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ORIGINAL ARTICLE

^{18}F-Labeled wild-type annexin V: comparison of random and site-selective radiolabeling methods

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Abstract Early stage apoptosis is characterized by the externalization of phosphatidylserine (PS) from the inner leaflet of the plasma membrane to the outer periphery. Consequently, PS represents an excellent target for non-invasive imaging of apoptosis by positron emission tomography. Annexin V is a 36 kDa protein which binds with high affinity to PS. Radiolabeling of wild-type annexin V with fluorine-18 (^{18}F) can be accomplished via random acylation of 23 amine groups (22 lysine residues and one N-terminal amine) with [^{18}F]SFB or site-specific alkylation reaction on cysteine residue at position 315 with maleimide-containing prosthetic groups like [^{18}F]FBEM. The effect upon random and site-directed ^{18}F labeling of annexin V was studied with EL4 mouse lymphoma cells. Both, randomly and site-selectively radiolabeled annexin V demonstrated comparable binding to apoptotic EL4 cells. This finding suggests that the ^{18}F radiolabeling method has no significant effect on the ability of ^{18}F-labeled wild-type annexin V to bind PS in apoptotic cells.

Keywords Wild-type annexin V · Fluorine-18 · Apoptosis · [^{18}F]SFB · [^{18}F]FBEM

Introduction

Non-invasive monitoring of tumor cell death in vivo is an important clinical need to assess therapeutic response. Different cell death pathways have been studied extensively, and molecular imaging of cell death after treatment with radiation or chemotherapy has the potential to provide detailed information on cellular transformations and dynamics of tumors before, during, and after therapeutic interventions (Smith and Smith 2012).

Apoptosis (programmed cell death) is characterized by a number of morphological and biochemical changes that can be targeted for molecular imaging purposes (Neves and Brindle 2014). In the early stages of apoptosis, phosphatidylserine (PS), a membrane phospholipid, is externalized from the inner leaflet to the outer leaflet of the cell membrane. In a healthy cell, PS is completely confined to the inner leaflet of the cell. Induction of apoptosis and subsequent increase in cytosolic Ca^{2+} concentration leads to redistribution of phospholipids and exposure of PS, which is recognized by macrophages and other phagocytes that engulf and remove the apoptotic bodies (Martin et al. 1995). PS is a well-characterized biomarker for early-stage apoptosis, and a promising molecular target for apoptosis imaging probes (Blankenberg 2008).

The most commonly used PS-targeting agent is annexin V, an endogenous, 36 kDa, intracellular protein that binds to PS with nanomolar affinity (K_d ~2 nM) in the presence of Ca^{2+} (Schaper and Reutelingsperger 2013; Tait et al. 1994). Annexin V modified with fluorescent dyes is used as a standard staining technique to assess apoptosis in vitro. Annexin V has also been labeled with various radionuclides to give radiotracers for molecular imaging of apoptosis in vivo using single-photon emission computed tomography (SPECT) and positron emission tomography (PET). Annexin V and annexin V mutants, generated for easier bioconjugation, were radiolabeled with ^{125}I, ^{123}I, ^{111}In and ^{99m}Tc for SPECT imaging of apoptosis (Vangestel et al. 2011). The most successful and extensively studied of these

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is ^{99m}Tc-HYNIC-annexin V, which was first reported by Blankenberg et al. (1998). Usefulness of ^{99m}Tc-HYNIC-annexin V for assessment of tumor response to therapy was demonstrated in a wide array of preclinical (Blankenberg et al. 1998; De Saint-Hubert et al. 2011) and clinical studies (Belhocine et al. 2002). However, ^{99m}Tc-HYNIC-annexin V shows high non-specific uptake in the kidneys, liver and spleen, resulting in a poor tumor-to-background ratio and limitations for imaging of apoptotic cells around these areas. Moreover, SPECT is also less sensitive than PET.

Therefore, radiolabeling of annexin V with a positron-emitting radionuclide for PET imaging of apoptosis is more desirable as it would provide higher quality images and allow for more sensitive imaging. Positron-emitting radionuclides for annexin V labeling include ^{124}I, ^{64}Cu, ^{18}F and ^{68}Ga (Lehner et al. 2014; Wängler et al. 2011; Keen et al. 2005; Wuest et al. 2015). Among the reported PET radiotracers based on annexin V, ^{18}F-labeled annexin V has received the most attention due to the favorable properties of ^{18}F for PET imaging.

^{18}F has a physical half-life of 109.8 min, high positron emission (97 %), low positron energy of 0.64 MeV resulting in high image resolution and lower radiation dose, and ^{18}F can be produced in large amounts with high specific activity by a small biomedical cyclotron (Smith et al. 2012).

Radiolabeling of annexin V with ^{18}F is most commonly accomplished via a random labeling approach involving the NHS-containing prosthetic group *N*-succinimidyl-4-[^{18}F] fluorobenzoate ([^{18}F]SFB), which can react with any of the 22 lysine residues and N-terminus present in annexin V. This labeling approach has been carried out by several groups (Yagle et al. 2005; Zijlstra et al. 2003), and the ability of [^{18}F]SFB-labeled annexin V to image apoptosis in vivo has been demonstrated in various preclinical models (Yagle et al. 2005; Murakami et al. 2004). However, only one group has reported the utility of [^{18}F]SFB-labeled annexin V for imaging therapy-induced apoptosis as demonstrated in head and neck squamous cell cancer UM-SCC-22B tumor xenografts upon treatment with doxorubicin (Hu et al. 2012. Tait et al. suggested that prosthetic group [^{18}F]SFB can conjugate to any of the 23 primary amines present on annexin V, some in close proximity of the active binding site of the protein. Such a non-specific, amine-directed modification of annexin V not only results in a poorly characterized radiotracer, but also might disrupt crucial binding interactions as demonstrated in a recent report describing that amine-directed chemical modification of annexin V reduced its membrane-binding activity even at low stoichiometries (Tait et al. 2006). In addition, if annexin V is conjugated with low specific activity [^{18}F]SFB at multiple sites, this could lead to disproportional signaling by low-affinity molecules in PET imaging (Li et al. 2008). These concerns led to the generation of annexin V mutants, or "second generation annexin V", which contain an additional short amino acid sequence on the N-terminus bearing a cysteine residue for easy, site-specific labeling by thiol-reactive prosthetic groups (Lahorte et al. 2004). However, availability of cysteine-containing second generation annexin V is limited. On the other hand, commercially available wild-type annexin V also contains a single chemically accessible cysteine residue in the 315-position of the protein. No loss of binding affinity was observed for annexin V when Cys315 was deleted or replaced with thiol-free amino acids (Tait et al. 2000). Thus, Cys315 is not required for functionality of annexin V, as modification of this residue does not alter its biological activity. Maleimide-containing prosthetic groups, which react with free thiol groups, can be used to target this single cysteine residue and thus offer a controlled, site-selective approach to radiolabel wild-type annexin V. However, to the best of our knowledge, there is only one report exploiting this radiolabeling approach for wild-type annexin V (Wuest et al. 2008).

In the present study, we describe random and site-selective labeling of wild-type annexin V with short-lived positron emitter ^{18}F, using [^{18}F]SFB as amine-reactive prosthetic group and *N*-[2-(4-[^{18}F]fluorobenzamido)ethyl]-maleimide ([^{18}F]FBEM) as thiol group-reactive prosthetic group. We examined the effect of both bioconjugation methods on PS-binding ability of ^{18}F-labeled annexin V using a cell binding assay. In this assay, we compared the binding of [^{18}F]SFB- and [^{18}F]FBEM-labeled annexin V to EL4 mouse lymphoma cells that were treated with camptothecin to induce apoptosis. We also examined binding of ^{18}F-labeled annexin V to apoptotic lymphoma cells as a function of Ca^{2+} concentration, since annexin V binding to PS is strongly Ca^{2+}-dependent (Tait et al. 2004).

Materials and methods

General

Purified recombinant human annexin V (1 or 5 mg, lyophilized) was purchased from BioVision (Milpitas, USA), reconstituted in phosphate-buffered saline (PBS), pH 7.4, to 1 mg/mL, and made into 100 μL aliquots which were kept at −20 °C. Water was obtained from a Barnstead Nanopure water filtration system (Barnstead Diamond Nanopure pack organic free RO/DIS). All chemicals and reagents were obtained from Sigma-Aldrich® unless otherwise stated. 18F was produced at the Edmonton PET Center using an Advanced Cyclotron System TR 19/9 cyclotron. Radio-TLC was performed using EMD Merck F254 silica gel 60 aluminum backed thin layer chromatography (TLC) plates

(Bioscan AR-2000). Semipreparative high performance liquid chromatography (HPLC) was performed on a Gilson system consisting of a 321 pump and a 171 diode array detector, and a Berthold Technologies Herm LB 500 was used as radio detector. UV absorbance was monitored at 254 nm.

Rotary evaporation of product solvents was carried out using a Buchi HB 140 Rotavpor-M with a Fisher Maxima C Plus Model M8C pump. Quantification of radioactive samples during chemistry was achieved using a Biodex ATOMLAB™ 400 dose calibrator. Reaction parameters were carried out with an Eppendorf Thermomixer®. Centrifugation of samples was carried out with a Hettich Zentrifugen Rotina 35R or a Fisher Scientific Mini Centrifuge.

Radio-sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on IDGel™-Express IR121S-20 precast gels (12 % tris buffer), which were run in a Bio-Rad Mini PROTEAN® Tetra Cell with running buffer.

Gels were run with a constant current of 30 mÅ for approximately 1 h, and Radio-SDS-page gels were scanned on a Fujifilm BAS 5000 phosphor imager and analyzed by AIDA Image Analyzer Software Version 4.50. The gels were then stained with Coomassie® Brilliant Blue R-250 (Bio-Rad) for 30 min at 37 °C, and left in destaining solution overnight. Tryptan Blue stained cells were counted using a Bio-Rad TC10™ Automated Cell Counter. A PerklinElmer 2480 Automatic Gamma Counter WIZARD2® was used for the cell binding assays. Flow cytometry was carried out using a Fluorescence Activated Cell Sorter (BD FACSCaliber™) bench top analyzer. For fluorescence confocal microscopy, a Zeiss LSM 710 confocal microscope was used. Protein content quantification was achieved from absorbance measurements using a Molecular Devices Spectramax 340PC microplate reader. In-house preparations of buffers were as follows: Phosphate-buffered saline (PBS) (140 mM sodium chloride, 2.7 mM potassium chloride, 5.4 mM sodium phosphate dibasic, 570 μM potassium phosphate monobasic, pH 7.4), borate buffer (50 mM sodium borate, pH 8.4) 2.5 mM Ca^{2+} binding buffer (10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (pH 7.4), 140 mM sodium chloride, 2.5 mM calcium chloride), 1.25 mM Ca^{2+} binding buffer (10 mM HEPES (pH 7.4), 140 mM sodium chloride l, 1.25 mM calcium chloride), running buffer for SDS-PAGE (25 mM Tris, 192 mM glycine, 0.1 % SDS), Destaining solution (10 % glacial acetic acid, 20 % methanol, 70 % deionized water).

Radiosynthesis of [18F]SFB

The prosthetic group [18F]SFB was prepared via a three-step procedure using a remotely controlled GE TRACERlab™ FX automated synthesis module according to Mäding et al. (2005). Quality control of module-synthesized [18F]SFB was achieved by radio-TLC. To increase the radiochemical purity of [18F]SFB for radiolabelling, 1 mL of [18F]SFB in acetonitrile was purified by HPLC using a Phenomenex LUNA® C18(2) (100Å, 250 × 10 mm, 10 μm) column and the following gradient: solvent A water; solvent B acetonitrile. 0 min 15 % B, 8 min 50 % B, 9–25 min 70 % B. The flow rate was 3 mL/min. At a retention time of about 15 min, the broad [18F]SFB peak was collected into a 50 mL pear-shaped flask, and solvent was removed by a rotary evaporator. The dry [18F]SFB was then used for radiolabeling wild-type annexin V.

Radiosynthesis of [18F]FBEM

[18F]FBEM was synthesized manually from [18F]SFB in a modified version of the procedure reported by Cai et al. (2006). 3 mg of *N*-(2-aminoethyl)maleimidetrifluoroacetate salt (the amine) was dissolved in 500 μL of borate buffer (pH 8.4) and reacted with 500 μL of [18F]SFB (obtained from the GE TRACERlab™ FX module) for 20 min at 50 °C.

[18F]FBEM was purified by HPLC using the same column and gradient that was used for [18F]SFB purification, and it also eluted with a retention time of about 15 min. The purified [18F]FBEM solution was placed on a rotary evaporator until all solvent was removed.

Labeling of wild-type annexin V with [18F]SFB

An annexin V aliquot (100 μg in 100 μL of either PBS (pH 7.4) was warmed to room temperature and added to the dry [18F]SFB (150–200 MBq) in a 50 mL pear-shaped flask, vortexed vigorously for about 30 s, and then transferred to a microcentrifuge tube. Another 50 μL of PBS was used to rinse the walls of the flask and added to the reaction mixture. The reaction mixture was then placed on the thermoshaker at 30 °C for 30 min to produce [18F]SFB-annexin V. [18F]SFB-annexin V was purified by size exclusion chromatography using either a PD-10 desalting column (GE Healthcare) or a 10DG desalting column (Bio-Rad Econo-Pac®) eluted with PBS into 250 μL fractions (8 drops per fraction). The collected high molecular weight fractions containing the highest activity were directly used for cell binding studies. For quality control, a 5 μL sample of the crude reaction mixture and a 5 μL sample of purified [18F]SFB-Annexin V were reduced with dithiothreitol (DTT) at 95 °C for 5 min and subjected to SDS-PAGE. The SDS-PAGE gels were then analyzed by phosphor imaging and staining/destaining.

Labeling of wild-type annexin V with [18F]FBEM

Once [18F]FBEM (~200 MBq) was manually synthesized from [18F]SFB and evaporated to dryness, the same

procedure that was used to label wild-type annexin V with [^{18}F]SFB was also used for [^{18}F]FBEM. Like [^{18}F]SFB-annexin V, [^{18}F]FBEM-annexin V was purified by size exclusion chromatography and directly used for cell binding studies. SDS-PAGE was carried out on crude and purified samples for quality control.

Cell cultures

EL4 murine T-cell lymphoma cells (TIB-39™), which readily undergo apoptosis when introduced to cytotoxic agents, were purchased from the American TypeCulture Collection (ATCC®) (Manassas, VA, USA) and maintained in RPMI 1640 medium (prepared from Gibco® powder) supplemented with 10 % fetal bovine serum (FBS) (Gibco®), 2 mM L-glutamine (Invitrogen) and 1 % penicillin/streptomycin (Invitrogen). Cell density was maintained in the range of 2×10^5 cells/mL to 2×10^6 cells/mL, and cell growth medium was added or changed every 2 days. Cells were incubated at 37 °C in a humidified incubator with a 5 % (v/v) CO_2 atmosphere (ThermoForma Series II Water Jacketed CO_2 Incubator).

Induction of apoptosis in EL4 cells and flow cytometry

(s)-(+)-Camptothecin (Sigma®), a DNA-topoisomerase I inhibitor, was used to induce apoptosis in EL4 cells. In order to determine the optimal concentration of camptothecin to induce sufficient apoptosis, flow cytometry was used to determine apoptosis levels in EL4 cells treated with increasing doses of the drug. The FITC-Annexin V Apoptosis Detection Kit 1 (BD Pharmingen™) was used to detect apoptotic cells. From this kit, fluorescein isothiocyanate conjugated annexin V (FITC-annexin V) was used to stain apoptotic cells, while propidium iodide (PI) was used to stain necrotic cells. The use of PI allowed for discrimination between apoptotic and necrotic cells. A 6-well plate (Corning®) was prepared with 3 mL of RPMI medium containing 3 million cells (cell density of 1 million cells/mL) in each well. Each well had a different concentration of camptothecin: 0.5, 1.0, 1.5, 1.75 and 2.0 μM, and the control well contained just NaOH (9 μL of 1.0 M NaOH solution) without camptothecin. The cells were left to incubate for 24 h at 37 °C. The next day, the cells were pelleted by centrifugation, washed twice with PBS, and resuspended in binding buffer (BD Pharmingen™) to a cell density of 1 million cells/mL. A 100 μL aliquot of each cell solution was transferred to its own flow cytometry tube. To each tube, 3 μL of annexin V-FITC and 3 μL of PI was added. 4 tubes of control cells were prepared: one unstained, one stained with FITC-annexin V only, one stained with PI only, and one stained with FITC-annexin V + PI.

The tubes were vortexed, covered with aluminum foil and left to incubate in the dark for 15 min at room temperature. 400 μL of binding buffer was then added to each tube, and apoptotic and necrotic cells in each tube were counted by a fluorescence activated cell sorter.

Fluorescence confocal microscopy

Fluorescence confocal microscopy was used to image the apoptosis-inducing effect of camptothecin on EL4 lymphoma cells. Only two groups of cells were imaged by fluorescence confocal microscopy: control (untreated) cells, and cells treated with 1.5 μM camptothecin, the optimal concentration to produce sufficient apoptosis levels determined by flow cytometry. These cells were prepared and treated using the same method that was used in the flow cytometry studies, 1 day prior to fluorescence imaging. Like the fluorescent staining done in the flow cytometry studies, FITC-annexin V and PI were used to detect apoptotic cells and necrotic cells, respectively. After 1 day of treatment, cells were pelleted by centrifugation, washed once in PBS and once in binding buffer, and then re-suspended in binding buffer to a cell density of 2 × 105 cells/mL. 30 μL FITC-annexin V and 30 μL PI was added to 500 μL of this cell solution, and the cells were incubated in the dark at room temperature for 15 min. Cells were then spun down and resuspended in 1 mL binding buffer, and 500 μL of each cell solution was added to its own well of a Nunc™ Lab-Tek™ II Chambered Coverglass System. The cells were then viewed and imaged using a confocal microscope available in the Cell Imaging Facility of the Cross Cancer Institute (Edmonton, Alberta).

Cell binding assay of [^{18}F]SFB-annexin V and [^{18}F] FBEM-annexin V in EL4 cells

24 h prior to the cell binding assay, two T75 flasks were prepared containing 15 mL of EL4 cells at a cell density of 1 million cells/mL. To one flask, 9 μL of the 2.5 mM stock solution of camptothecin was added to the 15 mL of media to produce a camptothecin concentration of 1.5 μM. To the control flask, 9 μL of 1.0 M NaOH solution was added. To prevent direct contact of 1.0 M NaOH and 2.5 mM camptothecin with the cells, NaOH/camptothecin was first added to the media, which was then added to the cells. Control and treated cells were then left to incubate for 1 day at 37 °C. On the day of experiment, control and treated cells were pelleted by centrifugation, growth media was removed, and cells were washed once with PBS, and once with 2.5 mM Ca^{2+}-containing binding buffer. Cells were resuspended in binding buffer to 5 × 106 cells/mL. Using two 12-well plates (one for control cells and one for treated cells), 300 μL of cell solution was added to each well.

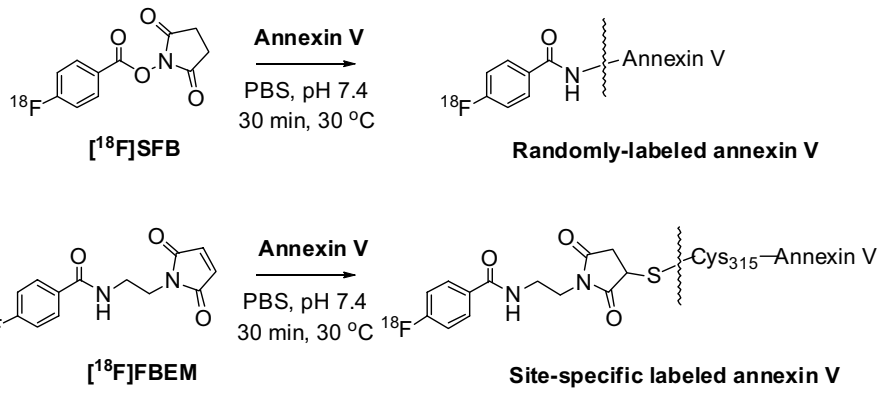


Fig. 1 Random and site-specific labeling of annexin V with [^{18}F]SFB and [^{18}F]FBEM

The ^{18}F-labeled annexin V tracer being investigated was diluted in binding buffer to 0.5 kBq/μL, and 200 μL of this ^{18}F-annexin V solution was added to each well in order to get approximately 0.1 MBq/well.

Tracer was not added to control and treated cells that were to be used for protein quantification. Cells were left to incubate at room temperature for 1, 15, 30 or 60 min. At each time point, the cells of a well were transferred to a microcentrifuge tube, rinsing the well with an additional 500 μL of binding buffer and adding this to the tube. Cells were centrifuged for 2 min at 1500 rpm in order to form a cell pellet. The supernatant was removed along with any unbound tracer. The pellet was resuspended in 500 μL of binding buffer, and this solution was transferred to a new tube to remove unbound tracer that was non-specifically bound to the tube. Cells were spun down to get a pellet, and the supernatant removed. The pellet-containing tubes were placed in a gamma counter to measure the amount of radiotracer bound to the cells. Calculated uptake values were expressed as percentage of total activity normalized to mg of the cellular protein content. In order to address the effect of Ca^{2+} concentration, another set of cell binding assay was carried out using a lower Ca^{2+} concentration, 1.25 mM, which is suggested to be more representative of physiological Ca^{2+} levels (Blankenberg 2008). To determine protein content, cells (control and treated) were lysed with CelLytic™ M (300 μL) for 10 min at 4 °C, centrifuged at 4 °C and 10,000 rpm for 8 min, and left in the freezer for at least one night. Following the instructions of bicinchoninic acid (BCA) BCA™ Protein Assay Kit (Pierce™), standards containing diluted albumin (BSA) were prepared at concentrations of 0, 50, 100, 200, 300, 400, 600, and 800 μg/mL. A 25 μL aliquot of each standard was pipetted into a 96-well microplate (Corning®) in descending concentration, along with 25 μL of the unknown protein content sample, thawed to room temperature. 7 mL of BCA Reagent A was thoroughly mixed with 140 μL of BCA reagent B, and a multichannel pipette was used to dispense 200 μL of this solution into a 96 well microplate that contained the protein content samples. The microplate was left to incubate for 25 min at 37 °C (Fisher Scientific Isotemp Incubator Model 546) and then the protein content was determined using an absorbance microplate reader.

Data analysis

All data are expressed as mean ± standard error of the mean (SEM) from 3 or more experiments. All graphs were constructed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). K_d and B_{max} values were calculated using a nonlinear regression (curve fit) analysis (GraphPad Prism 5.0). Statistical differences were considered significant if $P < 0.05$, and were tested using the unpaired *t* test.

Results and discussion

Radiolabeling of wild-type annexin V with [^{18}F]SFB and [^{18}F]FBEM

Random labeling of the 23 primary amine groups accessible in wild-type annexin V according to an acylation reaction with prosthetic group [^{18}F]SFB gave ^{18}F-labeled annexin V in decay-corrected radiochemical yields of 9 ± 1 % ($n = 7$, based upon [^{18}F]SFB).

Site-specific labeling of Cys315 in wild-type annexin V was achieved by an alkylation reaction with [^{18}F]FBEM to afford ^{18}F-labeled annexin V in decay-corrected radiochemical yields of 4 ± 2 % ($n = 5$, based upon [^{18}F]FBEM). Both prosthetic groups were purified by HPLC prior to the reaction with wild-type annexin V. The synthesis route of randomly labeled and site-specific labeled [^{18}F]annexin V is given in Fig. 1.

Both ^{18}F-labeled annexin V derivatives were obtained at high radiochemical purity >95 % after purification with size exclusion chromatography. Figure 2 shows the results of Coomassie stained SDS-PAGE and radio-SDS-PAGE

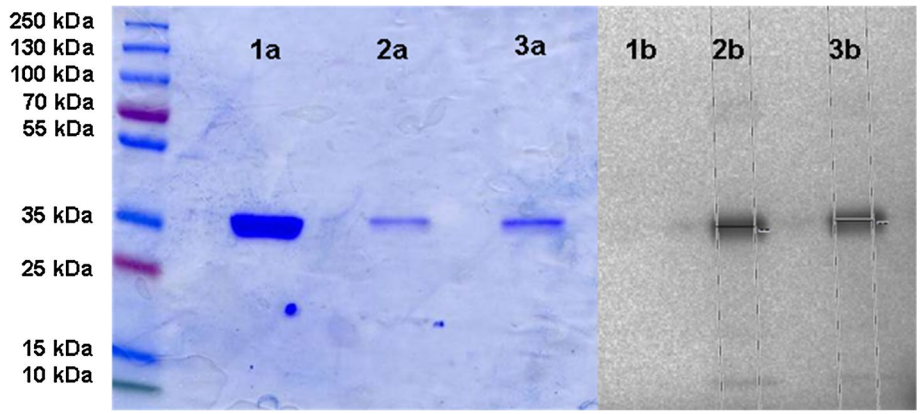


Fig. 2 SDS-PAGE analysis of wild-type annexin V (*lanes 1a* and *1b*), purified randomly labeled annexin V (*lanes 2a* and *2b*), and purified site-selectively labelled annexin V (*lanes 3a* and *3b*)

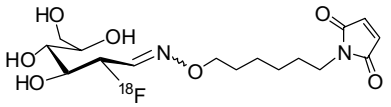
analysis of wild-type annexin V as reference, and purified ^{18}F-labeled annexin V derivatives.

Conjugation of prosthetic groups [^{18}F]SFB and [^{18}F]FBEM to wild-type annexin V proceeded under mild conditions (PBS, pH 7.4, 30 min, 30 °C), and no degradation was observed during the radiosynthesis of [^{18}F]annexin V as confirmed by SDS-PAGE analysis. Isolated radiolabeled protein corresponds with the expected molecular weight of wild-type annexin V derivatives of 36 kDa.

Radiolabeling of wild-type annexin V with [^{18}F]SFB has been reported by several groups over the past decades, and our results from this study further confirm the feasibility of using prosthetic group [^{18}F]SFB to radiolabel annexin V with ^{18}F according to this random labeling method (Blankenberg et al. 1998; Yagle et al. 2005; Zijlstra et al. 2003; Lahorte et al. 2004). Although we obtained lower radiochemical yields (9 %) of ^{18}F-labeled annexin V than other groups, this can be explained by the significantly smaller amount (0.1 mg) of wild-type annexin V used in our reactions. Other groups reported radiochemical yields between 42 and 70 % using much higher annexin V amounts in the range of 1 mg of protein (Yagle et al. 2005; Zijlstra et al. 2003; Toretsky et al. 2004). However, our obtained radiochemical yield of 9 % was comparable to the reported 10 % radiochemical yield by Murakami and colleagues, who also used small amounts (0.1 mg) of wild-type annexin V for the radiolabeling with [^{18}F]SFB (Murakami et al. 2004). Toretsky et al. (2004) described a linear relationship ($r^2 = 0.89$) between protein concentration and radiochemical yield of [^{18}F]annexin V, which provides a good explanation for the obtained radiochemical yields.

The fairly high costs for wild-type annexin V make the use of small amounts of the protein during the radiolabeling reaction highly desirable. Moreover, smaller amounts of protein provide radiolabeled annexin V at higher specific radioactivity.

Successful bioconjugation of the single cysteine residue at position 315 in wild-type annexin V was recently reported by our group (Wuest et al. 2008). Glucose-based



[^{18}F]FDG-MHO

Fig. 3 Structure of [^{18}F]FDG-MHO

maleimide prosthetic group [^{18}F]FDG-maleimidehexyloxime ([^{18}F]FDG-MHO) gave ^{18}F-labeled annexin V in radiochemical yields of 43–58 % starting from 0.1 mg of wild-type annexin V. This result makes [^{18}F]FDG-MHO a highly suitable prosthetic group for bioconjugation of wild-type annexin V. However, [^{18}F]FDG-MHO is a rather large prosthetic group in comparison to [^{18}F]SFB (Fig. 3).

In addition to the maleimide group it also contains a hydrophilic sugar moiety prone to modify the pharmacokinetic properties of [^{18}F]FDG-MHO-labeled annexin V differently than [^{18}F]SFB-labeled annexin V which lacks such a polar substitution pattern. These differences in size and pharmacokinetic profile make a direct comparison between the random labeling method using [^{18}F]SFB and a site-directed labeling approach with [^{18}F]FDG-MHO challenging. On the other hand, maleimide-containing compound [^{18}F]FBEM is an alternative thiol-selective prosthetic group which is more comparable to the size of [^{18}F]SFB. This consideration also applies to [^{18}F]FBEM-labeled annexin V (Fig. 1).

Thus, [^{18}F]FBEM is a suitable prosthetic group for a comparative study with acylation agent [^{18}F]SFB despite the rather low radiochemical yield of 4 % for the bioconjugation reaction to wild-type annexin V.

Induction of apoptosis in EL4 cells with camptothecin

Characterization of randomly and site-specific ^{18}F-labeled annexin V derivatives was performed in murine lymphoma EL4 cells, which were treated with camptothecin to induce apoptosis. Flow cytometry was used to assess the effect of

the camptothecin concentration on the level of apoptosis induced in EL4 cells. The results are given in Fig. 4.

The data shown in Fig. 4 revealed a small amount of basal apoptosis (17 %) in the untreated control cell population due to the unstable nature of the cell line. This baseline level of apoptosis in EL4 cells has been previously reported (Guo et al. 2009). Treatment with 1.5 μM of camptothecin induced the highest increase in apoptosis levels relative to untreated cells, as 56 % of the cells were stained with FITC-annexin V. Apoptosis levels reached a plateau at higher camptothecin concentrations (1.75 and 2.0 μM) resulting in 60 and 57 % apoptosis rates, respectively.

Based on these results, we selected a drug concentration of 1.5 μM to induce apoptosis in EL4 cells for the cell binding assay. Drug-induced apoptosis in EL4 cells was confirmed with confocal microscopy. Confocal microscopy images of camptothecin-treated EL4 cells stained with FITC-labeled annexin V and propidium iodide are shown in Fig. 5.

Fluorescence confocal microscopy images clearly demonstrate the successful induction of apoptosis in EL4 cells upon treatment with 1.5 μM of camptothecin as shown by the green stain from FITC-annexin V binding to PS in the treated cell population.

Cell binding using EL4 cells with [18F]SFB-annexin V and [18F]FBEM-annexin V

Cell binding studies with untreated (control) and drug-treated EL4 cells were used to characterize PS-binding properties of [18F]SFB- and [18F]FBEM-labeled annexin V. Both radiotracers showed significantly higher binding of 18F-labeled annexin V derivatives to EL4 mouse lymphoma cells treated with the apoptosis-inducing agent camptothecin (1.5 μM) compared to untreated cells over time. After 60 min, a fourfold higher uptake of [18F]SFB-annexin V to treated EL4 cells was found compared to non-treated cells (Fig. 6).

The Ca^{2+} concentration is known to be critical for PS-binding of annexin V. It was shown that annexin V binding to PS is strongly Ca^{2+}-dependent and declines rapidly over a range of 2.5–1.25 mM (Tait et al. 2004). To address this issue, we conducted another set of cell binding studies using Ca^{2+} concentrations of 1.25 and 2.5 mM. A Ca^{2+} concentration of 1.25 mM was recently suggested to be representative of Ca^{2+} levels in vivo (Tait et al. 2006).

Binding of [18F]SFB-annexin V to both control (untreated) EL4 cells (Fig. 7) and cells treated with 1.5 μM camptothecin (Fig. 8) was significantly decreased by reducing the Ca^{2+} concentration from 2.5 to 1.25 mM.

As expected, lowering the Ca^{2+} concentration from 2.5 to 1.25 mM significantly reduced binding of [18F]SFB-annexin V to both treated and untreated EL4 cells.

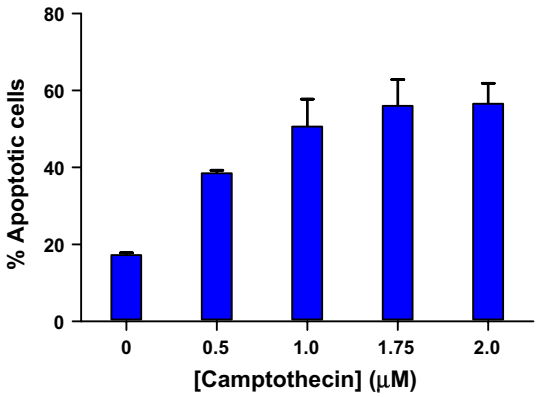


Fig. 4 Proportion of EL4 cells undergoing apoptosis when treated with increasing concentrations of camptothecin, as determined by flow cytometry with FITC-labeled annexin V ($n = 3$)

However, the lower Ca^{2+} concentration did not diminish the preferential binding of [18F]SFB-annexin V to cells treated with apoptosis-inducing agent compared to untreated cells. After 60 min, binding of the radioligand was three-times higher in treated cells versus control cells.

Our observed elevated binding of [18F]SFB-annexin V to apoptotic EL4 cells confirms previous reports on the feasibility of using randomly labeled [18F]SFB-annexin V for the detection of apoptosis. The PS-binding ability of [18F]SFB-annexin V was described in various other cell lines. Grierson and colleagues demonstrated PS-binding of [18F]SFB-annexin V through a cell binding assay using red blood cells (RBCs) with exposed PS (Grierson et al. 2004), while Zijlstra et al. (2003) demonstrated 60 % increased binding of [18F]SFB-annexin V to UV-irradiated Jurkat T-cells compared to non-irradiated cells. Another study by Toretsky et al. (2004) demonstrated 88 % more binding of [18F]SFB-annexin V to TC32 sarcoma cells treated with etoposide compared to untreated cells . When using site-specific labeled [18F]FBEM-annexin V, we observed a 2.6-fold increase in binding of the radioligand to treated EL4 cells compared to untreated cells (Fig. 9).

Results of PS-binding shown in Figs. 6 and 9 confirm that both randomly labeled [18F]SFB-annexin V and site-specific labeled [18F]FBEM-annexin V retained the ability to detect apoptotic cells in vitro. Both radiotracers demonstrated significantly higher binding to EL4 cells treated with 1.5 μM of camptothecin compared to untreated cells. Binding to apoptotic cells in terms of extent and kinetics was comparable for both 18F-labeled wild-type annexin V derivatives.

This result suggests that random and site-selective radiolabeling methods with wild-type annexin V provide radiotracers with comparable PS-binding capabilities. In our experiments, no significant differences upon PS binding in EL4 cells for both 18F-labeled annexin V derivatives were

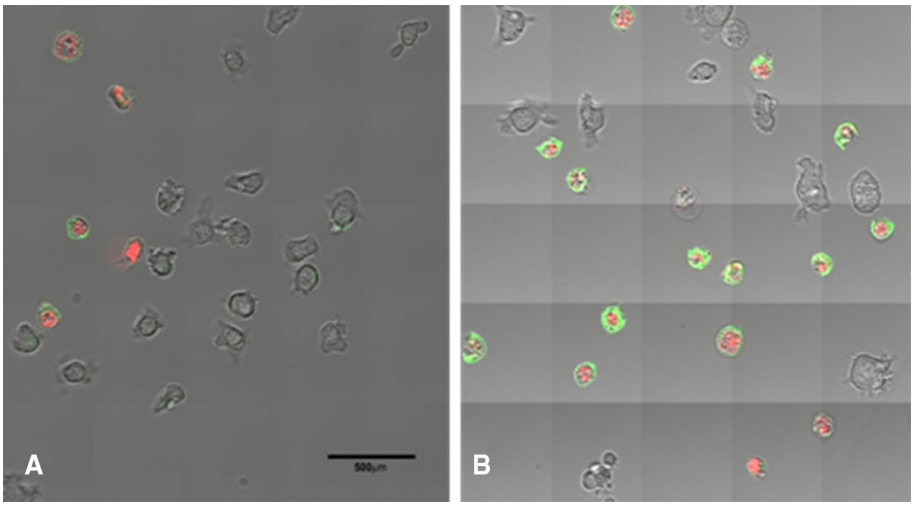


Fig. 5 Fluorescence confocal microscopy images of **a** Control (untreated) EL4 cell population; **b** EL4 cell population treated with 1.5 μM of camptothecin. *Green stain*—FITC-annexin V (apoptosis); *red stain*—propidium iodide (necrosis) (color figure online)

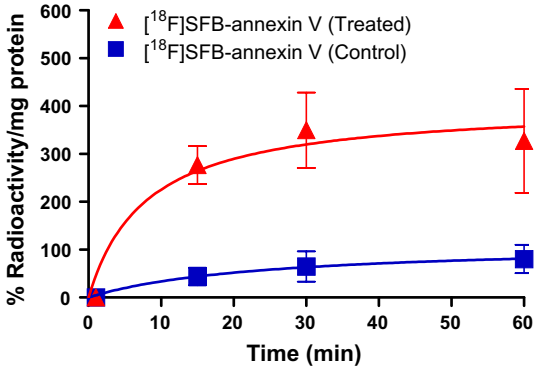


Fig. 6 Cell binding of randomly labeled [^{18}F]SFB-annexin V to untreated (control) EL4 cells and EL4 cells treated for 24 h with 1.5 μM of camptothecin ($n = 3$)

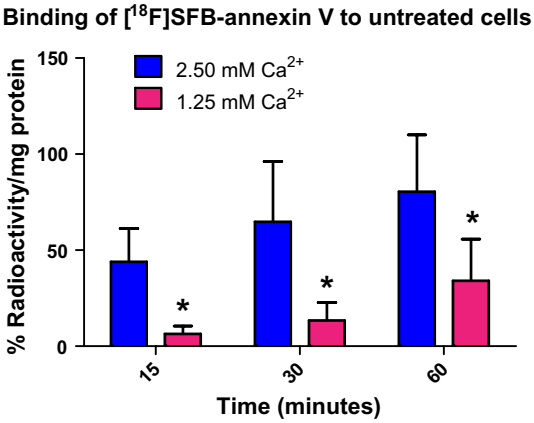


Fig. 8 Comparison of cell binding of [^{18}F]SFB-annexin V to camptothecin-treated EL4 cells in the presence of higher (2.5 mM) and lower (1.25 mM) concentrations of Ca^{2+} ($n = 3$)

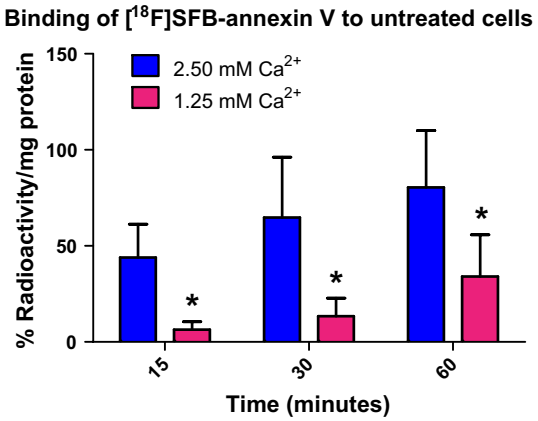


Fig. 7 Comparison of cell binding of [^{18}F]SFB-annexin V to untreated EL4 cells in the presence of higher (2.5 mM) and lower (1.25 mM) concentrations of Ca^{2+} ($n = 3$)

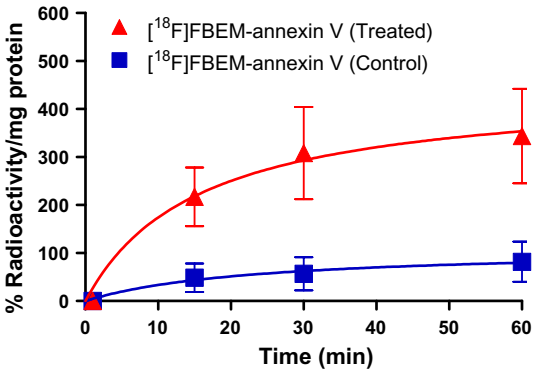


Fig. 9 Cell binding of site-specific labeled [^{18}F]FBEM-annexin V to untreated (control) EL4 cells and EL4 cells treated for 24 h with 1.5 μM of camptothecin ($n = 3$)

noticeable. However, this finding is in contrast to a comparative study reported by Tait et al. (2006).

In their study, the authors found that amine-directed random modification of annexin V substantially reduced its ability to bind to PS-expressing RBCs, though it did not diminish it completely. They also reported that site-selective conjugation of an additional cysteine residue present in the modified amino-terminal sequence of Ala–Gly–Gly–Cys–Gly–His of annexin V-128 (a second generation annexin V) resulted in an increased binding of the protein to RBCs. They also showed that the more conjugated annexin V is, the lower its PS-binding ability becomes. However, Grierson et al. (2004) determined that even high [^{19}F]SFB:annexin V molar ratios (32:1) during a 15 min incubation time for the conjugation reaction resulted in a rather low 2.1 average incorporation level of fluorobenzoic acid into annexin V without loss of PS-binding properties. Only extension of the reaction time from 15 to 60 min resulted in a conjugated product with compromised PS-binding capabilities.

Our described conjugation method is using HPLC-purified [^{18}F]SFB and [^{18}F]FBEM at high specific activity. Moreover, mild reaction conditions (PBS, 30 min, 30 °C, pH 7.4) during the conjugation reactions enable introduction of the radiolabel while preserving the structural and functional integrity of wild-type annexin V as demonstrated by the found comparable PS-binding ability for both, randomly labeled and site-specific labeled annexin V derivatives. However, given the rather low radiochemical yields for [^{18}F]FBEM-annexin V (4 %) combined with the lengthier and more complex synthesis of maleimide-based prosthetic group [^{18}F]FBEM, we propose to use the random labeling technique with [^{18}F]SFB to prepare ^{18}F-labeled wild-type annexin V. Radiolabeling of wild-type annexin V with the readily available prosthetic group [^{18}F]SFB provides ^{18}F-labeled annexin V as a radiotracer for PS-binding in apoptotic cells in reasonable radiochemical yields of around 10 % while using only small amounts of wild-type annexin V (0.1 mg) as starting material.

Conclusion

In conclusion, wild-type annexin V was successfully radiolabeled with ^{18}F starting from rather low amounts of the protein (0.1 mg) using both amino-targeting and thiol-targeting bioconjugation methods, though with modest radiochemical yields. When compared with well-characterized, randomly labeled [^{18}F]SFB-annexin V, site-specific labeled [^{18}F]FBEM-annexin V showed no difference in the ability to detect apoptotic cells. Since both radiolabeling methods did not have any significant effect on PS-binding ability of wild-type annexin V, it is more practical to use the simpler and more economical random labeling technique with prosthetic group [^{18}F]SFB to radiolabel this protein with ^{18}F.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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