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Site-specifically ^{11}C-labeled Sel-tagged annexin A5 and a size-matched control for dynamic in vivo PET imaging of protein distribution in tissues prior to and after induced cell death

Qing Cheng [a], Li Lu [b,c], Jonas Grafström [c], Maria Hägg Olofsson [d], Jan-Olov Thorell [c,e], Erik Samén [c,e], Katarina Johansson [a], Hanna-Stina Ahlzén [a], Stig Linder [d,1], Elias S.J. Arnér [a,2], Sharon Stone-Elander [a,c,e,*,3]

[a] Division of Biochemistry, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm, Sweden
[b] Karolinska Experimental Research and Imaging Center, Department of Comparative Medicine, Karolinska University Hospital, Stockholm, Sweden
[c] Department of Clinical Neuroscience, Karolinska Institutet, Stockholm, Sweden
[d] Department of Oncology-Pathology, Cancer Centrum Karolinska, Karolinska Institutet, Stockholm, Sweden
[e] PET Radiochemistry, Neuroradiology Department, Karolinska University Hospital, Stockholm, Sweden

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ABSTRACT

Background: Radiolabeled annexin A5 (AnxA5) is widely used for detecting phosphatidylserine exposed on cell surfaces during apoptosis. We describe here a new method for labeling AnxA5 and a size-matched control protein with short-lived carbon-11, for probing the specificity of in vivo cell death monitoring using positron emission tomography (PET) imaging.
Methods: AnxA5 and the control protein were recombinantly expressed with a C-terminal "Sel-tag", the tetrapeptide –Gly-Cys-Sec-Gly–COOH. The proteins were then labeled either fluorescently for in vitro corroborations of binding behaviors or with ^{11}C for dynamic in vivo PET studies.
Results: AnxA5 demonstrated retained calcium-dependent binding to apoptotic cells after the C-terminus modification. The control protein showed no functional binding. The ^{11}C-ligands demonstrated similar in vivo pharmacokinetic behavior in healthy mice except for higher uptake in kidney and higher intact elimination to urine of AnxA5. After inducing hepatic apoptosis, however, the uptake of labeled AnxA5 in the targeted tissue increased compared to baseline levels while that of the control protein tended to decrease.
Conclusions: These data suggest that the combined use of these two tracers can facilitate differentiating specific AnxA5 binding and its changes caused by induced cell death from uptake due to non-specific permeability and retention effects at baseline or after therapy.
General significance: The Sel-tag enables rapid and mild reactions with electrophilic agents giving site-specifically labeled proteins for multi-probe analyses. The combined use of ^{11}C-labeled AnxA5 and a size-matched control protein with dynamic PET can be useful for evaluating drug effects on target as well as off-target tissues.

1. Introduction

Key issues for the successful optimization of personalized medical care are the abilities to correctly diagnose the disease, to choose the most appropriate therapy and to evaluate the therapeutic response — all on an individual basis. Molecular imaging by positron or single photon emission tomography (PET or SPECT, respectively) with nano- to picomolar sensitivities can be used to non-invasively localize diseased tissue based on its underlying biochemical features and to, over time, monitor the effects of therapeutics directly in the diseased tissue [1–3]. New imaging tracers ranging from small molecules, to regulatory peptides, proteins, antibody fractions and monoclonal antibodies are under evaluation as diagnostic molecular imaging biomarkers [4]. Furthermore, polypeptide probes with high affinity and selectivity for an increasing number of targets are being selected by molecular display techniques [5–7]. PET has typically used the small, rapidly equilibrating molecules as tracers while the larger molecules have been used in SPECT imaging. Intermediate-sized polypeptides may also clear fairly rapidly from the circulation [8,9]. Properly labeled, they can therefore potentially be used to visualize targets with relatively low background interference very soon after administration. This may be desirable in many situations and crucial when trying to observe rapidly progressing disease processes (e.g. [10]). Finding an appropriate balance between tracer clearance and delivery rates to target tissues can thus involve changing both the

* Corresponding author at: Department of Neuroradiology, PET Radiochemistry, R3:00, Karolinska University Hospital, SE-17176 Stockholm, Sweden. Tel.: +46 8 517 75331; fax: +46 8 517 77072.
E-mail addresses: Stig.Linder@ki.se (S. Linder), Elias.Arner@ki.se (E.S.J. Arnér), sharon.stone-elander@karolinska.se (S. Stone-Elander).
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[2] On Sel-tag technology.
[3] On radiolabeling and PET imaging.

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structures of the probes and/or the chosen radionuclide according to the intended use. The labeling strategies needed may therefore vary throughout any tracer development and implementation process, suggesting a need for the development of novel radiolabeling methodologies of proteins for a range of different radionuclides.

Tracer substances for SPECT are labeled with long-lived ($t_{1/2}$ hours–days) radiometals and halogens. PET primarily uses short-lived ($t_{1/2}$ 2–110 min) "bioisotopes", although more long-lived positron-emitting radionuclides are also available [11,12]. Dynamic PET scanning with shorter-lived nuclides can be used to study tracer pharmacokinetics, to visualize multiple aspects that affect the delineation of diseased tissue and to reveal overall as well as individual parametric effects from therapy [13,14]. In the high throughput demands of clinical imaging, however, both SPECT and PET more often rely upon longer-lived radionuclides for "injecting outside the scanner, wait and then scan" protocols. Multiple scans of a given individual will then require waiting until the radiotracer has cleared and/or decayed, which may be up to days depending on the radionuclide or its in vivo behavior. Furthermore, the dose exposures from retained, long-lived radionuclides may become a limiting factor for repeated monitoring over time.

The short-lived PET radionuclides are not only a good match with the typically rapid pharmacokinetics of smaller polypeptides, but are also favorable for longitudinal studies. Though the use of ^{68}Ga ($t_{1/2}$ 68 min) is growing with increasing availability of generators, the most commonly used are ^{18}F ($t_{1/2}$ 110 min) and ^{11}C ($t_{1/2}$ 20 min), which are produced many times daily by cyclotrons at PET research, production and clinical imaging facilities. ^{18}F is used in clinical PET for snapshot imaging at discrete time-points and observations over several hours, next-day repeated imaging and can be transported within several hours from the production site. ^{11}C gives generally lower radiation doses and allows sequential tracer injections after 2–3 h (particularly important during validation phases and in clinical research protocols when performing e.g. Scatchard analyses, drug challenges to probe the specificity of new tracers, multi-tracer studies to probe different biochemical features of the tissue or when monitoring rapid changes in target tissues).

Labeling polypeptides with ^{18}F has been achieved using a number of primarily amino- and thiol-reactive radiolabeling precursors, while successful labeling with ^{11}C has only been reported for a few proteins that could tolerate either rather harsh labeling conditions or the insertion of a radiolabeling tag at multiple and random labeling positions [15]. We have previously presented a method for rapidly, mildly and site-specifically labeling proteins with ^{11}C. This method is based on the recombinant insertion of a tetrapeptide –Gly-Cys-Sec-Gly–COOH motif, called a "Sel-tag" (ST) [16–18] at the C-terminus of a protein (Fig. 1A). Upon its reduction, a highly nucleophilic selenocysteine (Sec) residue (Fig. 1B) is exposed that can react with electrophilic reagents. The method for Sel-tag based radiolabeling was developed using several different proteins and was recently also successfully utilized with a 6 kDa Affibody molecule [19]. Using this Affibody molecule, excellent detection of lesions with over-expressions of HER2 was possible 30–60 min after i.v. tracer injection. Furthermore, specificity of binding could be demonstrated in a second scan a few hours later by pre-blocking with unlabeled protein in each individual tumor-bearing mouse, thereby minimizing the influence of inter-individual differences [19]. Here we hypothesized that this labeling technique might also be utilized for PET imaging of even larger proteins with appropriately rapid pharmacokinetics in vivo and where a ^{11}C-label could be advantageous for longitudinal studies. For this study, we chose annexin A5 (AnxA5), which has often been evaluated for the detection of cell death, using a multitude of different labeling methodologies that may serve as benchmarks for the results of our current approaches.

The ability to monitor cell death over time can be very important, both for early detection of developing pathology and for making decisions about therapeutic response in target tissues such as tumors in order to avoid excessive systemic exposure to drugs with toxic side-effects. Currently the most widely-used imaging biomarkers for monitoring cell death are based on AnxA5. This endogenous 36 kDa protein binds with high affinity to phosphatidylserine (PS) which is externalized from the cytosolic side to the surface of the cells when they are dying (apoptotic) or dead (late apoptotic or necrotic) [20,21]. AnxA5 is characterized by very rapid initial blood clearance after intravenous (i.v.) administration [22,23]. The AnxA5-based tracers that have been described typically show low target/non-target uptakes in animal models as well as in patients and finding the optimal time for scanning can therefore be difficult [24]. Therapeutic monitoring with AnxA5 tracers needs baseline scanning as a reference point and incremental changes in both positive and negative directions have been interpreted as indications of drug efficacy [20,25], even though it is expected that increasing cell death would lead to increased tracer uptake. Enhanced permeability and retention (EPR) effects in tumors caused by leaky vessels and poor lymphatic drainage [26] can be deliberately used to enhance the deposition of macromolecular therapeutics [27,28], but can also confound imaging results that are attempting to estimate the degree and variations in binding interactions [29]. The contribution of

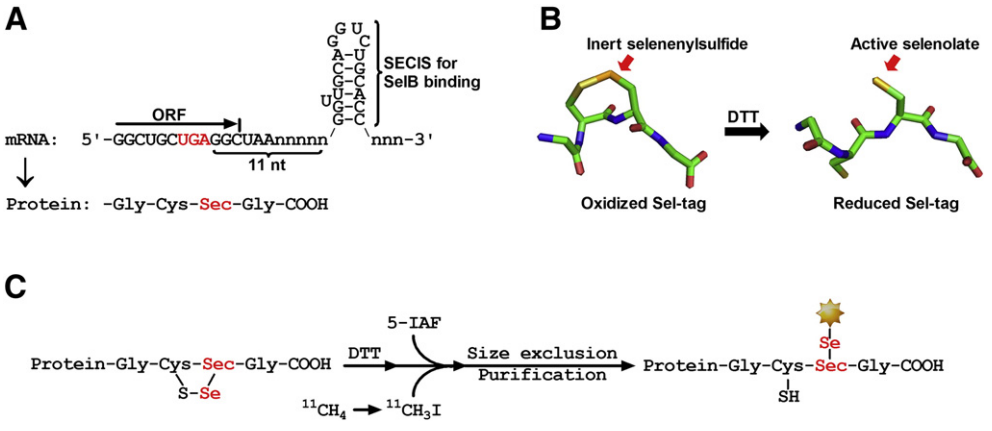

Fig. 1. Proteins can be tagged in the C-terminus for subsequent labeling through the Sec residue. (A) Scheme showing the principle for production of recombinant Sel-tagged proteins, using introduction of a sequence encoding the Sel-tag at the 3′-end of the open reading frame (ORF), followed by a bacterial-type selenocysteine insertion sequence (SECIS) element in the mRNA with a 11 nt long stem and a loop region binding the *E. coli* SelB elongation factor for Sec insertion (17). (B) Model of the inert oxidized Sel-tag with a selenenylsulfide at the C-terminus of a Sel-tagged protein (left) and its reduction by DTT thereby exposing the reactive selenolate of Sec (right), which subsequently can be targeted with electrophilic compounds for site-specific labeling. The figure shows the corresponding X-ray crystal structure of the C-terminus of oxidized and reduced thioredoxin reductase, which natively carries the –Gly-Cys-Sec-Gly motif of the Sel-tag [59]. (C) Flow scheme for the syntheses of the labeled proteins.