Nuclear Imaging of Bispecific Antibodies on the Rise

Borna Roohani*[1,2], Aldred Shane Mendez*[1,2], Mann Dangarwala[2], Samantha Katz[1], and Bernadette Marquez-Nostra[2]

[1]*Yale PET Center, Department of Radiology and Biomedical Imaging, Yale University, New Haven, Connecticut; and* [2]*Department of Radiology, University of Alabama at Birmingham, Birmingham, Alabama*

Bispecific antibodies (bsAbs) are engineered to target 2 different epitopes simultaneously. About 75% of the 16 clinically approved bsAbs have entered the clinic internationally since 2022. Hence, research on biomedical imaging of various radiolabeled bsAb scaffolds may serve to improve patient selection for bsAb therapy. Here, we provide a comprehensive overview of recent advances in radiolabeled bsAbs for imaging via PET or SPECT. We compare direct targeting and pretargeting approaches in preclinical and clinical studies in oncologic research. Furthermore, we show preclinical applications of imaging bsAbs in neurodegenerative diseases. Finally, we offer perspectives on the future directions of imaging bsAbs based on their challenges and opportunities.

Key Words: bispecific antibody; PET; SPECT; bsAb; immuno-PET

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Since 2022, about 75% of all 16 currently approved bispecific antibodies (bsAbs) have been granted permission by different health departments worldwide for use in the treatment of various diseases (*1*). Compared with monospecific antibodies, bsAbs have multiple mechanisms of action within one molecule to elicit the desired therapeutic effects. They can simultaneously target 2 different tumor-associated antigens to improve tumor-targeting selectivity and enhance receptor-mediated inhibitory functions. bsAbs such as bispecific t-cell engager molecules (BiTE) can also target 2 different cell types, bringing together T cells and tumor cells to modulate the immune system to kill tumor cells (*2*). Furthermore, bsAbs can be used in the central nervous system (CNS), with one arm of the bsAb binding to a receptor on the blood–brain barrier (BBB), thereby enhancing transcytosis into the brain, whereas the other arm binds to a molecular target in the brain (*3*).

Noninvasive imaging techniques that combine the specificity of antibodies with the sensitivity of PET (immuno-PET) or SPECT (immuno-SPECT) can serve as companion diagnostic tools to help select patients likely to respond to bsAb treatment (Fig. 1A). There are 2 different targeting approaches to the imaging of bsAbs. The first approach is direct targeting, in which the bsAb is conjugated to a metal chelator and subsequently labeled with a radioisotope (e.g., ^{111}In and ^{89}Zr) (Fig. 1B). Direct targeting may be used to determine the biodistribution of the bsAb and assess target engagement. This approach is especially useful to evaluate the heterogeneity of target expression in the metastatic setting in oncology or to evaluate CNS targets in neurodegenerative diseases. The second approach is pretargeting, in which the radioisotope is tagged to a small molecule called a hapten, which then binds to the engineered bsAb (*4*). This approach involves 2 or 3 steps, which are administered in this sequence: first, the bsAb is administered to allow antibody binding to its target in vivo, second, a clearing agent may be needed to sequester the unbound and circulating bsAb, and third, the radiolabeled hapten is administered to bind to the bsAb in vivo. Figure 1C shows an example of a 2-step sequence. Pretargeting is particularly advantageous in lowering the overall dosimetry to the body. Here, we review the recent trends in imaging of bsAbs since 2019 in several types of cancer and in neurodegenerative diseases.

SELECTION OF STUDIES

We searched 2 online databases: PubMed and Scopus. A comprehensive overview illustrating the process of selecting the studies can be found in Figure 2. We identified 42 original research papers evaluating radiolabeled bsAb imaging using PET or SPECT, with most focusing on oncology. Of the selected studies, 33% applied bsAb imaging to neurodegenerative diseases.

BSAB FORMATS

There are a vast number of different bsAb formats. A comprehensive review on bsAb structure and terminology has been provided by Brinkmann and Kontermann (*5*). Here, we describe some examples of bsAb formats that were evaluated in the reviewed imaging studies. One example is a bsAb with a knob-in-hole format, which maintains the general Y-shaped structure of a typical antibody with an estimated molecular weight of about 150 kDa. It comprises 2 distinct antigen-binding fragments (Fabs), each targeting a different epitope. Each Fab is connected to its respective CH2 and CH3 domains, forming the Fc region that incorporates the knob-in-hole modification in its CH3 domain, where one heavy chain has a "knob" (a large amino acid residue such as tyrosine) and the other a complementary "hole" (a small amino acid residue such as threonine) (*5*). This design ensures correct heavy-chain pairing and formation of the desired bsAb. Figure 3A illustrates this structure. The function of the Fc region is to engage the immune system, including complement activation, antibody-dependent cellular cytotoxicity, or opsonization. The latter 2 defense mechanisms occur when the Fc region binds to cell-bound Fc receptors (*6*). Furthermore, FcRn-mediated recycling was shown to be responsible for the long elimination half-life of antibodies. These mechanisms, along with biophysical properties of bsAbs, contribute to their enhanced therapeutic efficacy. In contrast, the absence of the Fc region reduces the size of the bsAb, permitting faster blood clearance, enhanced tumor penetration, and increased epitope accessibility (*7*). Figure 3B

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For correspondence or reprints, contact Bernadette Marquez-Nostra (bmarqueznostra@uabmc.edu).
*Contributed equally to this work.
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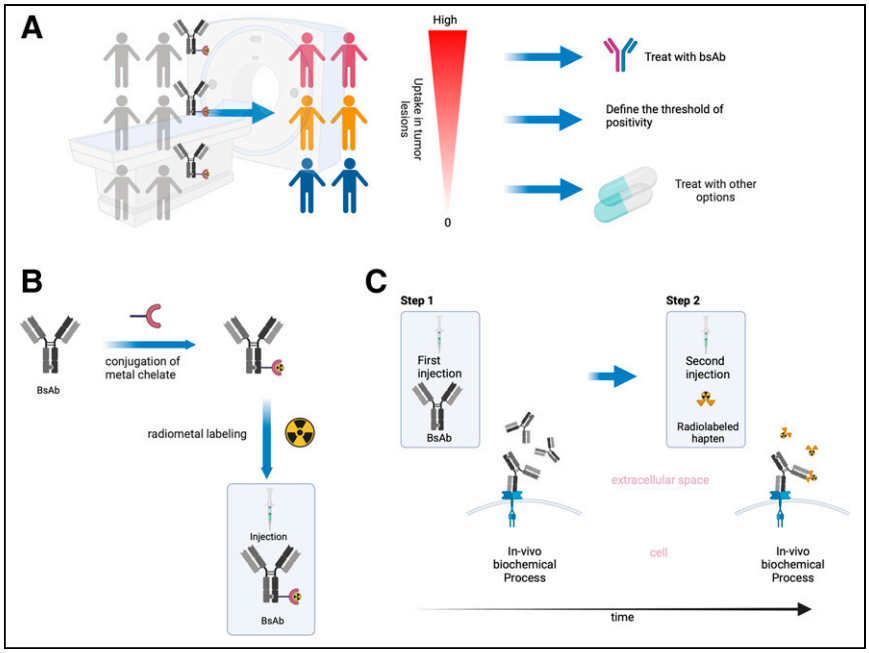

FIGURE 1. (A) Representation of potential patient selection for treatment by PET imaging with radiolabeled bsAb. (B) General schematic for conjugation and radiolabeling of bsAb before administration in vivo (1 step). (C) General pretargeting approach requiring at least 2 steps and injections typically given hours apart. First step is for bsAb to bind to its target in vivo and allow unbound bsAb to clear from bloodstream. Second step is for radiolabeled hapten to bind to bsAb in vivo.

shows an example of a di–single-chain variable fragment (scFv) format with a molecular weight of about 50 kDa.

PRECLINICAL IMAGING

Preclinical Imaging of Cancer

Of 23 studies, 78% used direct targeting and 22% used pretargeting approaches. Table 1 shows a list of these studies categorized by the type of disease and targeting approach. The molecular target, targeting vector, and radiolabeled agents can be differentiated to provide a streamlined visual representation of these studies.

Studies that used direct targeting with radiolabeled bsAbs showed the biodistribution and target engagement of bsAbs with different

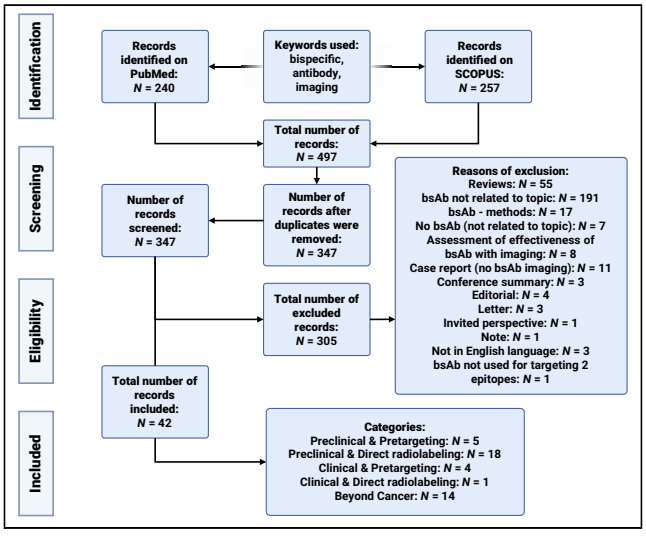

FIGURE 2. Selection process for included articles.

mechanisms of action, including inhibiting cancer cell signaling pathways, engaging 2 different immune cells, and BiTEs (*2*). Most studies in this section were performed with radiolabeled BiTEs. One example from this group is the study by Crawford et al. (*2*), in which [^{89}Zr]Zr-DFO-REGN4018 targeted MUC16 on ovarian cancer cells and CD3 on T cells as a companion imaging agent to REGN4018. Studies on monkeys showed that REGN4018 exhibited no toxicity, an increase in serum cytokines, substantial antitumor activity, and accumulation in the spleen and lymph nodes, likely due to the presence of CD3-positive T lymphocytes in these lymphoid organs (*2*).

Studies that used the pretargeting approach focused on image-guided resection in colorectal cancer, theranostics for peritoneal carcinomatosis and neuroblastoma, or evaluation of a nanocarrier for breast cancer (*4*,*8*–*10*). The theranostic studies were most advanced, showing high specificity of the pretargeting system for their respective targets and rapid clearance of the radiolabeled hapten (*4*,*9*,*10*). These studies demonstrated high image quality for the diagnostic component and a high therapeutic index for the therapy component. Furthermore, quantitative SPECT imaging was used to show effective delivery of the therapeutic agent and provide dosimetry estimates (*4*). Both direct targeting and pretargeting approaches pave the way for further progress toward precision medicine.

Preclinical Imaging of the CNS

Radiolabeled bsAbs have been expanded to image molecular targets in neurodegenerative diseases. One of the greatest difficulties for therapy and imaging of the CNS and its pathologic abnormalities is crossing the BBB. In general, antibodies are too large to cross an intact BBB. One approach to overcome this issue is to target the transferrin receptor (TfR), which is expressed on the apical cell surface of endothelial cells. Binding of one Fab arm to the TfR enables transcytosis of the whole bsAb, thus reaching the interstitium of the CNS (Fig. 3C). The other Fab arm could, for instance, bind the amyloid β-protein (Aβ) to image its accumulation in the brain of patients with Alzheimer disease. Eleven studies adopted this approach (*7*,*11*–*20*). In addition to Aβ, another target has been used as a biomarker for imaging Alzheimer disease: the Triggering Receptor Expressed on Myeloid cells 2 (*21*).

Gustavsson et al. (*11*) showed that imaging contrast in the brain can be improved by increasing clearance rates of radiolabeled bsAb from the brain. Their strategy was to decrease the size of the bsAb (e.g., the use of bsAb fragments). The scFv8D3, composed of the variable region of an antibody that is connected by a flexible linker, can penetrate the brain via its TfR-binding site; it was fused to the Aβ protofibril binding Rmab158. The resulting Rmab158-scFv8D3 bsAb was shown to have a faster blood clearance than the Rmab158 (*11*). Schlein et al. (*13*) evaluated Bapi-Fab8D3, an antibody targeting both Aβ and TfR, with an engineered FcRn binding mutation to enhance clearance, revealing a superior brain-to-blood ratio for Bapi-Fab8D3. Despite the relatively faster clearance, brain

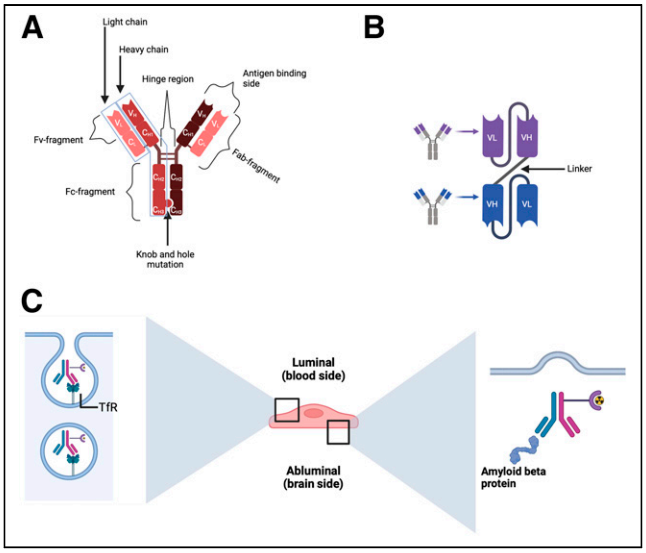

FIGURE 3. (A) Anatomy of bsAb. (B) Example of di-scFv, a bsAb without the Fc region. (C) Transcytosis of anti-TfR/anti-Aβ bsAb.

penetration was shown to be more efficient with the TfR-binding moiety (*11*). Other studies confirmed this effect (*7*,*11*,*14*,*15*,*21*). However, other efforts to further increase blood clearance rates by conjugating mannose to the RmAb158-scFv8D3 or chasing the bsAb with a clearing agent systematically did not accelerate the blood clearance rate from the brain relative to their unconjugated bsAb. Schlein et al. (*12*) attributed this phenomenon to the conjugate's prolonged binding to its TfR. Faresjö et al. (*16*) showed that a decrease in size and a decrease in TfR avidity are important factors for fast parenchymal delivery, but these characteristics did not affect the rate at which the bsAb cleared from the nervous tissue. Further studies are needed to optimize the pharmacokinetic properties of radiolabeled bsAbs in the CNS.

Currently, Alzheimer disease is imaged with ^{11}C-labeled Pittsburgh compound B, a clinically established PET tracer that binds Aβ. This radiotracer was shown to target only insoluble deposits of Aβ. In contrast, bsAb can specifically target soluble and nonfibrillar Aβ that undergoes more dynamic changes over the course of disease and its treatment (*17*). Meier et al. (*17*) investigated the clinically established [^{11}C]Pittsburgh compound B compared with ^{124}I- and ^{125}I-labeled RmAb158-scFv8D3, an asymmetric bsAb

TABLE 1
Preclinical Radiotracers Used in Direct Targeting and Pretargeting Approaches

Tumor or disease	Radiolabeled agent	bsAb	Molecular target	Reference
Direct targeting				
Melanoma	[^{131}I]I-KN046	KN046	PDL1, CTLA4	(*28*)
Breast cancer brain metastasis	[^{89}Zr]Zr-DFO-HER3-HBP-DOX	Nanoparticle (HBP) biodistribution	HER3/PEG	(*29*)
	[^{89}Zr]Zr-mesothelin HLE BiTE	BiTE	CD3, mesothelin	(*30*)
	[^{89}Zr]Zr-DFO-bintrafusp	Bintrafusp	PDL1, TGF-betaRII	(*31*)
	[^{89}Zr]Zr-DFO-amivantamab	Amivantamab	CMET, EGFR	(*32*)
	[^{89}Zr]Zr-DFO-muS110	muS110	CD3, EpCAM	(*33*)
Breast cancer	[^{89}Zr]Zr-Df-ATG-101	AT-101	PD-L1, 4-1BB	(*34*)
Liver cancer	[^{89}Zr]Zr-DFO-ERY974	ERY974	CD3, GPC3	(*35*)
Prostate cancer	[^{64}Cu]Cu-NODAGA-bsAb	bsAb	PSMA, CD3	(*36*)
	[^{89}Zr]Zr-DFO-PSMAxCD3	PSMAxCD3	PSMA, CD3	(*37*)
	[^{89}Zr]Zr-DFO-DLL3-scFv	DLL3-scFv	CD3, DLL3	(*38*)
Pancreatic ductal adenocarcinoma	[^{111}In]In-CD3xTRP1	CD3xTRP1	CD3, TRP1	(*39*)
Ovarian cancer	[^{89}Zr]Zr-DFO-REGN4018	REGN4018	CD3, MUC16	(*2*)
Colon carcinoma	[^{64}Cu]Cu-DOTA-dbBITE	dbBITE	CEA, CD3	(*40*)
	[^{89}Zr]Zr-DFO-IBI322	IBI322	PDL1, CD47	(*41*)
Colorectal cancer	[^{89}Zr]Zr-FAP-4–1BBL	FAP-4-1BBL	4-1BB, FAP	(*42*)
	[^{89}Zr]Zr-DFO-10G1	10G1	CD3, RON	(*43*)
	[^{124}I]I-AK104	AK104 (cadonilimab)	hPD1, CTLA4	(*44*)
Pretargeting				
Colorectal cancer	[^{111}In]In-RDC018	TF2	CEA, HSG	(*8*)
	[^{86}Y]Y-DOTA-Bn	Anti-GPA33/anti-DOTA	GPA33, DOTA	(*4*)
	[^{111}In]In-Pr	DOTA-PRIT	GPA33, DOTA	(*9*)
Neuroblastoma	[^{86}Y]Y-DOTA	P53-SADA-bsAb	Antiganglioside GD2, DOTA	(*10*)
Breast cancer	[^{64}Cu]Cu-MeCOSar-NHS-PEG	anti-PEG/anti-EGFR	PEG, epidermal growth factor receptor	(*45*)

that targets the TfR and Aβ protofibrils. This study concluded that the tracer detected changes in Aβ levels in the brain after treatment with β-site amyloid precursor protein cleaving enzyme 1 inhibitor (NB-360), whereas [^{11}C]Pittsburgh compound B did not detect such change. Furthermore, Fang et al. (*7*) showed that a di-scFv, [^{124}I]3D6-8D3, displayed higher sensitivity than [^{11}C]Pittsburgh compound B for binding to soluble neurotoxic Aβ. Others have used ^{89}Zr and ^{18}F for radiolabeling these bsAbs (*18–20*).

Other neurodegenerative diseases, such as Parkinson disease and multiple-system atrophy, have been imaged in a study with an ^{124}I-labeled bsAb targeting the α-synuclein protein next to TfR via PET (*22*). The study with ^{124}I-labeled bsAbs for αSYN imaging demonstrated successful in vitro binding to pathologic αSYN and increased the concentration of the radioligand in the brain in vivo. However, PET imaging showed no significant difference in signal intensity between αSYN transgenic and wild-type mice. This study underscores the challenges in developing imaging agents for intracellular protein aggregates such as αSYN (*22*).

Imaging molecular targets of the CNS with radiolabeled bsAbs is a novel approach to visualize neurologic pathologies with high specificity. This strategy allows intravenous injection of the tracer, which overcomes the need for invasive intracranial routes for injection.

CLINICAL RESEARCH

Direct Targeting

Recent clinical studies have determined the biodistribution and targeting capabilities of radiolabeled bsAbs in patients. A notable example is the work by Moek et al. (*23*), who conducted the first-in-human study that evaluated [^{89}Zr]Zr-N-SucDf-AMG 211, a Fab-based BiTE that targets the carcinoembryonic antigen (CEA) and CD3, in 9 patients with advanced gastrointestinal adenocarcinomas. CEA, which is overexpressed on the cancer cell surface, and CD3 (T cells) were targeted in this phase 1 clinical trial involving 5 patient cohorts. The first group received only the tracer. Groups 2 and 3 received 1.8 and 4.8 mg, respectively, of unlabeled AMG 211 as a blocking agent before imaging. These 2 groups were imaged at 28 d after treatment of AMG 211 (6.8 and 12.8 mg/d). All groups received 200 μg of [^{89}Zr]Zr-N-SucDf-AMG 211. The optimal dose of the blocking agent was determined to be 1.8 mg of unlabeled AMG 211. Although the sample size was small, results showed higher uptake of the tracer in lymphoid tissues, where T cells reside. CD3-mediated uptake in the spleen and the bone marrow had an SUV_{max} of 3.2 and 1.8, respectively. Tumor uptake varied by a factor of 5 within tumor lesions of the same patient and by a factor of 9 across all imaging cohorts. Intra- and interpatient heterogeneity of tracer uptake was explained by different CEA expression levels and differences in tissue permeability. This study demonstrates the need for precision imaging with radiolabeled BiTEs.

Pretargeting

The studies focusing on breast cancer, medullary thyroid cancer, and colorectal metastases used the bsAb TF2, an engineered trivalent antibody that bivalently binds to CEA and monovalently to the histamine-succinyl-glycine-hapten [^{68}Ga]Ga-IMP288 (*24–26*). This particular tracer was developed to improve diagnostic imaging of metastatic lesions to overcome the limitations of [^{18}F]FDG, as described below (*25*).

Breast and Medullary Thyroid Cancer Metastases. Two clinical trials (NCT01730612 and NCT01730638) aimed to image metastatic breast cancer and metastatic medullary thyroid cancer. Twenty-three breast cancer patients and 22 medullary thyroid cancer patients have been recruited for both trials. Rousseau et al. (*24*) compared the pretargeting method with the standard [^{18}F]FDG PET for HER2-negative breast cancer patients. The total lesion sensitivity was 94.7% for immuno-PET, a 5.1% increase compared with [^{18}F]FDG PET. However, less than half of all lesions in the lung were not detected with immuno-PET, whereas 100% of those metastases were seen with CT.

Pichon et al. (*25*) included 3 medullary thyroid cancer and 5 breast cancer patients who had vertebral metastases and were eligible for stereotactic body radiotherapy. Pretargeted immuno-PET was compared with standard clinical imaging methods. The sensitivity of MRI for predicting the involvement of a vertebral segment was 74%, followed by 64% for the pretargeting approach. However, 11 vertebral metastases that were detectable with immuno-PET were negative on MRI. [^{18}F]F-DOPA-PET and [^{18}F]FDG PET also showed poor detection of vertebral metastases in several vertebrae. Additionally, the specificity of the tracer and the resulting increased contrast allow segmentation of vertebral metastases in different parts of the vertebrae for radiotherapy planning. These findings underscore the significance of pretargeting in detecting CEA-expressing tumor cells that may not be seen on anatomic imaging alone.

Bodet-Milin et al. (*26*) focused on imaging lesions in medullary thyroid cancer patients with this pretargeted immuno-PET method and compared their findings with results from amino acid imaging with [^{18}F]F-DOPA PET. The results of this trial resembled those of the breast cancer trial—the overall sensitivity of the immuno-PET was 27% greater than that of [^{18}F]F-DOPA PET. Compared with all other imaging modalities, pretargeted immuno-PET showed a higher detection rate, excluding the lungs, whereas CT detected 100% of all appearing lesions.

Colorectal Cancer Metastases. Touchefeu et al. (*27*) imaged 11 patients with pretargeted TF2/^{68}Ga-IMP288 and showed a safe and precise alternative to conventional imaging including endoscopic ultrasound, MRI, CT, and [^{18}F]F-FDG PET. Pretargeted PET was shown to have 100% specificity and a 100% positive predictive value, which are considerably higher than those for [^{18}F]FDG PET or the combination of endoscopic ultrasound, CT, and MRI. Despite these promising findings, SUV_{max}, metabolic tumor volume, and total lesion glycolysis did not exhibit significant differences. The positive predictive value per-lesion analysis was 82%, whereas the positive predictive values for combination endoscopic ultrasound/CT/MRI and [^{18}F]FDG PET were 87% and 100%, respectively. Given the smaller number of patients, these findings need to be validated in a larger patient group (*27*).

The potential clinical significance of imaging with radiolabeled bsAb is to serve as a companion diagnostic to its corresponding bsAb therapy for patient selection. Imaging with radiolabeled bsAb would give insight into its biodistribution and evaluate the degree of on-target accumulation to predict response and off-target accumulation to assess potential toxicity.

PERSPECTIVE

bsAb in Oncology

Invasive biopsy is the standard of care for pathologic assessment of therapeutic targets. In some cases, certain lesions are

difficult or not feasible to access. However, in all cases, only a small part of the tumor can be biopsied, and its molecular characteristics do not always translate to the rest of the tumor or to other metastatic foci. Conversely, every lesion in its entirety can be quantified for the antigen of interest through imaging with radiolabeled antibodies—monospecific and bispecific—allowing a more accurate analysis of the molecular heterogeneity within the whole lesion and across different lesions in the body.

Traditionally, treatment with monospecific antibodies can lead to drug resistance, which paved the way for the development of bsAbs to improve treatment efficacy. The rapid increase in approval of bsAb treatments in the past 5 y has prompted imaging of radiolabeled bsAbs to determine biodistribution and target engagement. Imaging with radiolabeled bsAbs offers comprehensive insights into molecular heterogeneity across lesions and enables personalized therapy decisions. Currently, 2 active clinical trials are listed on clinicaltrials.gov. The first is for PET imaging with ^{89}Zr-labeled bsAb targeting PDL1 and 4-1BB (phase 1, NCT05638334) to assess whole-body biodistribution. The second is a radiotheranostic study using SPECT imaging with ^{111}In-labeled bsAb targeting the epidermal growth factor receptor and mesenchymal-to-epithelial transition factor and α-therapy with the ^{225}Ac-labeled bsAb to assess safety, dosimetry, and maximum tolerated dose (phase 1, NCT06147037).

Independently, PET or SPECT imaging with radiolabeled bsAbs can inform on total antigen expression and drug delivery. However, it cannot capture the entire therapeutic mechanism of action of bsAbs. We may need to know the extent of the recruitment of immune cells and the change in receptor expression for constructs that bind to tumor-associated receptors. Multiplex or multimodal imaging may be necessary because of the diverse formats and functions of bsAbs under development.

bsAb in the CNS

The development of radiolabeled bsAbs for imaging CNS pathologies represents a significant advancement in neurodegenerative disease research. By targeting the TfR to overcome the BBB, these bsAbs enable noninvasive visualization of key biomarkers such as Aβ in Alzheimer disease. Recent studies have demonstrated that optimizing the design of bsAbs through size reduction and affinity modulation can enhance brain uptake and clearance, improving imaging contrast. As this technology continues to evolve, it will provide valuable insights into various neurodegenerative conditions, improving diagnosis and treatment monitoring without the need for invasive procedures.

CONCLUSION

bsAbs have advanced molecular imaging by addressing the limitations of traditional monospecific antibodies. This review covers the development and application of bsAbs used in SPECT or PET imaging, comparing direct targeting and pretargeting approaches. We found that most studies in this review focused on applications in oncology whereas some studies are emerging to address neurodegenerative diseases. bsAbs targeting the TfR have shown effectiveness in crossing the BBB for CNS imaging, a feat that was historically impenetrable. Smaller bsAb formats that incorporate TfR-binding scFv8D3 have demonstrated improved brain penetration and clearance. The potential of imaging bsAbs to provide insights into tumor heterogeneity and guide treatment planning is significant and bridges the gap between the rising availability and clinical use of bsAb therapeutics and molecular imaging.

DISCLOSURE

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