five Tzs were selected to be tested on their in vivo click performance. Whereas all selected structures clicked in vivo into the brain, [18F]18 displayed the most favorable characteristics with respect to brain pretargeting. [18F]18 is our lead compound for future pretargeted imaging studies based on BBB-penetrant monoclonal antibodies. Pretargeting beyond the BBB will allow us to image targets beyond the BBB that are currently not imageable. For example, soluble protein isoforms could be imaged. These proteins are valuable drug targets for several neurodegenerative diseases and can currently not be imaged. Imaging would allow for diagnosis of these diseases, identifying responders from non-responders or to monitor treatment. Consequently, imaging will provide valuable information to accelerate drug development and greatly benefit patient care.

Introduction

Positron Emission Tomography (PET) is a non-invasive molecular imaging method that relies on radiolabeled molecules and is routinely used for clinical diagnosis, treatment monitoring and drug development.1-4 The key advantages of PET over other molecular imaging techniques are its quantitativeness, high sensitivity, superior resolution and low radiation dose for patients.5-7 Therefore, the development of new PET tracers is essential, especially for the emergence of new treatment forms.8-10 Monoclonal antibodies (mAbs) are
particularly promising vectors for diagnostic imaging. This is because of their high target specificity and low non-displaceable binding.\textsuperscript{11-14} Recently, it became possible to use these vectors also for targets within the brain. Penetration of the blood-brain-barrier (BBB) can be achieved by several methods such as active transport, for example by utilizing the transferrin transporter, or by opening the BBB, for example by using focused ultrasound (FUS) based strategies.\textsuperscript{15-20} Despite these advances, the use of mAbs for diagnostic brain imaging is still in its infancy as imaging is hindered by the slow pharmacokinetics of mAbs. Although there are long-lived radionuclides compatible with these pharmacokinetics,\textsuperscript{21-24} respective radionuclides provide inferior image quality and result in high radiation burden for the patient.\textsuperscript{24}

Pretargeting allows to combine slow-circulating targeting vectors with short-lived PET radionuclides (Figure 1A).\textsuperscript{24-28} In this approach, a tagged mAb is injected first and given time to accumulate at target-rich sites and eliminated from blood circulation. Then, a radiolabeled small molecule (effector molecule) is injected, which possesses fast pharmacokinetics and can rapidly and selectively react with the tag of the beforehand administered mAb.\textsuperscript{29, 30} The use of \textit{trans}-cyclooctene (TCO) derivatives as chemical tags and 1,2,4,5-tetrazine (Tz) derivatives as effector molecules has become state-of-the-art for pretargeted PET imaging due to the ultra-fast kinetics of the inverse electron demand Diels–Alder (IEDDA) “click” cycloaddition between these two structures, its bioorthogonality and compatibility with multiple scaffolds.\textsuperscript{31-33}

\textbf{Figure 1.} A) Pretargeting concept beyond the BBB. In a first step, a BBB-penetrating TCO-tagged mAb is injected. The mAb is actively transported over the BBB and binds to its target. In a second step, a \textsuperscript{18}F-radiolabeled Tz is injected. The Tz clicks with the TCO-tagged mAbs thus imaging the selected target. B) Comparison between pretargeted and conventional imaging C) Strategy and workflow of this study to develop BBB permeable Tz imaging agent that is able to click in vivo to targets within the brain. The starting point was a Tz probe developed for cancer pretargeting. A library was designed based on this scaffold to have optimal parameters to cross the BBB and click in vivo. All the designed molecules were synthesized to evaluate labeling feasibility and in vitro stability. The \textsuperscript{18}F-Tzs were then tested for imaging contrast with in vitro autoradiography. Finally, compounds were injected in mice to evaluate the brain uptake. Metabolism was then evaluated for the best compounds.
Development of controlled drug delivery by means of Tz-triggered decaging of TCO-drug conjugates or vice versa (click-to-release) makes this chemistry even more attractive. Recently, we performed a systematic study of the relationship between the physicochemical properties of Tzs and their in vivo click performance in a colon tumor model. This study revealed that only hydrophilic Tzs with fast click kinetics were suitable for pretargeted imaging. Such Tzs are unsuitable for imaging of brain targets as their hydrophilicity prevents any reasonable BBB penetration. We hypothesized, however, that pretargeted imaging within the brain is possible as other blood supplying and excreting tissues are spatially separated from the brain. This should decrease background and possibly allow to image targets within the brain.

The aim of the present study was to explore the physicochemical parameters that influence the performance of Tzs for pretargeted brain imaging and apply this knowledge to develop a suitable imaging agent for this purpose (Figure 1A). We decided to develop $^{18}$F-radiolabeled Tzs. First of all, fluorine-18 ($^{18}$F) is widely considered to be the ideal PET radionuclide due to its convenient half-life (110 minutes), high positron branching ratio (97%) and short positron range in tissue (max. range 2.4 mm in H$_2$O). These properties altogether result in high-resolution images with low patient radiation dose. Secondly, $^{18}$F can be centralized produced in large scale and $^{18}$F-labeled radiopharmaceuticals consequently distributed for clinical use.

Thirdly, introduction of $^{18}$F into the wider Tz-framework can be used to fine-tune its reactivity while simultaneously, the physiochemical properties of the fluorinated framework can be manipulated with a second handle (Figure 1B).

We have recently developed methods to prepare highly reactive Tzs labeled with fluorine-18. These methods were used to label all Tzs in this study. The respective development strategy is displayed in Figure 1. In short, we designed and synthesized a panel of $^{18}$F-labeled Tzs guided by parameters reported to increase the chances to successfully develop a PET brain tracer. Selection of the parameters was inspired by the BBB score – a parameter developed to identify brain-penetrating molecules. Subsequently, the Tz panel was subjected to in vitro and in vivo screening. Based on the results, a subset of Tzs was selected for a final evaluation round in a pretargeted model. In this model, rats were intracerebrally injected with a non-internalizing TCO-polymer. This polymer was then - in a second step - targeted by selected Tzs. We decided to use this invasive model as it allows to solely study the in vivo performance of the Tz without challenges arriving from a brain targeting antibody, for example with respect to blood circulation, target engagement or metabolism. Obtained results were afterwards analyzed to identify possible relations between the in vivo performance and physicochemical parameters of the Tzs. The aim of this work was to identify a Tz most suited for pretargeted brain imaging.

**Results and Discussion**

**Design of the Tetrazine Library**

The development of brain imaging agents is often challenging. Good BBB permeability, acceptable non-displaceable binding, or sufficient metabolic stability are only some of the criteria that must be met. Multiple trade-offs between parameters that influence these criteria exist. For example, high lipophilicity increases brain uptake, but also its non-displaceable binding component. It is, therefore, essential to identify the right value for these parameters, i.e., the value that positively effects one criterion without disturbing another one. Nowadays, many physicochemical parameters can simply be estimated from their chemical structure. These estimations can be used to calculate a variety of composite scores. The CNS MPO and BBB scores, for example, are based on parameters such as MW, numbers of H-bond donors/acceptors,
Tracers, a homologation approach was employed stepwise increasing the length/bulk of the chosen side chain that addition of a single methyl group can dramatically affect the pharmacokinetics and distribution of CNS tracers, a homologation approach was employed stepwise increasing the length/bulk of the chosen side chain.

In this work, we carefully designed a set of 18 Tzs, which are predicted to be suitable brain imaging agents. According to the PET MPO and BBB score, they possess a probability of >75% to enter the brain (Figure 2). Design Tzs possess a rate constant ($k_t$) of >70,000 M$^{-1}$ s$^{-1}$ in PBS with “standard” TCO. We have recently shown that such rate constants increase drastically the in vivo click performance of Tzs for pretargeting. As typical brain target concentrations are in the order of 100 nM, aforementioned rate constants will also be sufficient for pretargeted imaging beyond the BBB (SI).

![Figure 2 A) Selected Tz structures. B) Radiolabeling of compounds [18F]1-18 and HPLC trace of [18F]18. C) in silico properties of Tzs along with key screening results are displayed.](image)

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Notes: *The distribution coefficient at physiological pH = 7.4 [clogD$_7.4$] was calculated using the software Chemicalize. 2The values were calculated according to ref. 51. 3Second order rate constants are estimated from measurements of the Tz structural classes with trans-cyclooctene (TCO) at 37 °C in PBS (see supporting information for details). 4Average radiochemical yield values from 22 synthesizes. Experimental procedures and other information about the $^{18}$F-labeling of Tzs is found in the SI Section S7 and SI Table S2. 5Cortex-to-cerebellum ratios measured in pretargeted autoradiography. 6 Determined in %ID/mL. 7Area under the curve (min×%ID/mL) for the whole brain uptake from 0 to 90 min post Tz injection. 8 Determined in min$^{-1}$. 9Not measured due to the poor solubility of compound 6 in PBS. 10RCPs not reported and in vitro/in vivo evaluation not performed due to fast decomposition of the formulated [18F]Tzs.

3-Fluorophenyl Tz derivatives possess rate constants >70,000 M$^{-1}$ s$^{-1}$. They can be labeled with fluorine-18 and modified with a second handle, i.e., their physicochemical properties can also be manipulated. Consequently, these scaffolds are ideal to develop a BBB penetrant Tz. Since previous studies have shown that addition of a single methyl group can dramatically affect the pharmacokinetics and distribution of CNS tracers, a homologation approach was employed stepwise increasing the length/bulk of the chosen side chain.
within five preselected Tz motifs (groups) (Figure 2).47,53,54 Linkers within those motifs were designed based on the possibility to synthesize them easily and on similarities to endogenous structures known to enter the brain.

**Synthesis and Characterization of the Tetrazine Library**
Tzs were synthesized via a Pinner-like synthesis. Briefly, required nitriles were either commercially available or synthesized. Corresponding Tzs were obtained using a sulfur catalyzed synthesis strategy reported by Qu et al.56 Rate constants were determined with TCO by pseudo-first-order measurements in DPBS at 37 °C by stopped-flow spectrophotometry.40,56 All tested Tzs displayed rate constant (k2) values above 70,000 M⁻¹s⁻¹ (SI).

**Radiolabeling**
Precursors were synthesized similar as described above followed by palladium-catalyzed stannylation (SI). Cu-mediated ¹⁸F-fluorination succeeded using our previously reported method (Figure 2B, SI Section S7).41 If necessary, protecting groups were removed quantitatively in a second step using acidic conditions. After purification, all products were formulated in ethanol / phosphate buffer (pH 7.4). Radiochemical yields (RCYs) were obtained in the order of 9-19% in a synthesis time of approx. 90 min. Molar activities (A_m) were in the order of 70-210 GBq/µmol, radiochemical purities (RCP) >92%. Stability studies revealed that all compounds - except for 5 and 6 - were stable for at least 2 hours. Because of the low stability, possibly caused by radiolysis, 5 and 6 were not evaluated in further experiments.

**Pretargeted Autoradiography**
Autoradiography allows to identify radioligands with suitable imaging contrast for further in vivo studies.58,59 As Tzs do not possess a native target within the brain, direct autoradiography cannot be carried out. In light of that, we developed an autoradiography protocol based on pretargeting (Figure 3A, SI Section S8). In short, brain slices from Tg-ArcSwe mice - a mouse strain with high content of beta-amyloid (Abeta) fibrils in the cortex 60 - were first incubated with TCO-modified 3D6 (anti-Abeta) mAbs. Excess of mAbs was then washed away. In a second step, these slices were incubated with ¹⁸F-Tzs. Structures clicked subsequently to Abeta-bound TCO-3D6. This strategy enabled us to visualize the binding of the mAb to Abeta, but more importantly it also allowed us to study the binding properties of applied ¹⁸F-Tzs, e.g., with respect to their displaceable binding component. In order to mimic the in vivo situation as close as possible, so called "no-wash" autoradiographic experiments were carried out.61 The uptake values in cortex (Cor, high binding region) and in cerebellum (Cer, low binding region) were determined. Cor/Cer ratios were used to rank ¹⁸F-Tzs - the higher the ratio the better the contrast (Figure 2,3). [¹⁸F]12 possessed the best ratio, whereas [¹⁸F]1 the worst (Figure 3B). Cor/Cer ratios inversely correlated with clogP (SI Figure S2) and showed - as expected - that more lipophilic Tzs possess a higher non-displaceable component.

**Brain Uptake**
Hydrophilic Tzs result in the best in vitro contrast, but more lipophilic Tzs are reported to enter the brain better.62 To investigate this trade-off and identify a Tz that would provide an optimal combination of imaging contrast and brain uptake, we assessed the brain uptake and washout of all ¹⁸F-Tzs in vivo by dynamic PET scans in healthy Long-Evans rats (SI Section S9). Time activity curves (TACs) for the whole brain were expressed in %ID/mL tissue. We expected that ¹⁸F-Tz that show high peak uptake in the brain followed by fast washout are most likely best suited for pretargeted imaging. High initial uptake increases the possibility of the Tz to bind to their targets and fast wash-out decreases background levels. Consequently, Tzs with these
properties should result in a high imaging contrast. We used the peak uptake as a measure of the brain uptake and the inverse area under the curve (AUC) as a measure for the washout. The ratio between uptake and washout (peak/AUC) was used to rank $^{18}$F-Tzs (Figure 2). This value describes best the interplay between high initial uptake and fast washout. As higher it is, as better the interplay. Interestingly, peak/AUC ratios strongly correlated with $	ext{clogP}$ and $	ext{clogD}_{7.4}$ within the same Tz motif (SI Figure S7). The most lipophilic Tzs from each class (2, 10, 14 and 18) displayed the best peak/AUC ratios.

Candidate Selection for Pretargeted Imaging beyond the BBB
In order to select the most promising Tzs, we plotted the in vitro Cor/Cer ratio against the in vivo peak/AUC ratio (Figure 3E). None of these ligands appeared to be ideal, i.e., neither had the highest rank in both categories simultaneously. Consequently, we decided to test the three most promising Tzs identified in this plot, namely, [$^{18}$F]12, [$^{18}$F]10 and [$^{18}$F]14 as well as two low ranking Tzs in order to verify that our model holds and can predict the in vivo performance of these structures.

![Figure 3](image_url)

**Figure 3.** A) Workflow of pretargeted autoradiography. B) Pretargeted autoradiography images for selected Tzs. C) Brain time-activity curves for selected Tzs. D) Structures of Tzs selected for further evaluation. E) Ranking of evaluated Tzs by in vitro contrast and in vivo brain uptake kinetics. Tzs selected for further evaluation are marked with filled symbols, relative rankings (in vitro/in vivo) are shown in parentheses. In vitro contrast for [$^{18}$F]18 was estimated by regression (see SI).

Pretargeted Imaging beyond the BBB
To evaluate the in vivo click performance of our Tzs, we used an invasive model based on intracerebral injection of a TCO-functionalized polymer (Figure 4A). As mentioned beforehand, this model circumvents challenges that arise when administering a targeting vector systemically. For example, no blood circulating mAb is present in the intracerebral injection model that could ‘click away’ a significant fraction of administered Tz. A detailed description of the model is reported in the Supplementary Information. In brief, Long-Evans rats were injected with TCO-decorated PeptoBrush polymer (100 µg polymer, 72 nmol TCO in 4 µL of 0.1M phosphate buffer at pH 7.2) into the right striatum. This polymer has been shown to be non-internalizing. Moreover, in rodent plasma, which is similar to interstitial fluid of the brain, at least 50% of the TCOs were stable for 24 h. Restoration of the BBB integrity 20 h after polymer injection was confirmed by MRI imaging with the gadolinium-based contrast agent ProHance (SI). To determine the retention of the injected TCO-PeptoBrush at the injection site, we labeled the polymer with indium-111 (100-200 kBq/mg
polymer, SI Section S10). 20-24 h after TCO-polymer injection, rats were injected with $^{18}$F-Tzs (10-30 MBq, 0.3-1.3 nmol) into the lateral tail vein and scanned in a PET scanner for 90 min. Radioactivity uptake in the right striatum was compared with the uptake in the left (polymer-free) striatum. After the scan, rats were sacrificed, their brains dissected into right and left hemispheres and gamma-counted 24 h later (to measure the $^{111}$In-counts after the decay of $^{18}$F). On average, 49.4±8.7% (n=14) of the injected $^{111}$In-activity was found in the injected brain hemisphere, while only 0.5±0.2% (n=14) was found in the control hemisphere, indicating good and consistent retention of the TCO-polymer (SI Figure S9). It is not known whether the observed loss of $^{111}$In-activity from the injection site reflects de-chelation of $^{111}$In or washout of the whole TCO-polymer. However, activity loss from the injection site was spread throughout the whole body. Therefore, local TCO concentration in the striatum is >1000-fold higher than elsewhere in the body (SI).

### Table C: Relative and absolute imaging contrast determined by PET

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<td>18</td>
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### Figure 4

A) Intracerebral injection model. B) In vivo uptake of $^{18}$F-Tzs in the left (TCO-injected) and right (TCO-free) striatum. C) Absolute and relative contrast between left and right striatum for selected Tzs.

Preferential $^{18}$F-uptake in the TCO-polymer pre-injected striatum was clearly visible for all investigated Tzs - both on summed images as well as on detected TACs (Figure 4B). We ranked $^{18}$F-Tzs in terms of their absolute and relative imaging contrast (Figure 4C). Relative imaging contrast was defined as the ratio between $^{18}$F-uptake in the TCO-polymer injected vs the non-injected striatum, while the absolute contrast was defined as the difference between the uptake values, expressed in %ID/mL. The best relative contrast was observed for $^{[18F]}10$, the best absolute contrast for $^{[18F]}1$ - however, with a huge variation from scan to scan. $^{[18F]}18$ showed high values in both categories and is because of this our prime candidate to be used in further studies. These results are not expected from our prediction model displayed in Figure 3E. For example, $^{[18F]}1$ was suggested to be the least promising compound whereas good in vivo click uptake was observed in pretargeted experiments.
Metabolism

Metabolism is another important factor that influences the ability of Tzs to ligate to their targets in vivo. We decided, therefore, to determine the metabolism profile of our selected tracers (SI Section S11). Tzs [¹⁸F]14 and [¹⁸F]10 were the least stable Tzs. Only <5% intact tracer was detected in plasma after 90 minutes. [¹⁸F]12 and [¹⁸F]1 showed the best stability, 50% of intact tracer could be detected. Interestingly, [¹⁸F]18 displayed an intermediate metabolism rate. Approx. 30% intact tracer could be detected (Figure 5A). All detected radiometabolites were much more hydrophilic than the parent (SI Figure S12) indicating that these metabolites do not contribute to the brain uptake as they are unlikely to pass the BBB. In general, rapid metabolism appeared to strongly influence the brain uptake of the studied Tzs – mainly due the fact that less intact ¹⁸F-Tz is available to diffuse into the brain (Figure 5B). Percentage of intact Tz correlated with brain uptake (SI Figure S13). This explains the higher uptake of [¹⁸F]1 and [¹⁸F]18 as well as the lower uptake of [¹⁸F]14 and [¹⁸F]10 (Figure 3E).

![Figure 5](image)

**Figure 5.** A) In vivo metabolic stability of selected Tzs in rat plasma. B) Metabolite-corrected plasma input curves for selected Tzs. C-D) Correlation of relative imaging contrast with cLogP and in vitro contrast, respectively. Dashed curve in panel D represents putative non-monotonic relationship between relative imaging contrast and in vitro contrast. E) Correlation of absolute imaging contrast with in vivo brain AUC. F) Correlation of absolute imaging contrast with in vivo metabolic stability of ¹⁸F-Tzs. Summary rankings of [¹⁸F]18 - the most suited Tz for pretargeted imaging across the BBB. Rankings are relative to all other Tzs. G) Tzs most suited for pretargeting beyond the BBB. [¹⁸F]18 was identified to possess the most ideal parameters. Rankings are relative to other Tzs.

The In Vivo Click Performance

In order to explain the observed in vivo click performance, we examined the data received from in silico calculations, pretargeted autoradiography, in vivo brain uptake and metabolism studies. In vitro contrast
from pretargeted autoradiography experiments inversely correlated with the relative imaging contrast (r 0.69, Figure 5D). We speculate that this inverse correlation was detected as tested 18F-Tzs are still within the optimal lipophilicity interval with respect to BBB uptake and non-displaceable binding. In this interval, increased lipophilicity would only result in improved BBB permeability and not majorly increase the non-displaceable binding component. Only after a certain lipophilicity threshold the relative imaging contrast would negatively be affected, and this threshold was not exceeded within this study. Consequently, relative in vivo imaging contrast correlated with clogP (r 0.88, Figure 5C).

Our initial assumption that in vivo click performances correlate with the brain uptake/washout - as determined by the Peak/AUC ratio of the tracer - did not reveal any clear trend (Figure S13). However, tracer washout (AUC values) strongly correlated with absolute imaging contrast (r 0.99, Figure 5E). Interestingly, absolute imaging contrast for all 18F-Tzs except for [18F]12 - also correlated to the metabolic stability (Figure 5E). Slow metabolism appeared to be beneficial for higher absolute imaging contrast. We believe that rapid metabolism results in less tracer available to enter the brain from the blood. This explains the low absolute imaging contrast of [18F]14 and [18F]10, for example. Both tracers possess a rapid metabolism (Figure 4 and S5A,B,F). In contrast, [18F]18 displays an intermediate fast metabolism profile (Figure 5A,B,F) and consequently, a higher absolute imaging contrast is observed. Absolute imaging contrast of [18F]12 is not aligned with this observation. Its slow metabolism is not resulting in a high absolute imaging contrast. We believe that [18F]12 is possible to polar for sufficient BBB permeability (Figure 2). Our results indicate that fast tracer washout is not necessarily required for high absolute imaging contrast in vivo. However, slow metabolism resulting in high brain uptake results in good imaging contrast as the tracers can continuously enter the brain (Figures 3E and 4B). Increasing lipophilicity - at least to clogP values of 1.55 - results in higher absolute imaging contrast. The imaging contrast increases also correlated well with the BBB / CNS MPO score (r 0.91-0.93, Figure S13).

**Conclusion**

We investigated structure-activity relationships of a library of 18 different 18F-Tzs, their in vitro imaging contrast and in vivo brain pharmacokinetics to predict their in vivo click performance on targets beyond the BBB. No clear relation could be identified. However, slow metabolism as well as high lipophilicity appear to beneficial for high brain uptake. [18F]18 showed the best imaging properties. High brain uptake combined with good imaging contrast are the key properties of this tracer to be our prime candidate to be used in further pretargeted imaging studies beyond the BBB (Figure 5G). In a next step, we will design BBB penetrating and TCO-modified mAbs that can be targeted and imaged with [18F]18. This possibility will ultimately enable pretargeted imaging of brain targets, such as protein isoforms, which can currently not be imaged.

**Associated Content**

**Supporting Information**

The Supporting Information for this work contains: Supplementary Tables and Figures, description of organic synthesis, measurement of click rate constants of Tzs, radiolabeling protocols, protocols for in vitro autoradiography and in vivo PET scans, in vivo radiometabolite analysis, examples of analytical HPLC and radio-HPLC traces, or NMR spectra.
Notes
The authors declare no competing financial interest.

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References


