Biodistribution of a $^{67}$Ga-labeled anti-TNF VHH single-domain antibody containing a bacterial albumin-binding domain (Zag)

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Introduction: Small domain antibodies (sdAbs) present high potential for both molecular in vivo imaging and therapy. Owing to the low molecular weight they are rapidly cleared from blood circulation, and new strategies to extend their half-lives are needed for therapeutic applications. We have selected a bacterial albumin-binding domain (ABD) from protein Zag to be fused to an anti-tumor necrosis factor (TNF) single variable-domain heavy-chain region antibody (VHH) to delay blood clearance, and evaluated the biodistribution profile of the fusion protein.

Methods: The anti-TNF VHH and the fusion protein VHH-Zag were conjugated to S-2-(4-isothiocyanatobenzyl)-1,4,7-triazacyclononane-1,4,7-triacetic acid (p-SCN-Bn-NOTA). The anti-TNF and albumin-binding properties of the conjugates NOTA-VHH and NOTA-VHH-Zag were assessed by enzyme-linked immunosorbent assay (ELISA). The radioconjugates $^{67}$Ga-NOTA-VHH and $^{67}$Ga-NOTA-VHH-Zag were obtained by reaction of $^{67}$GaCl$_3$ with the corresponding conjugates at room temperature. Biodistribution studies were performed in healthy female CD-1 mice.

Results: The immunoreactivity of the VHH-based proteins is preserved upon conjugation to NOTA as well as after radiometallation. The radiochemical purity of the radioconjugates was higher than 95% as determined by ITLC-SG after purification by gel filtration. The biodistribution studies showed that the Zag domain affected the pharmacokinetic properties of VHH, with impressive differences in blood clearance (0.028 ± 0.004 vs 1.7 ± 0.8 % I.A./g) and total excretion (97.8 ± 0.6 vs 25.5 ± 2.1 % I.A.) for $^{67}$Ga-NOTA-VHH and $^{67}$Ga-NOTA-VHH-Zag, respectively, at 24 h p.i.

Conclusion: The Zag domain prolonged the circulation time of VHH by reducing the blood clearance of the labeled fusion protein $^{67}$Ga-NOTA-VHH-Zag. In this way, the anti-TNF VHH in fusion with the Zag ABD presents a higher therapeutic potential than the unmodified VHH.

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1. Introduction

The great majority of therapeutic antibodies marketed are full-length Immunoglobulin G (IgG) molecules that trigger effector functions and present long serum half-life [1]. Indeed, their high molecular weight (~150 kDa) leads to long residence time in blood circulation. Additionally, IgG antibodies diffuse poorly into solid tumors, and clear slowly from the body [1]. Despite their recognized potential for in vivo imaging applications, those features lead to poor contrast, which strongly hampers the wide use of antibodies as tracers for target-specific molecular imaging [2,3]. The same holds true for targeted radionuclide therapy of solid tumors using immunoglobulins. Indeed, the long half-life results in irradiation of non-target organs and tissues, and slow extravasation and penetration into the tumor, limiting the radiotherapeutic effect [3,4]. Overall, the drawbacks associated to IgGs, together with the rapid progress in recombinant technologies led to the development of smaller engineered antibodies and proteins, such as single-chain variable fragments (scFvs), sdAbs and affibodies [1–5]. However, due to their lower molecular weight, these proteins present in general significantly shorter half-lives and very rapid blood clearance, which hampers therapeutic applications, e.g. requiring infusions or repeated injections to maintain a therapeutically effective dose over a prolonged period of time [6]. Aiming to address this issue, several approaches have been proposed to extend plasma half-lives of therapeutic proteins and, consequently, improve their overall
biological properties. Some of these strategies aim at increasing the hydrodynamic volume of the molecules, thus reducing renal filtration and degradation. Among the various explored approaches, the noncovalent association of a therapeutic protein to plasma proteins, namely to albumin, or fusion of ABDs, peptides or low-molecular-weight albumin binders emerged as quite promising [7–10]. Human serum albumin (HSA) is the most abundant blood plasma protein and is produced in the liver as a monomeric protein of 67 kDa. Albumin presents an extraordinary long circulation half-life of approximately 19 days in humans and 3 days in mice, as a direct result of its size and interaction with the FcRn mediated recycling pathway [8,11]. Furthermore, HSA accumulates in tumors as well as arthritic joints and is a valuable biomarker of many diseases, including cancer, rheumatoid arthritis, and ischemia, among others [11].

ABDs have also been used for improvement of the biological properties of radiolabeled affibodies for systemic radiotherapy, with remarkable reduction of renal accumulation of radioactivity [10,12,13]. More recently, Müller et al. reported a 177Lu-labeled folate conjugate containing an albumin-binding group (4-(p-iodophenyl)butyric acid derivative), which presented a significant increase of the tumor-to-kidney ratio of radioactivity compared to the radioconjugate where the albumin-binding group is absent.

We and others have been exploiting bacterial ABDs fused to antibody fragments to prolong the half-life of the resulting fusion proteins [7,8,14,15]. The study presented herein aims at assessing the biodistribution profile of an anti-TNF VHH fused to the Zag ABD (VHH-Zag) isolated from Zag protein, which is a C-related protein from the surface of Streptococcus zooepidemicus [16]. To achieve this goal, the fusion protein was conjugated to p-SCN-Bn-NOTA derivative to yield the conjugate NOTA-VHH-Zag, which was labeled with gallium-67 (67Ga). The tissue distribution of the radiolabeled fusion protein (67Ga-NOTA-VHH-Zag) was evaluated in CD-1 mice and the results were compared with those obtained with 67Ga-NOTA-VHH, prepared as control tracer.

2. Materials and methods

SEP-PAK SI cartridges were purchase from Waters. Amicon centrifugal filter (M.W. cut-off = 10 kDa) units and PD-10 columns were purchase from Millipore and GE Healthcare, respectively. Gallium-67 citrate was a gift from the nuclear medicine service of Hospital de Santa Maria (Lisbon, Portugal). Gallium-67 chloride was prepared from Gallium-67 citrate as described in the literature [17]. Diethylenetriaminpentacetic acid (DTPA) was purchased from Sigma Aldrich. Instant Thin Layer Chromatography (ITLC): Analysis was performed using ITLC-SG Varian and Whatman nº1 strips eluted with water.

Radioactivity distribution on the ITLC strips was detected by a radioactive scanner (Berthold LB 2723, Germany) equipped with 20 mm diameter Na(Tl) scintillation crystal. Radioactivity measurements were done on a dose calibrator (Alokia Curiemeter, ICG-3, Japan) or on a gamma-counter (Berthold LB 2111, Germany).

2.1. Construction of VHH and VHH-Zag fusion protein

DNA encoding the anti-TNF VHH 3E clone [18] was synthesised by Nzytech adding a Sfi restriction site at 5’ and 3’ ends, respectively, for cloning into the pComb3x plasmid. pComb3X contains a peptide leader (PL) and sequences encoding peptide tags for purification (6-His) and detection (HA). A fragment encoding the PL-VHH-HIS-HA was generated by PCR and subcloned into the expression vector pT7 (Sigma-Aldrich) into the KpnI and HindIII restriction sites. To construct the VHH-Zag fusion, a DNA fragment comprising the entire Zag albumin-binding domain of Streptococcus was generated using PCR and adding SpeI and Ncol restriction enzymes and cloned into the appropriately cut pT7-PL-VHH-HIS-HA vector. The VHH and VHH-Zag constructions were verified by sequencing.

2.2. Expression and purification of VHH and VHH-Zag

The VHH and VHH fused with the Zag (VHH-Zag) cloned in the pT7 expression vector were expressed in Escherichia coli BL21 (DE3) cells. Both VHH and VHH-Zag constructions were confirmed by sequencing. One liter of LB with 100 mg/mL ampicillin was inoculated with 10 mL of overnight culture of transformed BL21 (DE3) and grown to exponential phase (A600 = 0.6–0.8) at 37 °C. Protein expression was induced by addition of 1 mM isopropyl 1-thiol-4-β-D-galactopyranoside and bacteria were grown 16 h at 18 °C. Cells were harvested by centrifugation and resuspended in 50 mM HEPES, 1 M NaCl, 5 mM CaCl2, pH 7.5. The pellet was sonicated at 4 °C during 20 min and centrifuged at 10,000 rpm for 30 min at 4 °C. Supernatant was purified by immobilized metal affinity chromatography (IMAC), using HP HisTrap columns and the AKTA FPLC system from GE Healthcare [14,15]. After purification, the purity (>95%) of the eluted samples was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The SDS-PAGE analysis of the purified VHH and VHH-Zag samples is presented in Supporting information (Fig. 1S). ExPASy - ProtParam tool was used to estimate the extinction coefficient of VHH and VHH-Zag (εVHH = 34380 L mol⁻¹ cm⁻¹; εVHH-Zag = 38580 L mol⁻¹ cm⁻¹).

2.3. Conjugation of p-SCN-Bn-NOTA to VHH and VHH-Zag

The VHH-based antibodies were conjugated to p-SCN-Bn-NOTA as described in the literature [19]. Briefly, VHH or VHH-Zag in 0.5 M sodium carbonate buffer, pH 8.7 (1.5 mL), was added to p-SCN-Bn-NOTA (10-fold molar excess) and incubated for 2 h at room temperature. The coupling reaction was quenched by adjusting the pH to 7.0–7.4 using HCl, 1 M. The conjugate was then purified by gel filtration using a HiPrep™ 16/60 Sephacryl™ S-100 High Resolution column purchased from GE Healthcare and 50 mM HEPES, 200 mM NaCl, 5 mM CaCl2, pH 7.5, as eluent. The concentration of NOTA-VHH and NOTA-VHH-Zag was determined at 280 nm using Nanodrop ND-1000 spectrophotometer and the following corrected extinction coefficients: εVHH = 34380 L mol⁻¹ cm⁻¹; εVHH-Zag = 38580 L mol⁻¹ cm⁻¹ and molecular weights: MW (VHH) = 19.38 kDa; MW(VHH-Zag) = 25.2 kDa.

2.4. Radiolabelling

The pH of a fraction (0.5 mL) of 67GaCl3 eluted from a SEP-PAK cartridge was adjusted to 5 by adding sodium acetate up to a 0.26 M concentration. To 150 μL of this solution (20 MBq), NOTA-VHH or NOTA-VHH-Zag (14.3 – 16.4 nmol) in 50 μL of acetate buffer (0.1 M, pH = 5) was added and incubated for 5 min at room temperature. The products were purified by gel filtration on a disposable PD10 column. The final solutions were analysed by ITLC: 67Ga-NOTA-VHH, (Ry = 0) using system A and B; 67Ga-NOTA-VHH-Zag (Ry = 0) using system A and B.
2.5. ELISA assay

Human serum albumina (HSA), rat serum albumin (RSA), mouse serum albumin (MSA) (10 μg/well) and human TNFα (200 ng/well) were incubated overnight at 4 °C in 96-well plate. The remaining binding sites were blocked with 5% (w/v) soya milk/PBS1x during 1 h. The VHH and VHH-Zag recombinant antibodies conjugated with NOTA and the 67Ga-labelled antibodies (after decay – 10 half-lifes) were incubated for 1 h at room temperature. Detection was performed with HRP-conjugated anti-HA-tag antibody (Roche) using ABTS substrate. Absorbance was measured at 405 nm in an ELISA reader.

2.6. DTPA challenge

Aliquots of 67Ga-NOTA-VHH and 67Ga-NOTA-VHH-Zag were added to an aqueous solution of DTPA (900 μL, 10^{-3} M) in a 1:100 molar ratio, and incubated at 37 °C. The samples were analyzed by ITLC-SG at different time points (2 h, 4 h, 6 h and 24 h) before and after filtration through a disposable PD10 column.

2.7. Biodistribution studies

The in vivo biodistribution studies of 67Ga-NOTA-VHH and 67Ga-NOTA-VHH-Zag were performed in groups of 3 healthy female CD-1 mice (Charles River, Spain) weighing approximately 23–25 g each. All animal experiments were performed in accordance with the guidelines for animal care and ethics for animal experiments outlined in the National and European Law. Animals were intravenously injected into the tail vein with 100 μL of the radiolabeled compounds (1.5–3.5 MBq) and were maintained on normal diet ad libitum. At 1 h, 4 h, 24 h post-injection (p.i.) mice were sacrificed by cervical dislocation. The radioactivity administered to the animals was measured using a dose calibrator. The difference between the radioactivity in the injected and that in the killed animals was assumed to be due to total excretion. Tissues of interest were dissected, rinsed to remove excess blood, weighed, and their radioactivity was measured using a gamma counter. Blood and urine was also collected at the sacrifice time and analyzed by ITLC. Biodistribution results were expressed as the percentage of the injected activity per gram of tissue. Statistical analysis of the biodistribution data (t-test) was done with GraphPad Prism and the level of significance was set as 0.05.

3. Results and discussion

In the present study we have evaluated the impact of an albumin-binding domain isolated from the Zag protein, a G-related protein from the surface of Streptococcus zoöepidemicus, in the biodistribution profile of an anti-TNF VHH. Several in vivo studies have demonstrated the interest of fusing the ABD from streptococcal protein G to small recombinant antibody fragments to strongly extend their plasma half-lives [7]. Results reported by Stork and co-workers also demonstrated enhancement in the blood clearance and changes on the biodistribution pattern of a single-chain diabody (scDb) directed against carcinoembryonic antigen (CEA) and CD3 capable of retargeting T cells to CEA-expressing tumor cells [20].

The VHH and VHH fused with the Zag domain were expressed in Escherichia coli BL21 (DE3) cells, purified using IMAC, and analyzed by SDS-PAGE. In previous studies, evaluation of binding by ELISA assays indicated that the Zag fusion protein preserved its binding to human, rat and mouse albumin as well as to TNF. Moreover, the fusion VHH-Zag presented a nanomolar affinity to human, rat and mouse serum albumin (Kd = 0.42 – 40.6 nM) as confirmed by surface plasmon resonance results [14,15].
from the whole animal. Whereas 67Ga-NOTA-VHH was rapidly cleared, the decrease in blood clearance and in the radioactivity excretion rate is quite often a biological issue associated to radiolabelled antibody fragments and peptides. In contrast, 67Ga-NOTA-VHH-Zag presented an entirely different tissue distribution. There was a slow washout not only from blood but also from muscle and bone. Moreover, due to the slowest blood clearance, highly irritated organs such as liver, spleen, lung accumulated relevant sum of activity. The presence of ABD also shifted the excretion pathway from mainly renal to mainly hepatobiliary as indicated by the liver/kidney uptake ratio and the low excretion rate of 67Ga-NOTA-VHH-Zag when compared to 67Ga-NOTA-VHH. This behavior could also be partially assigned to the fact that albumin is biosynthesized in the liver, leading to a high local concentration of this protein and increased accumulation of the ABD-containing radioactive antibody fragment.

Biodistribution of 67Ga-NOTA-VHH and 67Ga-NOTA-VHH-Zag in CD1- mice (n = 3) at 1 h, 4 h and 24 h p.i.

<table>
<thead>
<tr>
<th>Organ</th>
<th>67Ga-NOTA-VHH</th>
<th>67Ga-NOTA-VHH-Zag</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% IA/g ± SD</td>
<td>% IA/g ± SD</td>
</tr>
<tr>
<td>Blood</td>
<td>0.6 ± 0.2</td>
<td>0.16 ± 0.01</td>
</tr>
<tr>
<td>Liver</td>
<td>4.0 ± 0.7</td>
<td>6.0 ± 0.6</td>
</tr>
<tr>
<td>Intestine</td>
<td>1.8 ± 0.5</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.26 ± 0.09</td>
<td>2.4 ± 0.3</td>
</tr>
<tr>
<td>Lung</td>
<td>0.8 ± 0.1</td>
<td>1.8 ± 0.3</td>
</tr>
<tr>
<td>Kidney</td>
<td>43.5 ± 3.1</td>
<td>17.2 ± 3.0</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.27 ± 0.03</td>
<td>0.25 ± 0.19</td>
</tr>
<tr>
<td>Bone</td>
<td>0.26 ± 0.03</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.17 ± 0.07</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>Total Excretion ( % I.A.)</td>
<td>71.3 ± 1.6</td>
<td>73.0 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>4 h</td>
<td>24 h</td>
</tr>
<tr>
<td></td>
<td>0.028 ± 0.004</td>
<td>97.8 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>8.3 ± 1.3</td>
<td>13.6 ± 1.4</td>
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<tr>
<td></td>
<td>17.6 ± 1.6</td>
<td>25.5 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>0.11 ± 0.01</td>
<td>20 ± 2.7</td>
</tr>
<tr>
<td></td>
<td>2.9 ± 0.6</td>
<td>1.7 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>0.041 ± 0.006</td>
<td>1.9 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>0.036 ± 0.001</td>
<td>7.6 ± 1.8</td>
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<tr>
<td></td>
<td>4.6 ± 1.1</td>
<td>6.1 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>2.0 ± 0.5</td>
<td>3.7 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>0.010 ± 0001</td>
<td>2.8 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>3.1 ± 0.4</td>
<td>0.5 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>0.013 ± 0.002</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>0.27 ± 0.01</td>
<td>7.9 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>2.1 ± 0.4</td>
<td>0.4 ± 0.2</td>
</tr>
</tbody>
</table>

Radioactivity accumulation in relevant organs and total radioactivity excretion are highlighted in bold characters and numbers.

PD10 column after filtration of the stability samples, which indicates the absence of colloidal species. Therefore, the species detected at Rf = 0 could be ascribed to 67Ga-NOTA-VHH or 67Ga-NOTA-VHH-Zag. These results were further corroborated by the low in vitro stability of the radioactive species that resulted from the direct labeling of VHH and VHH-Zag with 67Ga3+. Indeed, after incubation of those species with excess DTPA (2 h, 37 ºC) a significantly high degree of transchelation was observed with formation of a new species corresponding to 67Ga-DTPA.

Brought together, the stability assays clearly demonstrated that the radiolabeling of the antibody fragments with 67Ga3+ was achieved through NOTA complexation of the radiodiode and not through unspecific binding.

The anti-TNF-binding properties of both radiolabeled antibody fragments and the albumin-binding properties of 67Ga-NOTA-VHH-Zag was preserved, as indicated by ELISA assays after radioactive decay of the initial preparation (10 half-lifes) at room temperature (Fig. 2).

The in vivo distribution of 67Ga-NOTA-VHH and 67Ga-NOTA-VHH-Zag was evaluated in healthy CD-1 mice to assess the effect of the Zag domain on the pharmacokinetics profile of the radiolabelled VHH. The biodistribution results of both compounds expressed as percentage of injected activity per gram of organ (% IA/g ± SD) is presented in Table 1. The total radioactivity excretion is also presented as percentage of the total injected activity (% I.A.).

Analysis of the biodistribution results indicated that the introduction of the Zag domain affected dramatically the pharmacokinetic properties of radiolabelled VHH, with remarkable differences in the uptake and elimination rates from major organs as well as on the excretory route. Indeed, the most striking features are the pronounced decrease in blood clearance and in the radioactivity excretion rate from the whole animal. Whereas 67Ga-NOTA-VHH was rapidly cleared from the blood stream (0.6 ± 0.2% I.A./g, 0.16 ± 0.01% I.A./g, 0.028 ± 0.004% I.A./g at 1 h, 4 h and 24 h respectively) the Zag was evaluated in healthy CD-1 mice to assess the effect of the Zag domain on the pharmacokinetics profile of the radiolabelled VHH. The biodistribution results of both compounds expressed as percentage of injected activity per gram of organ (% IA/g ± SD) is presented in Table 1. The total radioactivity excretion is also presented as percentage of the total injected activity (% I.A.).

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The biodistribution profile of 67Ga-NOTA-VHH showed a rapid clearance of the radiotracer from most tissues since no relevant radioactivity accumulation in any major organ, except those related with the excretion paths was found. Although there was a small contribution of the hepatobiliary pathway in the elimination of the radiolabelled VHH, most of the activity was rapidly taken by the kidneys and excreted into the urine. However an important amount of activity (4.8 ± 0.4% I.A./g at 24 h p.i.) was retained in this organ which

4. Conclusion

In the present study we have determined the biodistribution profile of an anti-TNF antibody fragment in fusion with an ABD from Zag protein. We have shown that the fusion of this ABD can enhance the in vivo performance of single-domain antibodies, decreasing the blood clearance, renal retention and excretion, compared to the biodistribution profile of the unmodified antibody fragment. These findings demonstrate that the Zag fusion can be considered as a promising approach to improve the pharmacokinetics of therapeutic proteins and peptides.

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References


