



Article

Efficient ^{68}Ga Labeling of a B7-H3-Targeting Affibody Molecule via Acyclic Tris(hydroxypyridinone) Chelator: Effects on Biodistribution in a Preclinical Model

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Abstract

B7-H3 (CD276), an immune checkpoint protein, is overexpressed in malignant tumors, while its expression in normal tissues is low, and several B7-H3-targeting therapies are under clinical evaluation. Radionuclide molecular imaging offers a non-invasive method for determining B7-H3 expression levels and may aid in improved patient selection. The feasibility of the use of Affibody molecules for the visualization of B7-H3 was demonstrated earlier. The selection of an approach for routine labeling providing high radiochemical yields and reproducibility is, however, critical for successful clinical translation. The optimal combination of a targeting protein, chelator/linker, and radionuclide should provide high-contrast visualization. In this study, we evaluated an acyclic chelator, tris(3,4-hydroxypyridinone) (THP), for labeling of the Affibody molecule Z_{B7-H3_2} with ^{68}Ga and compared its impact on radiolabeling efficiency and targeting properties with the impact of the cyclic chelator 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA). Maleimide derivatives of THP and NOTA were site-specifically coupled to the C-terminal cysteine of Z_{B7-H3_2}. THP-Z_{B7-H3_2} was successfully labeled with ^{68}Ga within 5 min of incubation at room temperature, achieving a 100% radiochemical yield. NOTA-Z_{B7-H3} required heating at 60 °C for 10 min, and the radiochemical yield was lower. Both radioconjugates exhibited specific binding to B7-H3-expressing cells with similar binding strength, and both tracers demonstrated similar tumor uptake ($p > 0.05$) in a murine model. The biodistribution was similar, although [^{68}Ga]Ga-NOTA-Z_{B7-H3_2} provided slightly but significantly higher tumor-to-liver and tumor-to-spleen ratios. Nonetheless, the advantages of THP include rapid and mild radiolabeling with high efficiency, eliminating the need for heating and a post-purification step, which suggests a potential for streamlined clinical translation of Z_{B7-H3_2}.



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1. Introduction

B7 homolog 3 (B7-H3), also known as CD276, is a member of the B7 family of immune checkpoint proteins which regulate immune responses by balancing immune activation

and suppression to maintain self-tolerance. Structurally B7-H3 is a type I transmembrane glycoprotein that includes an extracellular immunoglobulin domain, a transmembrane region, and a short cytoplasmic tail. B7-H3 exists in two isoforms based on extracellular domain structure: 2IgB7-H3 contains a single pair of immunoglobulin variable (IgV) and immunoglobulin constant (IgC)-like domains (VC), whereas 4IgB7-H3 possesses two identical pairs of these domains (VC-VC). Both isoforms exist in humans, but 2IgB7-H3 predominates in mice [1]. Functionally, B7-H3 plays dual roles. From an immunological perspective, it exerts a dominant suppression effect on immune cell activity, a mechanism often exploited by cancer cells to evade immune surveillance. B7-H3 can also promote cancer progression by triggering cell proliferation, invasion, migration, metabolic modulation, and evasion of apoptosis [2].

Notably, high expression levels of B7-H3 have been observed in several cancer types, while there is limited expression of the target in normal organs and tissues, making B7-H3 a promising molecular target for precision-based treatments [3]. Importantly, overexpression of the B7-H3 protein is frequently correlated with poor prognosis, poor patient survival, and low efficacy of immune checkpoint-based treatments [4,5].

Several B7-H3-targeting agents have been developed using monoclonal antibodies (mAbs), including B7-H3-blocking mAbs, antibody-drug conjugates (ADCs), bispecific antibodies (BiAbs), chimeric antigen receptor (CAR) T cells, and radioimmunotherapeutics (RIT) [6]. Antibody-based immunotherapy has emerged as a promising approach to target B7-H3. Preclinical and clinical studies utilizing radiolabeled anti-B7-H3 antibodies for treating solid tumors have demonstrated efficient tumor targeting, high antitumor efficacy, and acceptable safety [7–10]. However, radionuclide imaging using full-length mAbs has certain limitations. Particularly, relatively slow tumor accumulation and slow clearance from the bloodstream results in optimal imaging contrast for the first several days after administration [11]. Non-immunoglobulin engineered scaffold proteins (ESPs), such as Affibody molecules, are potential alternatives to overcome limitations associated with the use of mAbs. The most important features, including the small size (6–7 kDa), high chemical and thermal stability, possibility of straightforward modification, and low-cost production, make Affibody molecules a suitable alternative to mAbs for molecular imaging [12]. Affibody molecules allow for high-resolution imaging within a few hours after administration in both preclinical [13] and clinical studies [14–18]. Importantly, Affibody molecules are safe and well-tolerated with no immunogenicity effect. Despite these advantages, data concerning Affibody-based imaging agents for the visualization of B7-H3 remain limited, highlighting the need for further investigations. Radionuclide molecular imaging provides a quantitative approach to evaluate B7-H3 expression levels *in vivo*, enabling the selection of patients with sufficiently high B7-H3 expression for targeted therapies. Moreover, the use of non-invasive nuclear imaging modalities can overcome several limitations associated with the use of biopsies [19]. Several preclinical studies on visualizing B7-H3 expression demonstrated feasibility of imaging of B7-H3-expressing tumors using Affibody molecules within 2–4 h after injection [20–24].

Positron emission tomography (PET) can provide high sensitivity, superior spatial resolution, and accurate quantification, making it a more attractive diagnostic tool compared to single-photon emission computed tomography (SPECT) [25]. Gallium-68 (^{68}Ga) is one of the positron-emitting radionuclides, with good availability from the $^{68}\text{Ge}/^{68}\text{Ga}$ generator. The short half-life ($T_{1/2} = 67.6$ min, β^+ abundance 90%, and $E_{\beta^+ \text{ max}} = 1880$ KeV) makes Gallium-68 a suitable radionuclide for same-day PET imaging using rapidly cleared imaging agents [26]. ^{68}Ga -radiopharmaceuticals based on Affibody molecules have been used for PET imaging of HER2-expressing lesions in patients with metastatic breast cancer [14,15,17,27]. The feasibility of preclinical PET imaging of B7-H3 expression with

improved imaging contrast has been reported using affinity-matured Affibody molecules labeled with ^{68}Ga [24]. A recently published clinical evaluation of a first generation B7-H3 specific Affibody molecule labeled with ^{68}Ga demonstrated utility for non-invasive visualization of B7-H3 expression in metastases of malignant tumors [18]. However, further labeling chemistry and imaging contrast optimization of the Affibody-based targeting agents are required to ensure that the best possible agent is translated into daily clinical practice.

To enable stable attachment of radiometals, e.g., ^{68}Ga , to biomolecules, bifunctional chelators are used. An optimal chelator should allow for efficient radiolabeling under mild conditions and have a minimal impact on the pharmacokinetics of a tracer. A critical requirement for the chelator is to maintain a stable complex with a radionuclide, with minimal transchelation to plasma proteins (e.g., transferrin) and ensuring reliable distribution in vivo [28]. Ga (III) can bind to transferrin in blood at two transferrin-binding sites with high thermodynamic stability ($\log K_1 = 21.43$ and $\log K_2 = 20.57$) [29]. This may result in detachment of the radiogallium label from an imaging probe and binding to transferrin. Therefore, the gallium-chelator complex in a radiopharmaceutical should be sufficiently inert to avoid transchelation in vivo. Macrocyclic chelators are commonly used for labeling peptides and proteins with ^{68}Ga [30]. Kinetic inertness of complexes of macrocyclic chelators with metal ions facilitates their use for labeling of targeting agents with radiometals. For example, the tetraaza macrocyclic chelator DOTA is commonly used in radiopharmaceutical chemistry [30,31]. The triaza-macrocyclic chelator NOTA has even more favorable features for labeling with ^{68}Ga , providing both high kinetic inertness and thermodynamic stability ($\log K_{\text{NOTA-Ga(III)}} = 31.0$ vs. $\log K_{\text{DOTA-Ga(III)}} = 21.3$) [32]. In our previous study, we evaluated labeling of the Affibody molecule $Z_{\text{B7-H3}_2}$ with ^{68}Ga using a NOTA chelator and found that it can visualize B7-H3-expressing tumors in mice with a high imaging contrast [24].

Optimization of labeling chemistry, including the selection of an appropriate and high-affinity chelator, is critical for ensuring robust radiometal coordination and achieving a clinically effective imaging probe. A possible alternative to NOTA is THP, where acyclic THP forms complexes with ^{68}Ga at room temperature and exhibits neutral pH within only a few minutes, permitting a straightforward radiolabeling process [33]. These favorable characteristics of THP enable its use for efficient ^{68}Ga -labeling of PET tracers [34], and several preclinical [35–38] and clinical [39,40] studies have shown a sufficiently stable coupling of ^{68}Ga to THP in vivo.

Although ^{68}Ga -based radiopharmaceuticals are increasingly used, the majority of commonly used chelators still require heating, long labeling times, and post-labeling purification, which complicates routine clinical implementation. The use of the THP chelator offers a distinct advantage by enabling rapid complexation of ^{68}Ga at neutral pH and at room temperature with high radiochemical yields. This efficient labeling directly addresses a key translational gap: the need for radiotracers that can be prepared reliably and reproducibly in routine clinical settings, including sites without advanced radiochemistry infrastructure. In this manuscript, we build on these advantages of THP to evaluate if THP-conjugated Affibody molecules can facilitate diagnostic procedures, thereby providing a practical route toward more accessible and scalable clinical translation.

This study compared the effects of substituting the macrocyclic chelator NOTA with the acyclic chelator THP on the biodistribution of ^{68}Ga -labeled Affibody molecules for imaging B7-H3 expression. To achieve our aim, maleimide derivatives of the THP or NOTA chelators (Figure 1) were site-specifically coupled to the Affibody molecule $Z_{\text{B7-H3}_2}$. In vitro experiments, including evaluation of specificity, affinity, and cellular processing, were performed. To evaluate the impact of this substitution on biodistribution, a head-to-head

comparison of the biodistribution of [^{68}Ga]Ga-THP-Z_{B7-H3_2} and [^{68}Ga]Ga-NOTA-Z_{B7-H3_2} Affibody molecules in the same batch of tumor-bearing mice was performed.

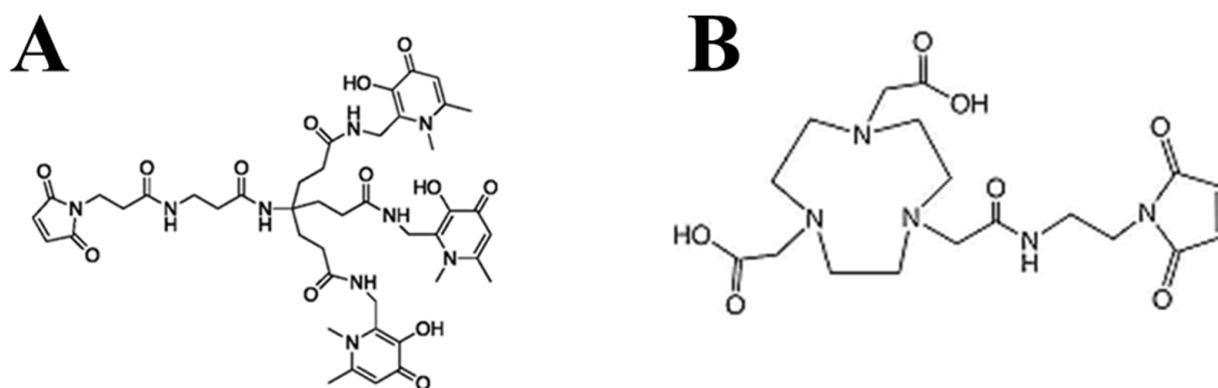


Figure 1. Structure of the maleimide derivatives of (A) THP and (B) NOTA chelators used for labeling with ^{68}Ga .

2. Results

2.1. Production, Purification, and Characterization of Anti-B7-H3 THP-Z_{B7-H3_2} and NOTA-Z_{B7-H3_2}

The His₆-tagged anti-B7-H3 Affibody molecule was expressed in *E. coli*. The clarified lysate was purified using a His GraviTrap IMAC column followed by purification by reverse-phase chromatography (RPC) and buffer exchange. TEV protease cleavage removed the N-terminal His₆-tag, and the anti-B7-H3 Affibody molecules containing a C-terminal cysteine were site-specifically conjugated with maleimide-THP and maleimide-NOTA. By using RP-UPLC-MS, the identity of each conjugated Affibody molecule was verified, and purity was determined (Table 1).

Table 1. RP-UPLC-MS analysis results for THP-Z_{B7-H3_2} and NOTA-Z_{B7-H3_2} ($n = 1$).

	Purity (%) Based on A220 Integration	Theoretical Mass (Da)	Determined Mass (Da)
THP-Z _{B7-H3_2}	97	7555.6	7556.9
NOTA-Z _{B7-H3_2}	97	7061.0	7061.6

2.2. Cell Assay

The binding of unlabeled THP-Z_{B7-H3_2} and NOTA-Z_{B7-H3_2} to B7-H3-expressing SKOV-3 cells was tested. Binding curves were plotted and are shown in Figure 2. EC₅₀ values were determined using the software GraphPad Prism (version 10.4.2) and are shown in Table 2. The data show that THP-Z_{B7-H3_2} had the same binding capacity as NOTA-Z_{B7-H3_2}.

Table 2. EC₅₀ determination from SKOV-3 cell binding.

	THP-Z _{B7-H3_2}	NOTA-Z _{B7-H3_2}
EC ₅₀ (nM)	1.5	1.5

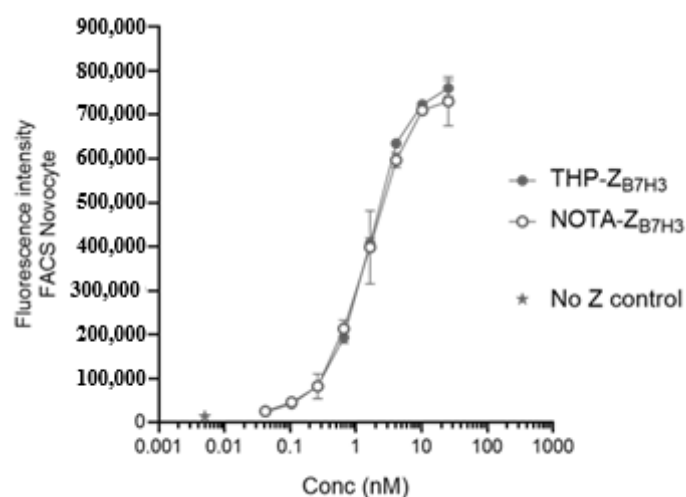


Figure 2. Binding of Affibody molecules to B7-H3-expressing SKOV-3 cells.

2.3. Radiolabeling and In Vitro Stability

Table 3 shows the results of radiolabeling of both THP- and NOTA-conjugated Affibody molecules with ^{68}Ga . According to measurements using instant thin-layer chromatography (iTLC), labeling of the THP-Z_{B7-H3_2} Affibody molecule with ^{68}Ga enabled a radiochemical yield of more than 99% already after 5 min of incubation at room temperature. Labeling of NOTA-Z_{B7-H3_2} for 10 min at 60 °C resulted in a radiochemical yield of $89.6 \pm 1.4\%$. For biodistribution, [^{68}Ga]Ga-NOTA-Z_{B7-H3_2} was purified by size-exclusion chromatography on NAP-5 columns, yielding radiochemical purities >98%. Molar activity values of [^{68}Ga]Ga-THP-Z_{B7-H3_2} and [^{68}Ga]Ga-NOTA-Z_{B7-H3_2} were 1–2 and 1.4 MBq/μg, respectively.

Table 3. Labeling of THP-Z_{B7-H3_2} and NOTA-Z_{B7-H3_2} with ^{68}Ga .

		Incubation Time, Min			Specific Activity, MBq/μg
		5	10	15	
THP-Z _{B7-H3_2} (n = 6)	Radiochemical yield, %	100 ± 0	99 ± 0	99.6 ± 0	1–2
NOTA-Z _{B7-H3_2} (n = 3)	Radiochemical yield, %	-	89.6 ± 1.4	-	1.4

The radio-HPLC chromatogram showed a retention time of 11.5 min for [^{68}Ga]Ga-THP-Z_{B7-H3_2} (Figure 3A), which matched the retention time of the non-labeled compound in the UV chromatogram (Figure 3B), confirming the efficient labeling of THP-Z_{B7-H3_2}. No free ^{68}Ga was observed in the radiochromatogram.

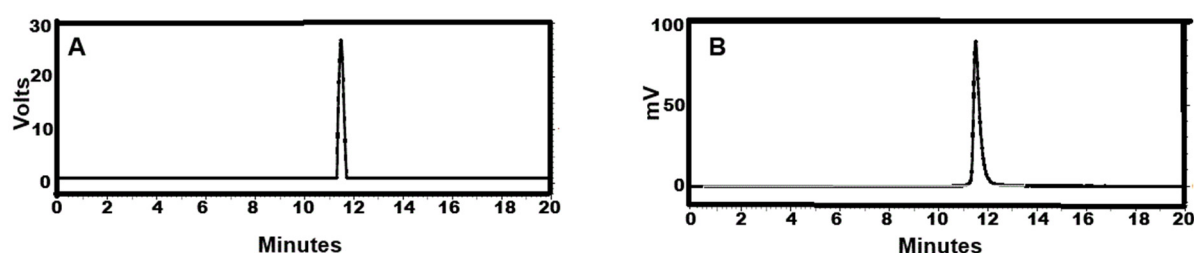


Figure 3. Reverse-phase HPLC chromatograms of (A) non-labeled-THP-Z_{B7-H3_2} and (B) [^{68}Ga]Ga-THP-Z_{B7-H3_2}. Retention times (Rt) are expressed in minutes (n = 2).

[^{68}Ga]Ga-THP-Z_{B7-H3_2} exhibited stability in murine serum, with $96.5 \pm 2.1\%$ and $91.8 \pm 1.1\%$ activity bound to the protein after 1 h and 2 h of incubation at 37 °C, respec-

tively, determined by iTLC. The results were comparable to the control samples (99.0 ± 1.4 and $95.8 \pm 1.1\%$, respectively, in PBS). Stability was further confirmed using the NAP-5 separation method, which demonstrated a protein-bound activity of $93.4 \pm 0.2\%$ and $95.9 \pm 0.2\%$ in murine serum and PBS, respectively, after 2 h of incubation (Table 4).

Table 4. In vitro stability of [^{68}Ga]Ga-THP-Z_{B7-H3_2} ($n = 3$).

Analysis Method	Protein-Bound Activity, %			
	In Murine Serum		In PBS	
	1 h	2 h	1 h	2 h
iTLC	96.5 ± 2.1	91.8 ± 1.1	99 ± 1.4	95.8 ± 0.4
NAP-5 separation	-	93.4 ± 0.2	-	95.9 ± 0.2

In-111 labeling of the DOTA-conjugated Affibody molecule was successfully achieved, yielding a radiochemical yield of 94%. This labeled compound was used for a half-maximal inhibitory concentration (IC₅₀) assay to determine the binding strength of THP-Z_{B7-H3_2} and NOTA-Z_{B7-H3_2}.

2.4. In Vitro Studies

In vitro binding specificity was assessed using a saturation assay. The data are presented as count per minute (CPM) per cell number (CPM/ 10^6) (Figure 4). There was a significant decrease ($p < 0.0001$) in binding of both [^{68}Ga]Ga-THP-Z_{B7-H3_2} and [^{68}Ga]Ga-NOTA-Z_{B7-H3_2} to SKOV-3 and BT-474 cell lines when cells were pre-saturated with an excess of the unlabeled Z_{B7-H3_2} compared with cells that were not treated with the unlabeled Z_{B7-H3_2}. There was no significant difference ($p > 0.05$) in the cell-bound activity of [^{68}Ga]Ga-THP-Z_{B7-H3_2} and [^{68}Ga]Ga-NOTA-Z_{B7-H3_2} in both cell lines.

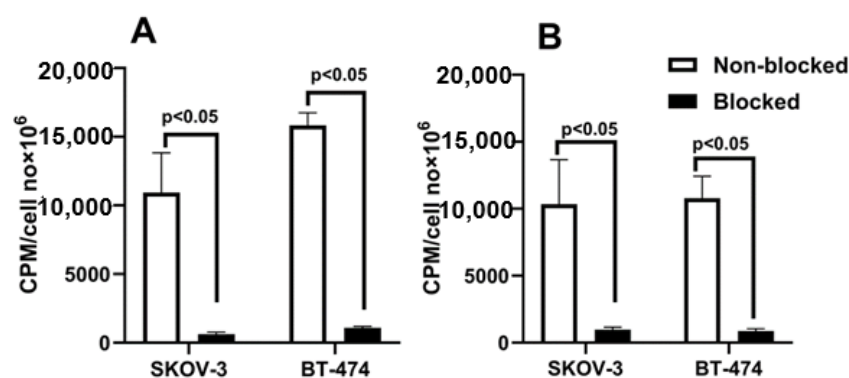


Figure 4. In vitro binding specificity of (A) [^{68}Ga]Ga-THP-Z_{B7-H3_2} and (B) [^{68}Ga]Ga-NOTA-Z_{B7-H3_2} in B7-H3-expressing SKOV-3 and BT-474 cell lines, respectively. The cells were incubated with 10 nM of the radiolabeled conjugates, while in the blocked groups, a 200-fold molar excess of unlabeled conjugates was added to block B7-H3. The data are presented as the mean count per minute value from three samples ($n = 3$)/cell number $\pm$ standard deviation.

To compare the relative binding strength of THP-Z_{B7-H3_2} and NOTA-Z_{B7-H3_2} Affibody molecules, they were loaded with natural gallium. IC₅₀ in SKOV-3 cells was measured using [^{111}In]In-SYNT179-DOTA as the competing ligand. Both compounds displaced [^{111}In]In-SYNT179-DOTA from B7-H3 in a concentration-dependent manner. The measured relative binding affinities were similar, with no statistically significant differences, as indicated by IC₅₀ values of 2.6 ± 0.4 nM and 3.2 ± 0.5 nM for THP-Z_{B7-H3_2} and NOTA-Z_{B7-H3_2}, respectively (Figure 5).

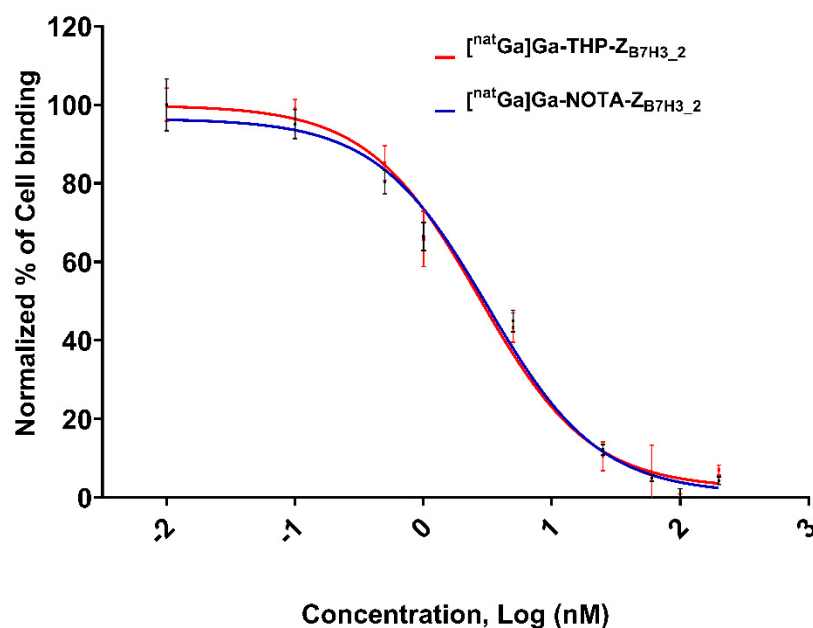


Figure 5. Inhibition of [^{111}In]In-SYNT179-DOTA binding to SKOV-3 cells by [$^{\text{nat}}\text{Ga}$]Ga-THP-Z_{B7-H3_2} and 3. [$^{\text{nat}}\text{Ga}$]Ga-Z_{B7-H3_2} Affibody molecules. Data are presented as mean values normalized to the highest value ($n = 3$) $\pm$ standard deviation.

The data concerning cellular processing of [^{68}Ga]Ga-THP-Z_{B7-H3_2} are shown in Figure 6. A similar binding pattern was observed in both cell lines. The total cell-associated bound activity for [^{68}Ga]Ga-THP-Z_{B7-H3_2} was 4.39 ± 0.18 and 6.30 ± 0.38 on SKOV-3 and BT-474. The internalization rate of [^{68}Ga]Ga-THP-Z_{B7-H3_2} was slow, with the internalized fraction accounting for $0.74 \pm 0.02\%$ and $0.70 \pm 0.03\%$ in SKOV-3 and BT-474, respectively, after 3 h of incubation. The total cell-associated bound activity for [^{68}Ga]Ga-NOTA-Z_{B7-H3_2} was 3.80 ± 0.11 and 4.6 ± 0.14 on SKOV-3 and BT-474, respectively. The internalized fraction was similar on both cell lines (0.77 ± 0.11 and $0.75 \pm 0.11\%$ of the cell-associated activity in SKOV-3 and BT-474, respectively) [24].

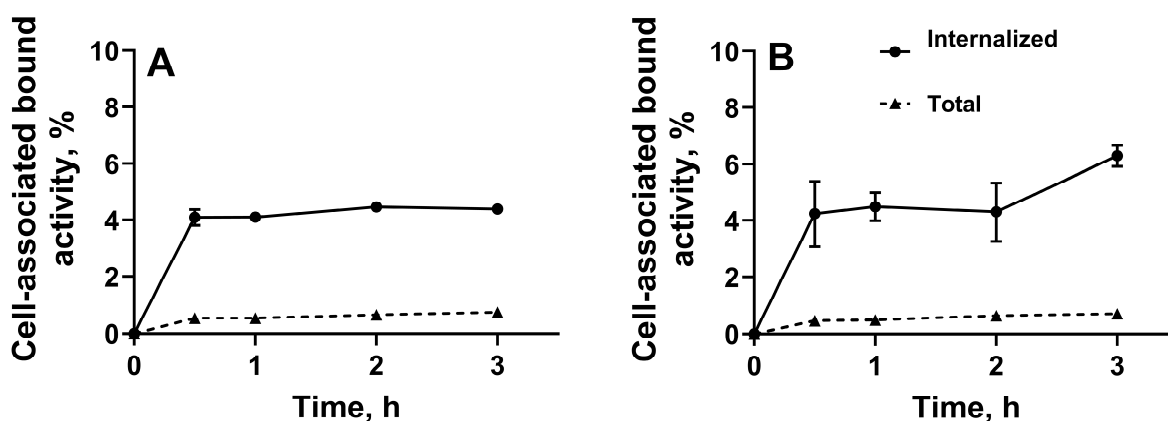


Figure 6. Cellular processing of [^{68}Ga]Ga-THP-Z_{B7-H3_2} in B7-H3-expressing (A) SKOV-3 and (B) BT-474 cells in vitro at 37 °C. The data are presented as the mean from three samples ($n = 3$) $\pm$ standard deviation (SD).

2.5. In Vivo Studies

In vivo specificity of B7-H3 targeting by [^{68}Ga]Ga-THP-Z_{B7-H3_2} was assessed by a comparison of its uptake in B7-H3-positive SKOV-3 and B7-H3-negative Ramos xenografts in mice (Figure 7). The uptake was much higher in SKOV-3 xenografts ($p < 0.05$) than in

B7-H3-negative Ramos xenografts. The data from the biodistribution measurement were in good agreement with the results of microPET imaging (Figure 7B).

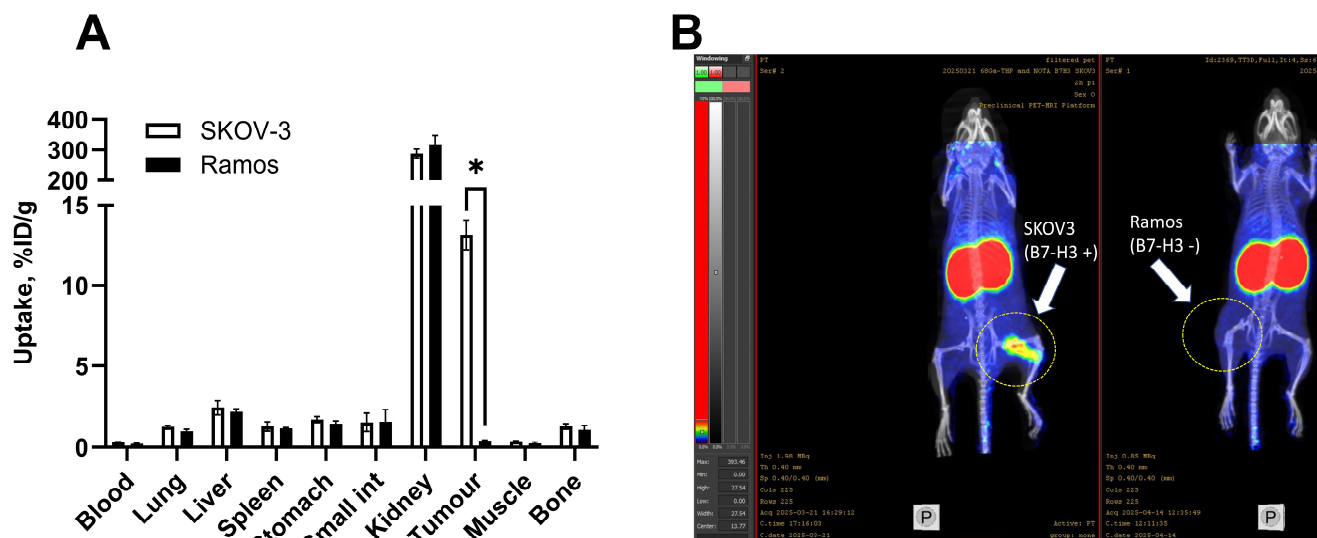


Figure 7. (A) Biodistribution of $[^{68}\text{Ga}]\text{Ga-THP-Z}_{\text{B7-H3}_2}$ in SKOV-3 (B7-H3-positive) and Ramos (B7-H3-negative) xenografts ($n = 4$) and (B) in vivo PET imaging of $[^{68}\text{Ga}]\text{Ga-THP-Z}_{\text{B7-H3}_2}$ 2 h post-injection ($n = 1$). An unpaired t -test was used to determine statistical significance. Asterisks (*) indicate statistically significant differences with $p < 0.05$.

The results of biodistribution measurements of $[^{68}\text{Ga}]\text{Ga-THP-Z}_{\text{B7-H3}_2}$ and $[^{68}\text{Ga}]\text{Ga-NOTA-Z}_{\text{B7-H3}_2}$ in SKOV-3 xenografts 2 h after injection were mostly similar. There was no significant difference in the uptake of both tracers in B7-H3-positive tumors. For the lung, liver, spleen, and bone, significantly ($p < 0.05$) lower uptake was observed for $[^{68}\text{Ga}]\text{Ga-NOTA-Z}_{\text{B7-H3}_2}$ (Figure 8A). $[^{68}\text{Ga}]\text{Ga-NOTA-Z}_{\text{B7-H3}_2}$ provided slightly but significantly ($p < 0.05$) higher tumor-to-liver and tumor-to-spleen ratios. The corresponding biodistribution and tumor-to-organ ratio data for both compounds are presented in Figure 8B. The imaging results are in agreement with the biodistribution data (Figure 9).

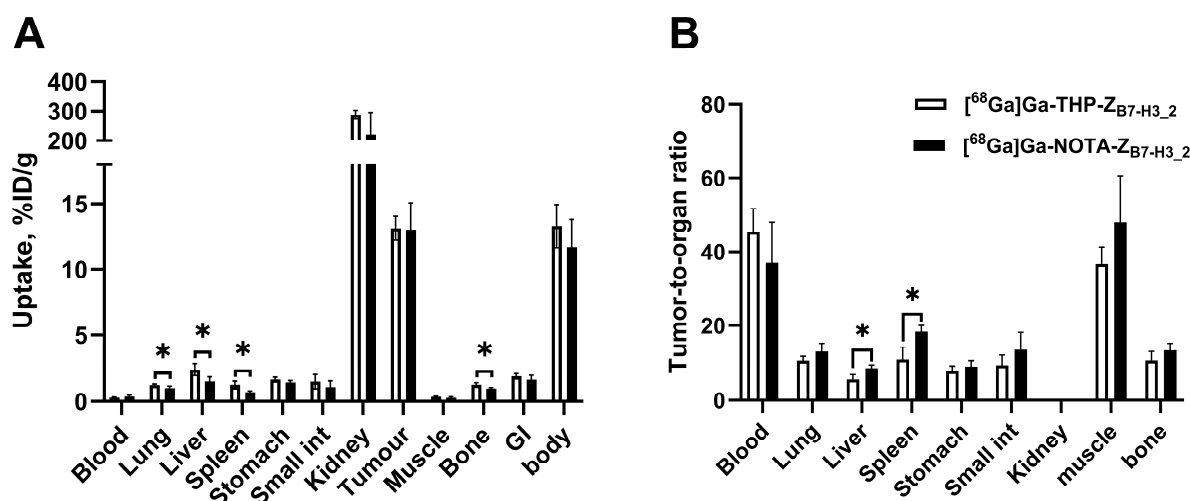


Figure 8. (A) Biodistribution and (B) tumor-to-organ ratios of $[^{68}\text{Ga}]\text{Ga-THP-Z}_{\text{B7-H3}_2}$ and $[^{68}\text{Ga}]\text{Ga-NOTA-Z}_{\text{B7-H3}_2}$ in SKOV-3 2 h after injection. Data are presented as $\%ID/g \pm SD$ ($n = 4$). Data for the GI tract and the rest of the body are presented as $\%ID$ per whole sample. An unpaired t -test was performed to determine statistical significance. Asterisks (*) indicate statistically significant differences with $p < 0.05$.

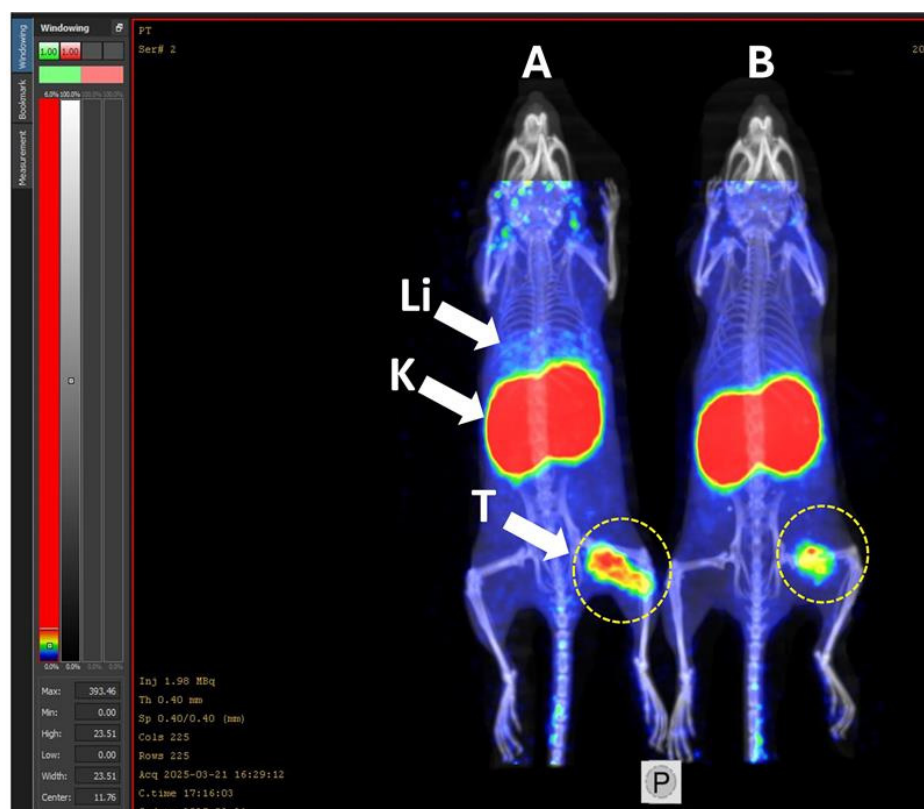


Figure 9. microPET/CT imaging of (A) $[^{68}\text{Ga}]\text{Ga-THP-Z}_{\text{B7-H3}_2}$ and (B) $[^{68}\text{Ga}]\text{Ga-NOTA-Z}_{\text{B7-H3}_2}$ in SKOV-3 xenografts 2 h after injection ($n = 1$ for each radioconjugate).

3. Discussion

Affibody molecules as non-immunoglobulin scaffold proteins have high potential for molecular imaging of different molecular targets [41,42]. They are amenable to manufacturing under GMP, and importantly, their safety and efficacy have been demonstrated in several clinical imaging studies [16,43]. The role of ^{68}Ga in driving the global expansion of clinical research and routine PET imaging has been considerable due to its several advantages [44]. The short-lived positron-emitting radionuclide ^{68}Ga , combined with the rapid kinetics of the small-sized Affibody molecules, enables harnessing the high sensitivity and resolution of radionuclide molecular PET imaging. The feasibility of preclinical imaging of B7-H3-expressing tumors using Affibody molecules labeled with different radionuclides, e.g., $^{99\text{m}}\text{Tc}$, ^{111}In , and ^{68}Ga , was successfully demonstrated earlier [20–24]. Radiometal coupling is typically achieved by attaching a chelator to the targeting agent. The choice of radionuclide (e.g., radiohalogen vs. radiometal), detection modality (PET or SPECT), and labeling chemistry can substantially influence the imaging property of a tracer. The labeling methods may alter the pharmacokinetics of a tracer, including binding affinity, cellular processing, and retention, as well as the biodistribution and clearance pathways of both the non-bound tracer and its radiocatabolites. This consequently influences imaging contrast, sensitivity, and optimal imaging time points [41,45].

A convenient, rapid, and reproducible radiopharmaceutical labeling method is crucial in clinical nuclear medicine, as streamlined preparation reduces operational complexity and minimizes the risk of handling errors. These features ensure the timely availability of high-quality PET tracers, ultimately improving workflow and supporting more reliable patient imaging outcomes. The THP chelator has emerged as one of the most efficient ligand systems for complexation with ^{68}Ga under mild conditions, making it highly attractive for clinical PET radiopharmaceutical development [34]. This approach enables labeling under

milder conditions of diverse biomolecules, including nanobodies and antibody fragments, resulting in radiotracers with high radiochemical yields and excellent stability. The clinical utility of THP-based radiotracers has been shown by promising results in predicting pathological responses to combination therapies and influencing clinical management [39,40]. To evaluate a potential clinical PET tracer for B7-H3 visualization, the maleimide derivative of the acyclic THP chelator was efficiently site-specifically coupled to the Z_{B7-H3_2} Affibody molecule for labeling with ⁶⁸Ga. ⁶⁸Ga labeling of the THP-conjugated Affibody molecule was straightforward, rapid, and highly efficient under mild conditions (e.g., at room temperature), and complete radiolabeling of THP-Z_{B7-H3_2} was achieved within 5 min (Table 3). Due to the high radiolabeling efficiency, [⁶⁸Ga]Ga-THP-Z_{B7-H3_2} was used without further purification. Importantly, sodium acetate is permitted, after appropriate dilution, for intravenous injection in humans both by the FDA and the European Medicines Agency. In contrast, radiolabeling of Z_{B7-H3_2} containing a macrocyclic NOTA chelator (NOTA-Z_{B7-H3_2}) required heating the labeling mixture and a longer reaction time. Still, the radiochemical yield was lower even after 10 min of incubation as compared with the yield of [⁶⁸Ga]Ga-THP-Z_{B7-H3_2}. Due to the lower radiochemical yield (<95%), purification was necessary for both in vivo studies and future clinical applications. [⁶⁸Ga]Ga-THP-Z_{B7-H3_2} demonstrated high stability, with a less than 10% release of free ⁶⁸Ga from the radioconjugate after up to 2 h of incubation in murine serum (Table 4). The rigid, pre-organized macrocyclic framework of NOTA facilitates the formation of a thermodynamically stable and kinetically inert complex with Ga³⁺. However, this structural rigidity can slow the metal coordination process, often necessitating elevated temperatures or prolonged incubation to achieve efficient complexation with macrocyclic chelators. These conditions can be unsuitable for heat-sensitive molecules, as they may be degraded under such conditions. On the other hand, the acyclic chelator THP has a flexible but stable structure, enabling relatively faster binding under milder conditions.

The findings from the in vitro binding specificity study demonstrated that [⁶⁸Ga]Ga-THP-Z_{B7-H3_2} specifically bound to B7-H3 (Figure 4). An estimation of binding strength from the specificity assay revealed comparable signal intensities for [⁶⁸Ga]Ga-THP- and [⁶⁸Ga]Ga-NOTA-Z_{B7-H3_2} in both SKOV-3 and BT-474, as illustrated in Figure 4. These results are consistent with the results of the IC₅₀ measurements. The similar IC₅₀ values confirmed that substitution of the macrocyclic chelator NOTA with the acyclic chelator THP does not impact the binding strength of the Z_{B7-H3_2} Affibody molecule to the living cells (Figure 5). The combined EC₅₀ and IC₅₀ data, in vitro processing, and in vivo uptake strongly support the conclusion that THP does not significantly alter the interaction with B7-H3. Several studies have demonstrated that minor modifications on molecular design, such as variations in the structure of the metal–chelator complex, the surface exposure of functional groups, and the local charge distribution, can significantly influence the biodistribution and targeting characteristics, e.g., the off-target interactions of small targeting agents used for imaging of different molecular targets [45]. Cellular processing (Figure 6) showed slow internalization, which is a typical pattern for Affibody-based probes, including [⁶⁸Ga]Ga-NOTA-Z_{B7-H3_2} [24]. The impact of the chelator character (acyclic THP vs. macrocyclic NOTA chelator) on biodistribution and targeting properties of B7-H3-expressing tumors was tested in vivo. Biodistribution data in tumor-bearing mice 2 h after injection (Figure 8A) demonstrated that the biodistribution of [⁶⁸Ga]Ga-THP- and -NOTA-Z_{B7-H3_2} was comparable across most organs. The tumor uptake was similar for both radioconjugates, resulting in a similarly high signal from the tumor. Several non-target organs showed a tendency toward higher uptake for [⁶⁸Ga]Ga-THP-Z_{B7-H3_2}. [⁶⁸Ga]Ga-NOTA-Z_{B7-H3_2} provided slightly but significantly higher tumor-to-liver and tumor-to-spleen ratios, resulting in better imaging contrast in the spleen and liver—an

important consideration for the detection of metastases (Figure 8B). PET/CT imaging confirmed the ex vivo results (Figure 9). In the present study, ex vivo biodistribution measurements provide a direct quantitative readout of tracer uptake in B7-H3-expressing tumors, which indirectly reflects the available receptor density under our experimental conditions. The SKOV-3 xenograft model used here has been characterized in previous studies, where B7-H3 expression levels were quantified [20]. Our ex vivo data show that [^{68}Ga]Ga-THP- $\text{Z}_{\text{B7-H3}_2}$ achieves tumor uptake comparable to the NOTA-based analogue despite differences in chelation chemistry, indicating that the THP-based tracer robustly reports target expression in a model with well-defined B7-H3 abundance. Although we did not perform new receptor-density quantification in this study, the combination of (i) established B7-H3 expression levels in SKOV-3 tumors, (ii) in vivo specificity confirmed by comparison with B7-H3-negative Ramos xenografts, and (iii) consistent ex vivo uptake patterns supports the conclusion that THP-based labeling preserves the ability of the Affibody molecule to reflect B7-H3 expression in vivo. Nevertheless, the THP chelator offers a practical advantage over NOTA by enabling rapid radiolabeling with high efficiency at room temperature, thereby simplifying the labeling process in a clinical radiopharmaceutical routine lab. A significant difference in tumor uptake was observed at 2 h post-injection in SKOV-3 and Ramos xenografts, demonstrating specific binding of [^{68}Ga]Ga-THP- $\text{Z}_{\text{B7-H3}_2}$ in vivo (Figure 7A). This finding was further confirmed by PET imaging (Figure 7B). The use of THP does not introduce additional safety concerns under the conditions applied in this study. THP has been used in several preclinical [33,35–38] and clinical [39,40] ^{68}Ga radiopharmaceuticals and has shown excellent in vivo stability and no adverse clinical effects at tracer-level doses. More than 100 patients have received THP-conjugated tracers in clinics, and no adverse clinical effects have been reported [39,40]. Moreover, the radiolabeling procedure itself is performed at room temperature and neutral pH, conditions that do not alter the structure or integrity of the Affibody molecule. Consistent with this, no signs of toxicity or abnormal behavior were observed in any of the animals during biodistribution or imaging studies. Formal toxicology studies will provide more precise evidence regarding this point. However, the available evidence supports the claim that THP conjugation and the associated labeling conditions do not pose additional risk in the experimental setting used here.

4. Materials and Methods

4.1. General

The majority of chemicals used in this study were of reagent grade and metal-free, except for those required for HPLC analysis, which were of HPLC grade and obtained from Merck, Sweden AB. Labeling buffers were prepared using high-purity Milli-Q water, which was further treated using the Chelex 100 resin (Bio-Rad Laboratories, Hercules, CA, USA) to eliminate metal contaminants. The radionuclide ^{68}Ga , used for labeling in the form of [^{68}Ga]GaCl₃, was acquired through elution from an IGG100 $^{68}\text{Ge}/^{68}\text{Ga}$ generator (Eckert & Ziegler, Berlin, Germany), using 0.1 M of metal-free HCl as the eluent. Additionally, non-carrier-added [^{111}In]InCl₃ (Curium Pharma, Hertogenbosch, The Netherlands) was used for labeling Affibody molecules containing the DOTA chelator in the IC₅₀ assay. Radioactivity measurements were conducted using a dose calibrator (IBC Dose Calibrator Comcer, Castel Bolognese (RA), Italy). The activity from cell and animal samples was measured using an automated gamma spectrometer equipped with a 3-inch NaI (TI) well detector (2480 Wizard, Wallac, Turku, Finland). For in vivo studies, radiolabeled compounds were purified using NAP-5 size-exclusion columns (Cytiva, Uppsala, Sweden) equilibrated with phosphate-buffered saline (PBS) (Biowest). Radiochemical yield and purity were assessed through iTLC on silica gel strips (Varian, Lake Forest, CA, USA)

with two mobile phases: 0.2 M of citric acid and pH 2, and 0.1 M of sodium citrate and pH 5–5.5 for ^{68}Ga -NOTA and -THP Affibody molecules, respectively. The acquired data were analyzed using the CR35 BIO Plus Storage Phosphor System with the AIDA image analysis software (ElysiaRaytest, Bietigheim-Bissingen, Germany) and the Cyclone Storage Phosphor system (Perkin-Elmer, Wellesley, MA, USA). To cross-validate radio-ITLC data, reverse-phase HPLC was conducted using an EliteLaChrom system (Hitachi, VWR, Darmstadt, Germany) consisting of an L-2130 pump, a UV detector (L-2400), and a radiation flow detector (Bioscan, Washington, DC, USA) coupled in series. A radio-HPLC analysis of the ^{68}Ga -labeled compound was performed using a Vydac RP C18 column (Avantor, Radnor Township, PA, USA, 300 Å; 3×150 mm; 5 µm). HPLC conditions were as follows: A = 10 mM of TFA/H₂O; B = 10 mM of TFA/acetonitrile; UV detection at 280 nm; gradient elution for 0–15 min at 5% to 70% B, 15–18 min at 70% to 95% B, and 19–20 min at 5% B; and a flow rate of 1.0 mL/min.

In vitro cell studies were performed using the B7-H3-expressing ovarian cancer SKOV-3 and breast cancer BT-474 cell lines, obtained from the American Type Culture Collection (ATCC). Ramos lymphoma cells (ATCCs) were used to establish B7-H3-negative xenografts. Cells were cultured in an RPMI medium (Flow Laboratories, Irvine, UK) supplemented with 10% of fetal bovine serum (20% of fetal bovine serum for BT-474), 2 mM of L-glutamine, 100 IU/mL of penicillin, and 100 mg/mL of streptomycin.

Statistical analysis was performed using the GraphPad Prism software version 10.4.2 for Windows (GraphPad Software, San Diego, CA, USA). A two-tailed unpaired *t*-test was used for comparison of the two sets of data. Differences were considered significant when the *p*-value was less than 0.05.

4.2. Production, Purification, and Characterization of Anti-B7-H3 THP-Z_{B7-H3_2} and NOTA-Z_{B7-H3_2}

The production of the unconjugated anti-B7-H3 Affibody molecule and the maleimide–NOTA conjugation to get NOTA-Z_{B7-H3_2} was performed as described earlier [24]. For the production of THP-Z_{B7-H3_2}, the unconjugated anti-B7-H3 Affibody molecules were conjugated with maleimide–THP (CheMatech, cat. no C119) at 3-fold molar excess in 0.2 M of sodium acetate, 0.5 mM of EDTA, and pH 6.5. The conjugation mix was incubated at 30 °C and 450 rpm for 60 min. After the incubation, 3-fold molar excess of L-cysteine (Sigma, cat. no C7352-25g) was added over maleimide–THP to deactivate the remaining excess of maleimide–THP. After the conjugation, the conjugation mix was buffer exchanged to 0.2 M of sodium acetate and pH 6.0. Identity of the produced NOTA-Z_{B7-H3_2} and THP-Z_{B7-H3_2} was confirmed and their purity was determined by RP-UPLC analysis using an Agilent 1290 Infinity UHPLC system equipped with DAD, single quadrupole MSD and a Zorbax 300SB-C8 Rapid Resolution HD column (Agilent Technologies, Santa Clara, CA, USA, P. N. 858750-906).

4.3. Cell Assay

The two Affibody molecules THP-Z_{B7-H3_2} and NOTA-Z_{B7-H3_2} were tested in terms of binding to B7-H3-expressing SKOV-3 cells. The cells were placed in a V-bottom 96-well plate (0.1×10^6 cells/well) and incubated at 4 °C for 1 h with Affibody molecules at decreasing concentrations from 26 nM to 40 pM. After $1 \times$ washing in PBS with 1% fetal bovine serum (FBS), binding of Affibody molecules was identified by an anti-Affibody polyclonal antibody (6 µg/mL) and incubated at 4 °C for 1 h, followed by an Alexa488-conjugated goat anti-rabbit IgG diluted at 1:2000 and incubated at 4 °C for 45 min. After $2 \times$ washing in PBS with 1% FBS, fluorescence intensity was measured by a flow cytometer (Novocyte). Each sample was tested in duplicate. Binding curves were plotted and EC50 values were determined using the software GraphPad Prism (version 10.4.2).

4.4. Radiolabeling and In Vitro Stability

For labeling with ^{68}Ga , THP-Z_{B7-H3_2} (40 μg) was mixed with 2 M of a sodium acetate buffer (100–120 μL and pH 6.0–6.5). A generator eluate (100–120 μL and 40–80 MBq) was added. The reaction mixture was thoroughly vortexed and incubated at room temperature for 5, 10 and 15 min. Upon completion of the incubation, the radiochemical yield of [^{68}Ga]Ga-THP-Z_{B7-H3_2} was determined using iTLC, with 0.1 M of sodium citrate (pH 5–5.5) as the mobile phase, and cross-validated by reverse-phase HPLC, as described earlier [20]. Labeling of the NOTA-conjugated anti-B7-H3 Affibody molecule with ^{68}Ga and the DOTA-conjugated Affibody molecule with ^{111}In was performed as described earlier [23,24].

The stability of [^{68}Ga]Ga-THP-Z_{B7-H3_2} was assessed by incubating a fraction of a fresh radioconjugate (2 μg) with murine serum or PBS (100 μL) as a control at 37 °C to mimic the concentration of the tracer in murine blood immediately after injection. At 1 and 2 h post-incubation, 1 μL of aliquots were taken to evaluate stability using iTLC, with 0.1 M of sodium citrate (pH 5–5.5) as the mobile phase. To further confirm its stability after 2 h, samples were passed through an NAP-5 column to separate high-molecular-weight compounds (over 5 kDa) and low-molecular-weight compounds (less than 5 kDa). Activities in both high- and low-molecular-weight fractions were measured to calculate the percentage of ^{68}Ga associated with the high-molecular-weight fraction (i.e., bound to Affibody molecules). The test was run in triplicate.

4.5. In Vitro Studies

B7-H3 expression levels were estimated to be 68,000, 45,000, and 250 receptors per cell for SKOV-3, BT-474, and Ramos, respectively [20]. Ovarian SKOV-3 and breast BT-474 cancer cell lines were used for cell studies representing B7-H3-expressing cell lines. The Ramos lymphoma cell line was used as a B7-H3-negative control. Cells were seeded in cell-culture dishes (35 mm in diameter) with a density of 10^6 cells/dish for the in vitro study. A set of three dishes was used for in vitro studies.

The binding specificity of ^{68}Ga -labeled Affibody molecules on B7-H3-expressing cells was tested using a saturation experiment, as described earlier [20].

For the IC₅₀ assay, SKOV-3 cells (5×10^5 per well) were seeded into 12-well plates one day before the experiment. On the day of the experiment, 250 μL of complete media were added to each well, followed by 250 μL of [^{nat}Ga]Ga-THP or NOTA-Z_{B7-H3_2} at increasing concentrations (final concentrations: 0.01, 0.1, 0.5, 1, 5, 25, 60, 100, and 200 nM) in the designated wells. For the control group, 250 μL of complete media were added. A total of 500 μL of [^{111}In]In-SYNT179-DOTA (final concentration 10 nM) were added into the respective wells. The wells were then incubated at 4 °C for 4 h. Following incubation, the supernatants were collected, and the wells were washed with PBS, with the wash fractions also transferred into the same vials. To detach the cells, a 0.25% trypsin-EDTA solution (500 μL) was added to each well, followed by incubation at 37 °C. The detached cells were then resuspended in 500 μL of the media, and the suspension was transferred into the corresponding vials. The radioactivity of the collected samples was measured using an automated gamma spectrometer, and the data were analyzed using GraphPad Prism 10.4.2. A nonlinear regression model was applied to generate the dose–response curve and determine the IC₅₀ value of THP- and NOTA-conjugated Affibody molecules.

The cellular processing of THP-Z_{B7-H3_2} in B7-H3-expressing cells during continuous incubation was studied using an acid-wash method [46]. The radiolabeled Affibody molecule (10 nM) was added to the cells and incubated at 37 °C in a humidified incubator for 1, 2, and 3 h.

4.6. In Vivo Studies

Animal studies were carried out in compliance with the national legislation on laboratory animal protection. Approval of the study was granted by the Ethical Committee for Animal Research in Uppsala (permit 5.8.18-00473/2021, approved 26 February 2021).

Biodistribution of ^{68}Ga -labeled Affibody conjugates was measured in BALB/C nu/nu mice bearing B7-H3-positive SKOV-3 xenografts. To establish xenografts, SKOV-3 cells (10^7 cells/mouse) were subcutaneously injected on the right hind leg of female BALB/c nu/nu mice in two groups (4 mice per group). For in vivo specificity, B7-H3-negative Ramos cells (6×10^6 cells/mouse) were subcutaneously implanted on the left hind leg of female BALB/c nu/nu mice (one group of 4 mice). Four mice per group were used in the biodistribution. The biodistribution was measured three weeks after cell implantation. The average animal weight was 18.4 ± 1.3 g. The average tumor weight was 0.2 ± 0.1 g and 0.3 ± 0.1 g for SKOV-3 and Ramos xenografts, respectively. The biodistribution was measured 2 h after injection. Two groups of mice (4 mice for each radiocojugate) were injected with [^{68}Ga]Ga-THP- $\text{Z}_{\text{B7-H3}_2}$ and [^{68}Ga]Ga-NOTA- $\text{Z}_{\text{B7-H3}_2}$ (0.28 nmol, 400 kBq, in 100 μL of PBS) separately, into the tail vein. To test B7-H3-mediated accumulation in vivo, one group of animals (4 mice) bearing B7-H3-negative Ramos xenografts was injected with the same peptide mass and activity of [^{68}Ga]Ga-THP- $\text{Z}_{\text{B7-H3}_2}$, and the biodistribution was measured 2 h after injection. Mice were euthanized by overdosing on an anesthetic solution (20 μL of solution per gram of body weight: ketamine, 10 mg/mL; Xylazine, 1 mg/mL), followed by a heart puncture. Blood, organs, and tissues were collected and weighed. The organ radioactivity was measured using a gamma counter along with three standards and syringes for each animal. Uptake values for organs were calculated as the percentage of the injected dose per gram tissue (%ID/g). A head-to-head biodistribution comparison of [^{68}Ga]Ga-THP- $\text{Z}_{\text{B7-H3}_2}$ Z_{B7H3} with [^{68}Ga]Ga-NOTA- Z_{B7H3} was measured in the same batch of mice.

To confirm biodistribution results, small-animal PET/CT imaging was performed. One SKOV-3-bearing mouse was intravenously injected with ^{68}Ga -labeled Affibody molecules (0.28 nmol and 2.5 MBq). To confirm in vivo specificity, one mouse bearing a Ramos xenograft was intravenously injected with the same peptide and activity dose. The mice were euthanized by CO_2 asphyxiation immediately before being placed in the camera. The mice were imaged at 2 h after injection using a PET/CT scanner (Mediso Medical Imaging Systems, Budapest, Hungary). CT acquisition was performed using nanoScan PET/CT (Mediso Medical Imaging Systems Ltd., Hungary) immediately after PET acquisition using the same bed position. The PET scans were performed for 30 min, followed by CT examination at the following parameters: a CT-energy peak of 50 keV, 670 A, 480 projections, and a 2.29 min acquisition time. The PET data were reconstructed into a static image using the Tera-TomoTM 3D reconstruction engine. CT raw files were reconstructed in real time using Filter Back Projection in the Nucline 2.03 Software (Mediso Medical Imaging Systems, Hungary). PET and CT files were fused and analyzed using the Nucline 2.03 Software (Mediso Medical Imaging Systems, Hungary) and are presented as maximum intensity projections (MIPs) in the RGB color scale.

5. Conclusions

An Affibody molecule containing the acyclic chelator THP was successfully labeled with ^{68}Ga . Substitution of the macrocyclic chelator NOTA with the acyclic chelator THP did not affect the specificity and binding strength of the Affibody molecule labeled with ^{68}Ga . The utilization of the THP chelator offers a distinct advantage by enabling rapid quantitative radiolabeling. The preliminary data indicate that the THP-conjugated Affibody molecule could be a promising candidate for future clinical translation.

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References

- Kontos, F.; Michelakos, T.; Kurokawa, T.; Sadagopan, A.; Schwab, J.H.; Ferrone, C.R.; Ferrone, S. B7-H3: An Attractive Target for Antibody-based Immunotherapy. *Clin. Cancer Res.* **2021**, *27*, 1227–1235. [\[CrossRef\]](#)
- Mielcarska, S.; Kot, A.; Dawidowicz, M.; Kula, A.; Sobków, P.; Kłaczka, D.; Waniczek, D.; Świętochowska, E. B7-H3 in Cancer Immunotherapy-Prospects and Challenges: A Review of the Literature. *Cells* **2025**, *14*, 1209. [\[CrossRef\]](#)
- Zhou, W.T.; Jin, W.L. B7-H3/CD276: An Emerging Cancer Immunotherapy. *Front. Immunol.* **2021**, *12*, 701006. [\[CrossRef\]](#)
- Picarda, E.; Ohaegbulam, K.C.; Zang, X. Molecular Pathways: Targeting B7-H3 (CD276) for Human Cancer Immunotherapy. *Clin. Cancer Res.* **2016**, *22*, 3425–3431. [\[CrossRef\]](#)
- Getu, A.A.; Tigabu, A.; Zhou, M.; Lu, J.; Fodstad, Ø.; Tan, M. New frontiers in immune checkpoint B7-H3 (CD276) research and drug development. *Mol. Cancer* **2023**, *22*, 43. [\[CrossRef\]](#) [\[PubMed\]](#)
- Koumrentziotis, I.-A.; Theocharopoulos, C.; Foteinou, D.; Angeli, E.; Anastasopoulou, A.; Gogas, H.; Ziogas, D.C. New Emerging Targets in Cancer. The Role of B7-H3. *Vaccines* **2024**, *12*, 54. [\[CrossRef\]](#)
- Burvenich, I.J.G.; Parakh, S.; Lee, F.T.; Guo, N.; Liu, Z.; Gan, H.K.; Rigopoulos, A.; O’Keefe, G.J.; Gong, S.J.; Goh, Y.W.; et al. Molecular imaging of T cell co-regulator factor B7-H3 with ⁸⁹Zr-DS-5573a. *Theranostics* **2018**, *8*, 4199–4209. [\[CrossRef\]](#) [\[PubMed\]](#)
- Ahmed, M.; Cheng, M.; Zhao, Q.; Goldgur, Y.; Sarah, M.; Cheal, S.M.; Guo, H.; Larson, S.M.; Cheung, N.V. Humanized Affinity-matured Monoclonal Antibody 8H9 Has Potent Antitumor Activity and Binds to FG Loop of Tumor Antigen B7-H3. *Biol. Chem.* **2015**, *290*, 30018–30029. [\[CrossRef\]](#) [\[PubMed\]](#)
- Modak, S.; Zanzonico, P.; Grkovski, M.; Slotkin, E.K.; Carrasquillo, J.A.; Lyashchenko, S.K.; Lewis, J.S.; Cheung, I.Y.; Heaton, T.; LaQuaglia, M.P.; et al. B7H3-Directed Intraperitoneal Radioimmunotherapy with Radioiodinated Omburtamab for Desmoplastic Small Round Cell Tumor and Other Peritoneal Tumors: Results of a Phase I Study. *J. Clin. Oncol.* **2020**, *38*, 4283–4291. [\[CrossRef\]](#)
- Kramer, K.; Pandit-Taskar, N.; Kushner, B.H.; Zanzonico, P.; Humm, J.L.; Tomlinson, U.; Donzelli, M.; Wolden, S.L.; Haque, S.; Dunkel, I.; et al. Phase 1 study of intraventricular ¹³¹Iomburtamab targeting B7H3 (CD276)-expressing CNS malignancies. *J. Hematol. Oncol.* **2022**, *15*, 165. [\[CrossRef\]](#)
- Kenanova, V.; Wu, A.M. Tailoring antibodies for radionuclide delivery. *Expert Opin. Drug Deliv.* **2006**, *1*, 53–70. [\[CrossRef\]](#) [\[PubMed\]](#)
- Ståhl, S.; Gräslund, T.; Karlström, A.E.; Frejd, F.Y.; Nygren, P.-Å.; Löfblom, J. Affibody molecules in biotechnological and medical applications. *Trends Biotechnol.* **2017**, *35*, 691–712. [\[CrossRef\]](#)
- Zhang, L.; Zhang, H. Recent advances of affibody molecules in biomedical applications. *Bioorgan. Med. Chem.* **2024**, *113*, 117923. [\[CrossRef\]](#)

14. Baum, R.P.; Prasad, V.; Müller, D.; Schuchardt, C.; Orlova, A.; Wennborg, A.; Tolmachev, V.; Feldwisch, J. Molecular imaging of HER2-expressing malignant tumors in breast cancer patients using synthetic ^{111}In - or ^{68}Ga -labeled affibody molecules. *J. Nucl. Med.* **2010**, *51*, 892–897. [[CrossRef](#)] [[PubMed](#)]
15. Sörensen, J.; Velikyan, I.; Sandberg, D.; Wennborg, A.; Feldwisch, J.; Tolmachev, V.; Orlova, A.; Sandström, M.; Lubberink, M.; Olofsson, H.; et al. Measuring HER2-Receptor Expression In Metastatic Breast Cancer Using [^{68}Ga]ABY-025 Affibody PET/CT. *Theranostics* **2016**, *6*, 262–271. [[CrossRef](#)] [[PubMed](#)]
16. Klint, S.; Feldwisch, J.; Gudmundsdóttir, L.; Bergstedt, D.K.; Gunneriusson, E.; Höidén-Guthenberg, I.; Wennborg, A.; Nyborg, A.C.; Kamboj, A.P.; Peloso, P.M.; et al. Izokibep: Preclinical development and first-in-human study of a novel IL-17A neutralizing Affibody molecule in patients with plaque psoriasis. *MAbs* **2023**, *15*, 2209920. [[CrossRef](#)]
17. Guo, X.; Zhou, N.; Liu, J.; Ding, J.; Liu, T.; Song, G.; Zhu, H.; Yang, Z. Comparison of an affibody-based molecular probe and ^{18}F -FDG for detecting HER2-positive breast cancer at PET/CT. *Radiology* **2024**, *311*, e232209. [[CrossRef](#)]
18. Xia, L.; Wu, Y.; Ren, Y.; Wang, Z.; Zhou, N.; Zhou, W.; Zhou, L.; Jia, L.; He, C.; Meng, X.; et al. A whole-body imaging technique for tumor-specific diagnostics and screening of B7H3-targeted therapies. *J. Clin. Investig.* **2025**, *135*, e186388. [[CrossRef](#)]
19. Alqahtani, F.F. SPECT/CT and PET/CT, related radiopharmaceuticals, and areas of application and comparison. *Saudi Pharm. J.* **2023**, *31*, 312–328. [[CrossRef](#)]
20. Oroujeni, M.; Bezverkhniaia, E.A.; Xu, T.; Liu, Y.; Plotnikov, E.V.; Karlberg, I.; Ryer, E.; Orlova, A.; Tolmachev, V.; Frejd, F.Y. Evaluation of an Affibody-Based Binder for Imaging of Immune Check-Point Molecule B7-H3. *Pharmaceutics* **2022**, *14*, 1780. [[CrossRef](#)]
21. Oroujeni, M.; Bezverkhniaia, E.A.; Xu, T.; Liu, Y.; Plotnikov, E.V.; Klint, S.; Ryer, E.; Karlberg, I.; Orlova, A.; Tolmachev, V.; et al. Evaluation of Affinity Matured Affibody Molecules for Imaging of the Immune Checkpoint Protein B7-H3. *Nucl. Med. Biol.* **2023**, *124–125*, 108384. [[CrossRef](#)]
22. Oroujeni, M.; Carlqvist, M.; Ryer, E.; Orlova, A.; Tolmachev, V.; Frejd, F.Y. Comparison of approaches for increasing affinity of affibody molecules for imaging of B7-H3: Dimerization and affinity maturation. *EJNMMI Radiopharm. Chem.* **2024**, *9*, 30. [[CrossRef](#)]
23. Tolmachev, V.; Papalanis, E.; Bezverkhniaia, E.A.; Rosly, A.H.; Vorobyeva, A.; Orlova, A.; Carlqvist, M.; Frejd, F.Y.; Oroujeni, M. Impact of Radiometal Chelates on In Vivo Visualization of Immune Checkpoint Protein Using Radiolabeled Affibody Molecules. *ACS Pharmacol. Transl. Sci.* **2025**, *8*, 706–717. [[CrossRef](#)]
24. Tolmachev, V.; Bezverkhniaia, E.A.; Papalanis, E.; Muhammad, A.M.B.; Verabyova, A.; Gunneriusson, E.; Klint, S.; Ryer, E.; Carlqvist, M.; Kazmierczak, W.; et al. Preclinical PET Imaging of B7-H3 Expression Using Affibody Molecules Labelled with Positron-Emitting Gallium-68. *ACS Pharmacol. Transl. Sci.* **2025**, *8*, 3509–3522. [[CrossRef](#)] [[PubMed](#)]
25. Zhu, A.; Lee, D.; Shim, H. Metabolic Positron Emission Tomography Imaging in Cancer Detection and Therapy Response. *Mol. Imaging Oncol.* **2011**, *38*, 55–69. [[CrossRef](#)]
26. Martiniova, L.; De Palatis, L.; Etchebehere, E.; Ravizzini, G. Gallium-68 in Medical Imaging. *Curr. Radiopharm.* **2016**, *3*, 187–207. [[CrossRef](#)] [[PubMed](#)]
27. Alhuseinalkudhur, A.; Lindman, H.; Liss, P.; Sundin, T.; Frejd, F.Y.; Hartman, J.; Iyer, V.; Feldwisch, J.; Lubberink, M.; Rönnlund, C.; et al. Human Epidermal Growth Factor Receptor 2-Targeting [^{68}Ga]Ga-ABY-025 PET/CT Predicts Early Metabolic Response in Metastatic Breast Cancer. *J. Nucl. Med.* **2023**, *9*, 1364–1370. [[CrossRef](#)] [[PubMed](#)]
28. Tsionou, M.I.; Knapp, C.E.; Foley, C.A.; Munteanu, C.R.; Cakebread, A.; Imberti, C.; Eykyn, T.R.; Young, J.D.; Paterson, B.M.; Blower, P.J.; et al. Comparison of macrocyclic and acyclic chelators for gallium-68 radiolabelling. *RSC Adv.* **2017**, *7*, 49586. [[CrossRef](#)]
29. Harris, W.R.; Pecoraro, V.L. Thermodynamic binding constants for gallium transferrin. *Biochemistry* **1983**, *22*, 292–299. [[CrossRef](#)]
30. Kostelnik, T.I.; Orvig, C. Radioactive Main Group and Rare Earth Metals for Imaging and Therapy. *Chem. Rev.* **2019**, *119*, 902–956. [[CrossRef](#)]
31. Price, T.W.; Greenman, J.; Stasiuk, G.J. Current advances in ligand design for inorganic positron emission tomography tracers ^{68}Ga , ^{64}Cu , ^{89}Zr and ^{44}Sc . *Dalton Trans.* **2016**, *45*, 15702–15724. [[CrossRef](#)]
32. Wadas, T.J.; Wong, E.H.; Weisman, G.R.; Anderson, C.J. Coordinating radiometals of copper, gallium, indium, yttrium, and zirconium for PET and SPECT imaging of disease. *Chem. Rev.* **2010**, *110*, 2858–2902. [[CrossRef](#)] [[PubMed](#)]
33. Berry, D.J.; Ma, Y.; Ballinger, J.R.; Tavaré, R.; Koers, A.; Sunassee, K.; Zhou, T.; Nawaz, S.; Mullen, G.E.D.; Hiderb, R.C.; et al. Efficient bifunctional gallium-68 chelators for positron emission tomography: Tris(hydroxypyridinone) ligands. *Chem. Commun.* **2011**, *47*, 7068–7070. [[CrossRef](#)]
34. Cusnir, R.; Imberti, C.; Hider, R.C.; Blower, P.J.; Ma, M.T. Hydroxypyridinone chelators: From iron scavenging to radiopharmaceuticals for PET imaging with gallium-68. *Int. J. Mol. Sci.* **2017**, *18*, 116. [[CrossRef](#)] [[PubMed](#)]
35. Ma, M.T.; Cullinane, C.; Imberti, C.; Torres, J.b.; Terry, S.Y.A.; Roselt, P.; Hicks, R.J.; Blower, P.J. New Tris(hydroxypyridinone) Bifunctional Chelators Containing Isothiocyanate Groups Provide a Versatile Platform for Rapid One- Step Labeling and PET Imaging with $^{68}\text{Ga}^{3+}$. *Bioconjug. Chem.* **2016**, *27*, 309–318. [[CrossRef](#)]

36. Nawaz, S.; Mullen, G.E.D.; Sunassee, K.; Bordoloi, J.; Blower, P.J.; Ballinger, J.R. Simple, mild, one-step labelling of proteins with gallium-68 using a tris(hydroxypyridinone) bifunctional chelator: A ^{68}Ga -THP-scFv targeting the prostate-specific membrane antigen. *EJNMMI Res.* **2017**, *7*, 86. [[CrossRef](#)]
37. Young, J.D.; Abbate, V.; Imberti, C.; Meszaros, L.K.; Ma, M.T.; Terry, S.Y.A.; Hider, R.C.; Mullen, G.E.; Blower, P.J. ^{68}Ga -THP-PSMA: A PET Imaging Agent for Prostate Cancer Offering Rapid, Room-Temperature, 1-Step Kit-Based Radiolabeling. *J. Nucl. Med.* **2017**, *58*, 1270. [[CrossRef](#)] [[PubMed](#)]
38. Ma, X.; Zhou, X.; Hu, B.; Li, X.; Yao, M.; Li, L.; Qin, X.; Li, D.; Yao, Y.; Hou, X.; et al. Preclinical evaluation and pilot clinical study of [^{68}Ga]Ga-THP-APN09, a novel PD-L1 targeted nanobody radiotracer for rapid one-step radiolabeling and PET imaging. *Eur. J. Nucl. Med. Mol. Imaging* **2023**, *50*, 3838–3850. [[CrossRef](#)]
39. Zhou, X.; Li, N. Efficacy of radiolabelled PD-L1-targeted nanobody in predicting and evaluating the combined immunotherapy and chemotherapy for resectable non-small cell lung cancer. *Eur. J. Nucl. Med. Mol. Imaging* **2025**, *7*, 2343–2354. [[CrossRef](#)]
40. Kulkarni, M.; Hughes, S.; Mallia, A.; Gibson, V.; Young, J.; Aggarwal, A.; Morris, S.; Challacombe, B.; Popert, R.; Brown, C.; et al. The management impact of ^{68}Ga -tris(hydroxypyridinone) prostate-specific membrane antigen (^{68}Ga -THP-PSMA) PET-CT imaging for high-risk and biochemically recurrent prostate cancer. *Eur. J. Nucl. Med. Mol. Imaging* **2020**, *47*, 674–686. [[CrossRef](#)]
41. Garousi, J.; Orlova, A.; Frejd, F.Y.; Tolmachev, V. Imaging using radiolabelled targeted proteins: Radioimmunodetection and beyond. *EJNMMI Radiopharm. Chem.* **2020**, *5*, 16. [[CrossRef](#)] [[PubMed](#)]
42. Tolmachev, V.; Orlova, A. Affibody Molecules as Targeting Vectors for PET Imaging. *Cancers* **2020**, *12*, 651. [[CrossRef](#)] [[PubMed](#)]
43. Eissler, N.; Altena, R.; Alhuseinalkhudhur, A.; Bragina, O.; Feldwisch, J.; Wuerth, G.; Loftenius, A.; Brun, N.; Axelsson, R.; Tolmachev, V.; et al. Affibody PET Imaging of HER2-Expressing Cancers as a Key to Guide HER2-Targeted Therapy. *Biomedicines* **2024**, *12*, 1088. [[CrossRef](#)] [[PubMed](#)]
44. Tanwar, K.S.; Pandey, M.K. Gallium-68-labeled radiopharmaceuticals: A review. *Chem. Soc. Rev.* **2025**, *54*, 7421. [[CrossRef](#)]
45. Tolmachev, V.; Orlova, A. Influence of labelling methods on biodistribution and imaging properties of radiolabelled peptides for visualisation of molecular therapeutic targets. *Curr. Med. Chem.* **2010**, *17*, 2636–2655. [[CrossRef](#)]
46. Wällberg, H.; Orlova, A. Slow internalization of anti-HER2 synthetic affibody monomer ^{111}In -DOTA-ZHER2:342-pep2: Implications for development of labelled tracers. *Cancer Biother. Radiopharm.* **2008**, *23*, 435–442.

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