Site-Specific Labeling of Annexin V with F-18 for Apoptosis Imaging

Xue He Li,†,‡ Jeanne M. Link,* ,‡ Svetlana Stekhova,†,‡ Kevin J. Yagle,‡ Christina Smith,§ Kenneth A. Krohn,‡ and Jonathan F. Tait§

Department of Radiology and Department of Laboratory Medicine, University of Washington Medical Center, Seattle, Washington 98195. Received April 22, 2008; Revised Manuscript Received June 5, 2008

Annexin V is useful in detecting apoptotic cells by binding to phosphatidylserine (PS) that is exposed on the outer surface of the cell membrane during apoptosis. In this study, we examined the labeling of annexin V-128, a mutated form of annexin V that has a single cysteine residue at the NH₂ terminus, with the thiol-selective reagent [18F]FBABM. Annexin V-128 showed increased uptake in rat liver proportional to apoptosis after apoptosis was chemically induced (15). The rapid blood clearance and urinary excretion of [18F]FAN suggest its potential as a PET imaging agent. This radiotracer, however, has its own disadvantages. The predominant renal clearance increases the radiation burden to the kidneys and bladder, and a patient would have to be catheterized if the diagnostic area of interest were in the abdomen (15). Moreover, this labeling method for annexin V is nonspecific, as the reaction is between the prosthetic group ([18F]SFB) and any available NH₂ groups in this protein. There are at least 23 potential sites for this reaction to occur in each annexin V molecule, some near the active binding site of the protein. A recent report confirms that amine-directed chemical modification of annexin V reduces its membrane-binding activity even at low stoichiometries (16). Multiple substitutions of annexin V with [18F]SFB could mean disproportional signaling by low-affinity molecules in PET imaging. Therefore, a site-specific method to label annexin V with only one substitution per molecule should have an advantage over the [18F]SFB-modified version.

An alternative strategy to label proteins involves targeting the free thiol groups that are present only in cysteine residues. Thiol reactive agents such as N-substituted maleimides and iodoacetamide can be used to modify proteins at cysteines at specific sites (17–20). Annexin V-128 is a mutated form of annexin V containing only one cysteine. The initiator Met at position one is deleted and a six amino acid extension containing one cysteine is added at the N-terminus. The N-terminus does not play a role in the binding activity of annexin V and so this modification does not change the binding affinity of annexin V-128 for PS. Site-specific labeled (with 99mTc) annexin V-128 derivatives showed twice as much in vivo apoptosis-specific uptake in rat liver models as did amine-derivatized forms of annexin V (16). A previous approach to label thiol-containing molecules with N-[4-([4-[18F]fluorobenzylidene]aminoxy)butyl]maleimide ([18F]FBABM) used a two-step/two-pot synthesis that was labor intensive and gave low yield with larger biomolecules such as 5′-S-ODN (21). In this paper, we report an improved synthesis and efficient purification of [18F]FBABM.
and the production of [¹⁸F]annexin V-128 ([¹⁸F]FAN-128) with this thiol-reactive reagent. The PS binding affinity of [¹⁸F]FAN-128 and its potential as a PET imaging agent for cell death are also discussed.

MATERIALS AND METHODS

General. All reagents and solvents were purchased from Aldrich Chemical Co. (Milwaukee, WI) as reagent grade and were used without purification unless otherwise noted. The labeling precursor, 4-trimethylammoniumbenzaldehyde triflate 1, was prepared as described by Haka et al. (24) and stored in a sealed vial over desiccant in the refrigerator. The coupling precursor, N-[4-(aminooxy)butyl]maleimide 3, as a HCl salt was synthesized according to Toyokuni et al. (21) with modifications and stored over desiccant in the refrigerator. Enriched (97%) [¹⁸O]water was obtained from Medical Isotopes, Inc. (Pelham, NH). Chromax 30-PS-HCO3. ¹⁸F separation cartridges were obtained from GE Medical Systems and C-18 SepPak (light) cartridges were obtained from Waters Corp. (Milford, MA). The first part of the radiosynthesis (the production of the radioprecursor [¹⁸F]FABAM) was performed using a GE Tracerlab FXFN automatic synthesis module. The second part of the radiosynthesis (the radio-labeling of annexin V-128) was done manually outside the GE box. ¹H NMR data were obtained with a Varian 300 MHz NMR instrument. Quality control of the purified radioactive product was performed on a Waters 2690 HPLC module coupled with an in-line Micromass ZMD mass spectrometer.

Preparation of Annexin V-128. Annexin V-128 (∼2 mg/mL in 20 mM HEPES sodium, 100 mM NaCl, pH 7.4) was prepared and purified as described (26). The protein was reduced with 1 mM diithiothreitol (DTT) for 15 min at 37 °C and then applied to a Sephadex G-25 column equilibrated with deoxygenated buffer (20 mM HEPES-sodium, 100 mM NaCl, pH 7.4) to remove the reductant. Protein concentration was measured by absorbance at 280 nm and the reduced protein was used for labeling within 6 h.

Synthesis of N-[4-([4-Dimethylaminobenzylidene)aminooxy]butyl]maleimide 4. A solution of 4-dimethylaminobenzaldehyde (17 mg, 0.11 mmol) and 3-HCl (20 mg, 0.09 mmol) in anhydrous DMF (1 mL) was stirred at r.t. for 16 h. The reaction mixture was then poured into water and extracted with diethyl ether. After drying over anhydrous Na2SO4, the ether reaction mixture was then poured into water and extracted with diethyl ether. After cooling to r.t., 1.5 mL of 45% ethanol in water (v/v) was added to the reaction vessel and the whole mixture was transferred to the HPLC loop. Semi-preparative HPLC was performed with a C18 column (Phenomenex Prodigy ODS(3), 5 μm, 250 × 10 mm) eluted with 45% ethanol/water (v/v) at 3 mL/min and 50 °C. The chemically and radiochemically (>99%) pure fraction containing [¹⁸F]FABAM was collected (∼12 mL; t½ 38–40 min). The chemical and radiochemical purity was assessed with a C18 analytical HPLC column (Phenomenex Inertisil ODS-3, 100 × 2.1 mm, 5 μm, eluted with 55% methanol/water (v/v) at 0.3 mL/min flow rate and 40 °C with in-line mass detection.

Labeling of Annexin V-128 with [¹⁸F]FABAM. Purified [¹⁸F]FABAM from the previous step (∼12 mL) was diluted with water (∼50 mL) and passed through a Waters C18 SepPak that had been preconditioned with 5 mL ethanol and 5 mL water. The SepPak was washed with additional 10 mL water and the excess water was expelled with a push of air. 2 mL of ether was then pushed through the SepPak to elute the product into a clean 5 mL glass test tube. The ether phase of the biphasic mixture was drawn out and transferred into a clean 5 mL BD Vacutainer test tube. The ether was then removed by evaporation under a gentle flow of argon at 50 °C. After cooling to r.t., approximately 0.7 mg freshly prepared annexin V-128 in 0.5 mL buffer (20 mM HEPES, 100 mM NaCl, pH 7.4) was carefully added to the residual film and incubated for 15 min with periodic gentle agitation. Labeled [¹⁸F]fluoroorannxin V-128 ([¹⁸F]-FAN-128) was isolated by a semi-preparative size-exclusion column (BioSep SEC-S 2000, 300 × 7.8 mm) eluted at 1 mL/min with 10 mM phosphate/150 mM NaCl (pH = 7). [¹⁸F]-FAN-128 was the first radioactive peak eluted from the column with a retention time of ∼7–9 min.

Cell-Binding Assay of [¹⁸F]FAN-128. The membrane binding affinity of [¹⁸F]FAN-128 was determined following the procedure described by Tait et al. (22, 16). Briefly, mixtures of red blood cells (RBCs) with exposed PS on the outer surface of the cell membrane (Couler “4C Plus” Cell Control, Fullerton, CA), [¹⁸F]FAN-128 (diluted to a final concentration of 1 nM) in buffer (100 mM NaCl, 50 mM HEPES-Na, pH 7.4, 0.02% NaN3, 1 mg/ml BSA) and CaCl2 at precise concentrations (between 0 and 3 mM) were prepared. The mixtures were incubated for 10 min at r.t. After centrifugation, the supernatant was discarded. Then the RBCs with bound [¹⁸F]FAN-128 were washed with the same buffer containing CaCl2 at the same concentrations and re centrifuged and the supernatant was discarded. Membrane-bound [¹⁸F]FAN-128 was then released with assay buffer containing 5 mM EDTA and counted in the gamma counter.

RESULT AND DISCUSSION

Chemistry. Our synthesis of [¹⁸F]FABAM builds on the approach reported by Toyokuni et al. (21) (Scheme 1). Briefly, tri fluoride 1 was heated with dried [¹⁸F]KF in anhydrous acetonitrile in the presence of Kryptox[2,2,2] to generate [¹⁸F]FBA 2,
impurities, which would compete with \([18F]FBABM\) for the reaction. Additionally, the slow rate of reaction associated with dilute solvent for 15 min in a closed reaction vessel in a GE Tracerlab FXN automated synthesis module. The solvent was heated under helium pressure (200 mbar). Determined from radio-HPLC chromatograms of the crude product. Data represent an average of 3 runs.

followed by the addition of maleimide 3 in methanol and purification by HPLC. Their two-step/two-pot synthesis of \([18F]FBABM\) was used to label the thiol-containing tripeptide glutathione (GSH) and a 5'-thiol-functionalized oligodeoxyribonucleotide (5'-ODN). This synthesis started with the generation of 2 followed by a C18 SepPak purification. After coupling to the heterobifunctional linker \(N\)-[4-(aminoxy)butyl]maleimide 3 using the oxime-O-ether formation reaction and HPLC purification, \([18F]FBABM\) was formulated in acetonitrile and successfully labeled GSH or 5'-ODN. This method, however, has disadvantages for routine protein labeling. First, the synthesis of \([18F]FBA\) was labor-intensive, with a two-pot setup and a C18 SepPak purification step in between. Second, although the labeling of GSH gave good yield (70% decay corrected), the yield in labeling larger biomolecules such as 5'-ODN (5% decay corrected) was low. The concentrations of the GSH or the 5'-ODN in these reactions were 6 mM and 0.1 mM, respectively. The radiochemical yield of radiolabeled peptides/proteins or other biomolecules via prosthetic groups typically decreases as the concentration of the biomolecule decreases. For an even larger molecule such as annexin V-128, which is 4-dimethylaminobenzaldehyde formed inevitably during the reaction. It is important to note that, because FBA is a volatile compound at elevated temperature, it is crucial to cool the reaction mixture to r.t. before opening the reaction vessel to add the coupling precursor.

The critical factor for optimal protein labeling with \([18F]FBA\) is the chemical purity of the product. We suspected that a maleimide side product was present that would compete with \([18F]FBA\) for reaction with thiol groups. HPLC-MS was used to identify this major contaminant as \(N\)-(4-dimethylaminobenzylidene)aminooxy)buty]maleimide (4) (estimated >100 µg from a typical run described above) derived from the 4-dimethylaminobenzaldehyde formed inevitably during the initial nucleophilic fluorination step (Scheme 2). In a typical run, about 300 µg 4-dimethylaminobenzaldehyde was detected after this first step. The identity and quantity was determined by HPLC-MS analysis, in comparison to an authentic sample. Our initial attempt to label annexin V-128 with \([18F]FBA\) containing 4 gave no yield, presumably due to a competition for the single thiol group by the vast molar excess of the alternative maleimide contaminant 4. In a typical labeling reaction, the molar ratio of FBA to annexin V-128 (generally 0.6 mg was used) assigned to possess the \(E\)-configuration (21) under our reaction condition. This was confirmed by coeluting with the cold standard of FBA, whose NMR spectrum revealed only one isomer, on HPLC. During the preparation of this manuscript, Berndt et al. reported a one-pot method to prepare a similar thiol-reactive labeling agent \(N\)-[6-(4-[18F]fluorobenzylidene)-aminooxy]hexyl]maleimide (\([18F]FBA\)) with similar yield to ours (23). Their method, however, includes an extra preparative step using solid-phase extraction on LiChrolut RP18 cartridge before semipreparative HPLC purification.

Table 1. Yield of FBA (2) under Different Reaction Conditions*  
| solvent | amt. of precursor 1 (mg) | temperature (°C) | yield (%) |
|---------|-------------------------|------------------|-----------|
| DMSO    | 8                       | 100              | 90        |
| THF     | 4                       | 80b              | 0         |
| CH3CN   | 2                       | 100b             | 10        |
| CH3CN   | 3                       | 100b             | 20        |
| CH3CN   | 4                       | 100b             | 75        |
| CH3CN   | 8                       | 100b             | 90        |

* All reactions were carried out by heating the precursor in 1 mL solvent for 15 min in a closed reaction vessel in a GE Tracerlab FXN automated synthesis module. b Heated under helium pressure (200 mbar). c Determined from radio-HPLC chromatograms of the crude product.
was approximately 1:1, whereas that of 4 was greater than 20:1. Attempts to remove the undesired 4-dimethylaminobenzaldehyde after the first step with a C18 SepPak were unsuccessful. The removal of 4 from the final product [18F]FBABM proved to be challenging. The calculated log P values for FBABM and 4 are 1.91 and 1.85, respectively (Molinspiration program: http://www.molinspiration.com/cgi-bin/properties). The two compounds coeluted using the HPLC method from the literature (21). We developed a useful separation by using Phenomenex Prodigy ODS(3) 5 µm 250 × 10 mm column. At 50 °C and 45% ethanol/water as the eluent at 3 mL/min, 4 was completely removed from [18F]FBABM (impurity tR(4) ~ 26–28 min, tR(FBABM) ~ 34–38 min). A typical semipreparative HPLC chromatogram is shown in Figure 1. No detectable amount of 4 was present in the purified [18F]FBABM product. We realize that, in this isocratic HPLC method, although a complete separation of impurity 4 was achieved, the long retention time of the [18F]FBABM is disadvantageous for routine production. We are currently investigating alternative purification method with gradient HPLC.

Annexin V-128 (26) contains one cysteine in its N-terminus. The single naturally occurring cysteine residue in its interior domain has been mutated to a serine in order to eliminate the possibility of modification by thiol-reactive prosthetic conjugating agents. Annexin V-128 is freshly reduced immediately prior to each labeling study with DTT to ensure that the free thiol is present. Annexin V-128. Wild-type annexin V has one internal cysteine residue that is present in the interior domain of wild-type annexin V is not readily accessible for maleimide binding. Therefore, we are able to conclude that the [18F]FBABM labeling of annexin V-128 is site-specific. The mutation of Cys316 to Ser in annexin V-128 may not be necessary for future imaging where the membrane is far from saturated with protein.

The purified [18F]FBABM was concentrated onto a C18 SepPak, washed with diethyl ether, and taken to dryness. After evaporation, the [18F]FBABM was incubated with annexin V-128 in HEPES buffer (pH = 7.4, 0.5 mL). It is noted that the number of liquid transfers and the contact surface area with the reaction vessel should be minimized to reduce unnecessary loss of annexin V-128 due to surface adsorption of the protein. Even using a careful transfer technique, approximately 1/3 of the total radioactivity is typically lost in transfers for injection onto the size-exclusion column (SEC). After HPLC purification, radiochemically pure

![Figure 1. A typical semipreparative HPLC chromatogram for [18F]FBABM purification.](Image)

![Figure 2. Typical binding curve of [18F]FAN-128 to PS-enriched membranes. Typical binding curve of [18F]FAN-128 as a factor of calcium concentration.](Image)

As a negative control, we repeated the reaction under the same condition using wild-type annexin V instead of mutated annexin V-128. Wild-type annexin V has one internal cysteine but it does not have a free thiol group on its N-terminus. In this reaction, the yield of [18F]labeled protein was negligible (<0.2%). This result shows that, under our reaction conditions, [18F]FBABM does not react with other nucleophiles such as the free NH2 groups in annexin V, and the single cysteine residue that is present in the interior domain of wild-type annexin V is not readily accessible for maleimide binding. Therefore, we are able to conclude that the [18F]FBABM labeling of annexin V-128 is site-specific. The mutation of Cys316 to Ser in annexin V-128 may not be necessary for future annexin V based tracer design.

[18F]FAN-128 PS Binding Assay. The in vitro membrane-binding affinity of [18F]FAN-128 was determined by erythrocyte binding using the method reported by Tait et al. (16). The commercially available RBCs had exposed PS on their outer surfaces of the cell membranes because of the preservatives used (27). Annexin binding to PS is calcium dependent. By titrating calcium instead of protein, this method allows an accurate measurement of annexin binding at <1% occupancy, a value similar to that for in vivo imaging where the membrane is far from saturated with protein. A typical binding curve for [18F]FAN-128 is presented in Figure 2. The binding affinity was measured by determining EC50 and slope from this experimental titration curve (22). The negative log of the binding constant, pK, is equal to −(n log10EC50 + log10[membrane]), where n is the number of Ca2+ ions interacting with each annexin V molecule, the EC50 is the molarity of Ca2+ at which cell binding is half of the maximal value, and [membrane] is the concentration of annexin V-binding sites in the assay (16). Seven batches of [18F]FAN-128 gave an average pK of 28.5 ± 1.8, an EC50 of approximately 1 mM, and a slope (Hill coefficient) between 7 and 8. This compares favorably with reported values for wild-type annexin V of a pK around 30, EC50 close to 1, and a slope of 8 (26, 16). These data indicate that the in vitro binding activity
of this site-specific, monosubstituted protein was not significantly different from that of the wild-type and an in vivo investigation of [18F]FAN-128 as an apoptosis imaging agent is warranted.

CONCLUSION

We have developed an improved, one-pot synthesis of the thiol-reactive labeling reagent [18F]FBABM. It gives satisfactory radiochemical yield and can be automated for routine production. The critical improvement was identifying and then developing a purification method to remove the impurity N-[4-[4-(dimethylamino)benzylidene)aminooxy]butyl]maleimide (4). The presence of this impurity in [18F]FBABM was dramatically decreasing the yield of labeled protein by competing for the available thiol groups. To our knowledge, this is the first time that this important issue has been addressed. We have also achieved an efficient synthesis of 18F-labeled annexin V-128 ([18F]FAN-128), a 36 kDa protein containing a single thiol group, with the [18F]FBABM radioprecursor. This maleimide modification of annexin V-128 does not compromise its membrane binding affinity.

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