A novel $^{18}\text{F}$-labeled clickable substrate for targeted imaging of SNAP-tag expressing cells by PET in vivo†

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Bioorthogonal covalent labeling with self-labeling enzymes like SNAP-tag bears a high potential for specific targeting of cells for imaging in vitro and also in vivo. To this end, fluorescent SNAP substrates have been established and used in microscopy and fluorescence imaging while radioactive substrates for the highly sensitive and whole-body positron emission tomography (PET) have been lacking. Here, we show for the first time successful and high-contrast PET imaging of subcutaneous SNAP-tag expressing tumor xenografts by bioorthogonal covalent targeting with a novel $^{18}\text{F}$-based radioligand in vivo.

A number of chemical and biochemical strategies for labeling specific types of cells have been developed in the past years. Several of these strategies have been used for targeted labeling of mammalian cells, e.g. in tumors. While labeling of cells in vitro can be often realized straightforwardly using cell-specific genetically or pathophysiologically epigenetically cellular targets, cell-specific targeted labeling for imaging in vivo is more intricate and often unsuccessful due to the lack of exclusivity of naturally expressed targets, unfavorable biodistribution of the chemical targeting unit, and its metabolism and excretion. Another important limitation of imaging cells in vivo is the physical detection principle of the various imaging strategies. Fluorescence-based targeting allows for in vivo targeting; however, the imaging read-out by microscopy is typically done ex vivo – preventing dynamic and serial imaging – or intravitaly with restricted surgical access to very confined tissue volumes of single organs. Fluorescence reflection imaging (FRI) provides whole-body imaging but is limited by penetration depth and scattering of light in organisms. In contrast, scintigraphic imaging methods such as SPECT or PET provide high sensitivity and quantitative, fully non-invasive whole-body imaging of organisms, which has also the unique capability of clinical translation. Scintigraphic imaging has been successfully used to target individual cells in vivo, e.g. using non-cell-specific metabolic markers such as $[^{18}\text{F}]$fluorodeoxyglucose ($[^{18}\text{F}]$FDG) or gene reporters such as thymidine kinase genetically introduced into cells or mouse models and targeted by $[^{18}\text{F}]$fluoroguanine ($[^{18}\text{F}]$FHBG). The so-called SNAP-tag stands out as a versatile genetic approach for irreversible covalent labeling of cells with, in principle, any type of reporter in a chemo-enzymatic strategy. It is a variant of the $O^6$-alkylguanine-DNA alkyltransferase suitable for labeling with $O^6$-benzylguanine (BG) derivatives which can be coupled with reporters at the $para$-position of the benzyl moiety. This self-labeling tag exhibits high specificity and kinetics for in vivo labeling of fusion proteins on cells with synthetic fluorophores and it has been used for in vivo imaging in mice using far-red emitting fluorescent dyes, such as BG-547,4 BG-8005 or novel charge-free fluorescence-switchable near-infrared (NIR) dyes. Non-invasive imaging of tumors in nude mice was successful after subcutaneous implantation of murine breast carcinoma cells engineered to express SNAP-GPI using intravenous injection of the infrared probe BG-782. Furthermore, the Cre-dependent expression of SNAP-tag in mice has been shown to be a powerful method for the genetic targeting of chemical indicators in vivo, as demonstrated by labeling in complex tissues in vivo with fluorophores from green to near infrared emission.7

However, as discussed above, the application of SNAP-tag-based biomedical imaging in vivo using fluorescent dyes might be restricted by post-mortem approaches (microscopy), invasivity (intravital microscopy), limited sensitivity due to tissue penetration and lack of absolute quantification due to scattering of light (FRI). Such challenges would be ideally addressed by a radioactive SNAP-tag substrate combined with scintigraphic, non-invasive and dynamic whole-body imaging by PET; however, radioactive SNAP-tag substrates have not been reported so far. Therefore, we aimed at the development of a novel $^{18}\text{F}$-labeled SNAP-tag...
Accordingly, we synthesized an (prop-2-yn)zosulfonate (was subsequently tosylated to give 2-azidoethyl-4-methylbenzylguanine) [\(^{18}\text{F}\)]SBG, a radioactive SNAP-tag substrate for PET imaging.

substrate, its characterization in vitro, and a proof-of-principle PET study in SNAP-tag expressing subcutaneous tumors in vivo (Fig. 1A).

Synthesis and labeling of [\(^{18}\text{F}\)]FTBG: For radioactive labeling of the SNAP-tag substrate we favored a copper-catalyzed azide–alkyne cycloaddition (CuAAC) which provides the required specificity, yield and velocity for introduction of [\(^{18}\text{F}\)]fluorine. Accordingly, we synthesized an O\(^{\prime}\)-benzylguanine with a terminal alkyne and 1-azido-2-[\(^{18}\text{F}\)]fluorethane (Fig. 1B).

To this end, the activated guanine 1 was prepared by reacting 6-chloroguanine with 1-methylpyrrolidine. A straightforward approach to introduce a terminal alkyne to the para-position of the benzyl moiety was to start from the symmetric 1,3-diphenylpropane. The activated guanine 1 was then reacted with propargylbromide and sodium hydride to give 4-benzenedimethanol and react one of the benzylic alcohol functions by semi-preparative HPLC equipped with a gamma-detector (Fig. 1A).

To obtain click reagent [\(^{18}\text{F}\)]4, a three step synthesis was necessary. First, the reaction of 2-bromoethanol with sodium azide was performed to obtain 2-azidoethanol which was subsequently tosylated to give 2-azidoethyl-4-methylbenzozosulfonate in 66% yield. Compound [\(^{18}\text{F}\)]4 was obtained via fluorination of 7 using [\(^{18}\text{F}\)]fluoride, Kryptofix\(^{\text{K}}\) 2.2.2 and potassium carbonate 71 ± 6% (d. c., n = 14). It was used for CuAAC with the PYBG (3) after distillation to yield the desired [\(^{18}\text{F}\)]FTBG (Fig. 2A). The reaction mixture was first purified by C18-solid phase extraction after ligand exchange of copper-complexes with [\(^{18}\text{F}\)]FTBG (Fig. 2B) using ammonia buffer. A second purification by semi-preparative HPLC equipped with a gamma-detector was necessary to obtain pure [\(^{18}\text{F}\)]5 13.6 ± 4.9% (d. c., n = 14, total yield over two steps of radiosynthesis). Furthermore, we synthesized BG-Dy549P1, a cell-impermeable fluorescent SNAP-tag ligand, to facilitate characterization of [\(^{18}\text{F}\)]FTBG on cells in vitro (see ESI†).

In vitro characterization of [\(^{18}\text{F}\)]FTBG: We first performed in vitro uptake experiments to study selectivity and binding characteristics of [\(^{18}\text{F}\)]FTBG to SNAP-tag epitopes expressed on the cell surface of transduced Gli36 glioblastoma cells (SNAP-tag\(^{+}\)). We found that [\(^{18}\text{F}\)]FTBG binds to epoietin on SNAP-tag\(^{+}\) cells with high selectivity and binding was saturated at high ligand concentrations (Fig. 2A, \(B_{\text{max}} = 170.6 ± 7.8 \text{ Bq}, K_{d} = 7784 ± 1605 \text{ Bq, non-linear regression analysis with one-side fit with total and non-specific binding}) as compared to SNAP-tag\(^{-}\) cells. (B) 1 × 10\(^{5}\) SNAP-tag\(^{+}\) cells were incubated with cell-permeable SNAP-Cell\(^{\text{K}}\) or non-permeable SNAP-Surface\(^{\text{K}}\) block substrates or were left untreated before 100 kBq [\(^{18}\text{F}\)]FTBG was added. Blocking resulted in significantly lower accumulation of radioactivity in the cells when compared to control cells (\(n = 4, \text{RM One-way ANOVA with Tukey’s test for multiple comparisons}, *p < 0.05\)).
non-transduced WT cells (SNAP-tag\textsuperscript{−}) was observed. In line with these findings, pre-dosing of SNAP-tag\textsuperscript{+} cells with non-radioactive cell-permeable SNAP-Cell\textsuperscript{R} or impermeable SNAP-Surface\textsuperscript{R} blocking substrates (to study cell permeability) prior to incubation with [\textsuperscript{18}F]FTBG led to a significantly reduced accumulation of the radioligand in the cells (Fig. 2B).

In addition, we studied cell permeability of [\textsuperscript{18}F]FTBG by confocal imaging of SNAP-tag\textsuperscript{+} cells. Cells were either pre-dosed with [\textsuperscript{18}F]FTBG for 30 min or left untreated followed by staining with the cell-impermeable SNAP-tag dye BG-Dy549P1 and the highly cell-permeable dye SNAP-Cell\textsuperscript{R} 647-SiR. Pre-dosing of cells with [\textsuperscript{18}F]FTBG largely abolished fluorescent labeling of both extra- and intracellular epitopes (Fig. 2D) while we observed bright staining of these structures in untreated SNAP-tag\textsuperscript{+} cells (Fig. 2C). This together with the above radiotracer uptake experiments suggests that [\textsuperscript{18}F]FTBG is cell-permeable.

**In vivo biodistribution:** Following successful evaluation of SNAP-tag-specific binding in vitro we aimed at studying biodistribution of [\textsuperscript{18}F]FTBG in vivo. In vivo biodistribution was determined by dynamic PET in 4 adult female C57/BL6 mice after intravenous injection. [\textsuperscript{18}F]FTBG showed a fast clearance from the blood and from non-targeted tissues such as muscle following the perfusion phase (Fig. 3A and B). In contrast, [\textsuperscript{18}F]FTBG accumulated in the liver and the kidneys (Fig. 3C), followed by a highly efficient and almost balanced net excretion to the gallbladder and the urinary bladder (Fig. 3D). We calculated a total clearance of 79.77 ± 4.48% Injected Dose (%ID) at 60 min, of which 49.78 ± 5.75% ID were excreted through liver and 50.22 ± 5.75% ID through kidney (mean ± standard deviation (SD)). The in vivo biodistribution data was corroborated by ex vivo counting of tissues and fluids harvested 90 min p.i. (ESI† Table S2).

**SNAP-tag targeted imaging in tumor xenografts using [\textsuperscript{18}F]FTBG:** Feasibility of targeting SNAP-tag by [\textsuperscript{18}F]FTBG in vivo was studied in Gli36 tumor cells transduced with a SNAP-tag fusion construct (SNAP-tag\textsuperscript{+}) versus wild type cells (SNAP-tag\textsuperscript{−}) (see ESI†). Tumor cells were injected subcutaneously into the left (SNAP-tag\textsuperscript{+}) and right (SNAP-tag\textsuperscript{−}) shoulders/flanks of female C57/BL6 mice (n = 6) and PET imaging was performed 7–10 days after tumor cell implantations. Upon intravenous injection of [\textsuperscript{18}F]FTBG a 60 min dynamic imaging acquisition was initiated. Following initial perfusion-related uptake of [\textsuperscript{18}F]FTBG in all tumors, [\textsuperscript{18}F]FTBG was quickly washed out from SNAP-tag\textsuperscript{−} tumors with a remaining mean activity concentration of 0.15 ± 0.08%ID per mL (Fig. 4B) at 60 min p.i. However, in contrast, [\textsuperscript{18}F]FTBG was retained in all SNAP-tag\textsuperscript{+} tumors with 0.38 ± 0.14%ID per mL (Fig. 4B and C) and a SNAP-tag\textsuperscript{−}/SNAP-tag\textsuperscript{+} tumor ratio of 2.5 at 60 min p.i. Fig. 4A shows PET images of tumors from individual animals 60 min p.i. with a significant uptake of [\textsuperscript{18}F]FTBG only in SNAP-tag\textsuperscript{+} tumors.

At the end of the PET scans, tumors were harvested, weighed and counted. Even more striking as compared to the in vivo VOI analysis, uptake in SNAP-tag\textsuperscript{−} tumors ex vivo (%ID per g) was

![Fig. 4](https://example.com/fig4.png)
7.94-fold higher than that of SNAP-tag− tumors (Fig. 5D). In addition, all tumors were cryo-fixated and sliced for histology (Fig. 5B) and autoradiography. Again, only SNAP-tag+ tumor tissues showed a high uptake whereas SNAP-tag− tumor tissues did only show background activity (Fig. 5A and C).

In conclusion, this study shows for the first time a radiochemical strategy for labeling of a SNAP-tag substrate by [18F]fluorine, its favorable binding characteristics and specificity in vitro, and successful bioorthogonal covalent targeting and imaging of SNAP-tag expressing tumor cells by PET in mice in vivo. This approach uniquely establishes SNAP-tag-based targeting for PET and thus also opens new avenues for multiscale imaging, i.e., the transfer and exchange of biological information from high-resolution microscopy to whole-body quantitative PET imaging. Future studies will define the overall sensitivity and specificity of this new approach compared to other SNAP-tag directed imaging strategies as well as to metabolite and gene reporter-based strategies and its application to various cell types including immune cells and stem cells.

Besides preclinical applications in disease models, translational attempts are foreseen. E.g., therapeutic CAR T cells could be genetically equipped by SNAP-tags to enable tracking of their distribution in the individual patient. Further, fusion of the SNAP-tag to antibody fragments has been employed to introduce a variety of labels or theranostic agents such as photosensitizers for future biomedical applications.13

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Conflicts of interest

There are no conflicts to declare.

References