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Site-specific ^{68}Ga-labeled Annexin A5 as a PET imaging agent for apoptosis

Matthias Bauwens[a,*,1], Marijke De Saint-Hubert[b,1], Ellen Devos[b], Niko Deckers[c], Chris Reutelingsperger[c], Luc Mortelmans[b], Uwe Himmelreich[d], Felix M. Mottaghy[e], Alfons Verbruggen[a]

[a]*Radiopharmacy, KULeuven, Belgium*
[b]*Nuclear Medicine, KULeuven, Belgium*
[c]*Biochemistry, Maastricht University, The Netherlands*
[d]*Biomedical NMR Unit/ MoSAIC, KULeuven, Belgium*
[e]*Nuklearmedizin, RWTH Aachen, Germany*

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Abstract

Purpose: Two variants of Annexin A5 (Cys2-AnxA5 and Cys165-AnxA5) were labelled with Gallium-68 in order to evaluate their biological properties.
Procedures: Biodistribution and pharmacokinetics of the radiotracers were studied with μPET in healthy mice and in a mouse model of hepatic apoptosis. μPET imaging after IV injection of the tracers in combination with μMRI was performed in Daudi tumor bearing mice before and after treatment with a combination of chemotherapy and radiotherapy.
Results: The biodistribution data indicated a fast urinary clearance with only minor hepatobilliary clearance, although a high retention in the kidneys was observed. Animals treated with anti-Fas showed a 3 to 8 times higher liver uptake as compared to healthy animals. Tumor uptake of ^{68}Ga-Cys2-AnxA5 and ^{68}Ga-Cys165-AnxA5 was low but significantly increased after therapy.
Conclusion: Both ^{68}Ga-Cys2-AnxA5 and ^{68}Ga-Cys165-AnxA5 show a clear binding to apoptotic cells and are promising tracers for rapid evaluation of cancer therapy.

Keywords: [^{68}Ga]; Annexin A5; Apoptosis; Therapy evaluation; PET

1. Introduction

Currently cancer treatment consists of either chemotherapy, radiotherapy, systemic radionuclide therapy or a combination of these methods. Follow-up of response to cancer therapy is crucial for an optimal treatment strategy to reduce the dose in responding patients or to change to another therapy type in non-responding patients. Anatomic imaging modalities such as magnetic resonance imaging (MRI) and computed tomography (CT) are less suitable for this purpose as they are based on tumor size reduction which occurs rather late after effective therapy and is not always conclusive [1].

Previous studies indicate that successful chemotherapy or radiotherapy induce apoptosis of neoplastic cells very early after therapy [2-4]. Therefore molecular imaging of apoptosis offers a direct and early measurement of response to cancer therapy [5,6]. One of the early characteristics of apoptosis is externalization of phosphatidylserine (PS) on cell membranes. Annexin A5 (AnxA5), a 35.8 kDa protein, binds with a high affinity to membrane-bound PS in a Ca^{2+}-dependent manner. Consequently, several radiolabeled AnxA5 derivatives have been developed over the last years. ^{99m}Tc-radiolabeled hydrazinonicotinamide-AnxA5

* Corresponding author. Laboratory of Radiopharmacy, KULeuven, 3000 Leuven, Belgium. Tel.: +3216343732; fax: +3216343891.
E-mail address: matthias.bauwens@pharm.kuleuven.be (M. Bauwens).
[1] M. Bauwens and M. De Saint-Hubert contributed equally to the study and should both be considered as first author of the article.

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(HYNIC-AnxA5) has been proposed for molecular imaging of apoptosis and first clinical data of ^{99m}Tc-HYNIC-AnxA5 have already been published [7-9]. Several other ^{99m}Tc-AnxA5 constructs, with varying synthesis pathways but generally similar biodistribution patterns, have also been reported [10,11]. However, AnxA5 derivates radiolabeled with a PET radioisotope would have a distinct advantage over those radiolabeled with a SPECT isotope, as resolution and sensitivity of PET imaging is superior to that of SPECT imaging. New AnxA5 derivatives are therefore often labeled with fluorine-18, a positron emitting radionuclide with favorable characteristics for radiolabeling (half life 110 minutes), imaging and radiation dosimetry (positron of median energy, Emax β^+=0.625 MeV). Several ^{18}F-labeled AnxA5 derivatives have been proposed and showed promising results in terms of tumor therapy follow-up [12-14]. However, their yield of radiochemical synthesis is rather low (less than 10% at the end of synthesis) due to the prolonged, multi-step procedures. Also, ^{18}F is only easily available for centers with an in-house or nearby cyclotron.

Gallium-68, another PET radioisotope, is less used even though it is currently easily available from ^{68}Ge/^{68}Ga generator systems. It has a half-life of 68 minutes and emits positrons with Emax β^+=1.9 MeV. So far, only one attempt was reported for the synthesis an AnxA5 derivative labeled with ^{68}Ga. In this case, ^{68}Ga-DOTA-thiocyanate was coupled to an amino group of AnxA5 [15].

Apart from the choice of radioisotope, the site at which the radiolabel is attached to the AnxA5 protein is of crucial importance. As all four domains of AnxA5 are required for optimal binding to apoptotic tissue, caution is necessary in amine-directed labeling strategies [10]. This is especially true in the case of a pre-labeling derivatization approach, where several metal chelators can be bound on the same AnxA5 molecule. The group of Tait et al. showed that site-specific labeling of AnxA5 outside the binding region of the protein improved the sensitivity for apoptosis compared to random labeling of amino residues [11,12]. Recently our group site-specifically labeled AnxA5 with $Tc(CO)_3$ for improved imaging of apoptosis [16].

Considering the usefulness of ^{68}Ga and the importance of site-specific labeling, we decided to develop ^{68}Ga-Dotamaleimide-Cys2-AnxA5 (^{68}Ga-Cys2-AnxA5) and ^{68}Ga-Dotamaleimide-Cys165-AnxA5 (^{68}Ga-Cys165-AnxA5). Cys2-AnxA5 and Cys165-Anx are variants of AnxA5 containing a single available cysteine residue at respectively the 2-position and the 165-position. Both cysteines are located at the concave side of AnxA5 opposite to the convex side that harbors the Ca^{2+}/phospholipid binding sites. Covalent coupling of large structures such as liposomes and iron nanoparticles via thiol-chemistry to Cys2-AnxA5 were found not to affect PS binding activity [17-19].

Several models were used to determine apoptosis-specificity of the labeled compounds. First, we induced apoptotic cell death both in vitro and in vivo by means of anti-Fas treatment. Secondly, we investigated the binding specificity of ^{68}Ga-Dotamaleimide-Cys*-AnxA5 in a mouse tumor model, before and after tumor treatment using a combination of chemotherapy and radiotherapy.

2. Material and methods

All chemicals used were of analytical or pharmaceutical grade. The following chemicals and materials were used: sodium acetate, trifluoroacetic acid, ultrapure water, 37 % hydrochloric acid (all from Sigma-Aldrich Chemie, Steinheim, Germany), Tris, (Merck, Darmstadt, Germany), Hepes (Acros, Geel, Belgium) and Dotamaleimide (Macrocyclics, Dallas, Texas, USA). ^{68}Ga was obtained from a titanium dioxide-based ^{68}Ge/^{68}Ga generator (Cyclotron, Obninsk, Russia). Radioactivity was measured using an ionization chamber based activity meter (Capintec Radioisotope Calibrator CRC-721, Ramsey, NJ, USA). For the purpose of ^{68}Ga purification, a mini-column for anion exchange chromatography was prepared manually by filling a small column (1 mm×3 mm) with strongly basic anion exchange resin (Dowex 1×8, Fluka, Sigma-Aldrich Chemie). The mini-column was conditioned by washing with 3 ml of 6 M HCl.

HPLC analyses were performed using an LC apparatus that consisted of a solvent pump (Hitachi Lachrom Elite L-2130 quaternary gradient pump, VWR International, Haasrode, Belgium), a UV detector (Hitachi L-2400 UV detector) at 254 nm and a 3-inch radiometric NaI(Tl) detector in series. HPLC analysis of ^{68}Ga-Dotamaleimide was performed using a Platinum EPS-C18 column (5 μm, 10 mm×150 mm) (Grace-Alltech, Lokeren, Belgium) and as the mobile phase gradient mixtures at a flow rate of 3 ml/minute. The mobile phase was programmed from 0.1 % trifluoroacetic acid (TFA) in water to 0.1 % TFA in acetonitrile (ACN) over the course of 20 minutes. The gradient was linear and started after running 0.1 % TFA in water for 3 minutes (HPLC 1).

HPLC analysis of ^{68}Ga-Dotamaleimide-Cys2-AnxA5 (^{68}Ga-Cys2-AnxA5) and ^{68}Ga-Dotamaleimide-Cys165-AnxA5 (^{68}Ga-Cys165-AnxA5) was performed using a BioSep-SEC-S 3000 column (Phenomenex, Utrecht, The Netherlands), with 10 mM phosphate buffer (pH 7) as the mobile phase at a flow rate of 1 ml/minute (HPLC 2).

2.1. Cys2-AnxA5 and Cys165-AnxA5 production

The variants Cys2-AnxA5 and Cys165-AnxA5 were produced by site directed mutagenesis of the cDNA of human AnxA5, expressed in E. coli and purified to homogeneity (purity >95%) at Maastricht University, the Netherlands [17]. In order to test whether the introduction of Cys2 and Cys165 affects Ca^{2+}-dependent binding to PS we measured binding isotherms at varying [Ca^{2+}]. Fig. 1 shows that Cys2-AnxA5 and Cys165-AnxA5 have binding isotherms similar to the wild type AnxA5 binding isotherm indicating that the substitutions do not affect affinity of Ca^{2+}-dependent PS binding.