Minireview

Molecular Advances in Pretargeting Radioimunotherapy with Bispecific Antibodies[1]

Chien-Hsing Chang, Robert M. Sharkey, Edmund A. Rossi, Habibe Karacay, William McBride, Hans J. Hansen, Jean-François Chatal, Jacques Barbet, and David M. Goldenberg[2]

IBC Pharmaceuticals, L. L. C., Morris Plains, New Jersey 07950 [C-H. C., E. A. R.]; Immunomedics, Inc., Morris Plains, New Jersey 07950 [C-H. C, W. M., H. J. H.]; Garden State Cancer Center, Belleville, New Jersey 07109 [R. M. S., H. K., D. M. G.]; and Inserm U463, 44093 Nantes, Cedex 01, France [J-F. C., J. B.]

Abstract

The use of antibodies against tumor-associated cell surface antigens for the targeted delivery of radionuclides was introduced >20 years ago. Although encouraging results have been achieved with radiolabeled antibodies in the management of hematopoietic malignancies, there remains a need for successfully treating solid tumors with this modality. One promising approach involving pretargeted delivery of radionuclides has been shown to be capable of significantly increasing the radioactive uptake in tumor relative to normal organs, thereby potentially improving the efficacy of both detection and therapy of cancer. Uncoupling of the radionuclide from the tumor-targeting antibody allows the relatively slow process of antibody localization and clearance to occur before a very rapid and highly specific delivery of the radioactive payload carried on a small molecule, such as a peptide. This minireview discusses the various strategies and advancements made since the concept of pretargeting was proposed in the mid-1980s, with emphasis on those comprising bispecific antibodies for cancer therapy. Critical aspects of these pretargeting systems for achieving higher tumor:nontumor ratios are considered. In addition, both preclinical and clinical results obtained from a pretargeting method known as the Affinity Enhancement System are presented. Future directions of pretargeting technology are also suggested.

Introduction

Targeted delivery of radionuclides for imaging and therapy of cancer, using antibodies against tumor-associated antigens or haptens, is an active area of ongoing investigation at both the experimental and the clinical levels (1). Since the introduction of the terms RAID[3] and RAIT, representing the use of radiolabeled antibodies for imaging and therapy, respectively, >20 years ago, the extensive efforts have resulted in the commercialization of several cancer-imaging agents based on RAID. The current prospects of establishing RAIT as a new therapeutic modality of cancer is also very promising because of the encouraging results obtained with radiolabeled antibodies in the management of hematopoietic neoplasms, especially non-Hodgkin's lymphoma (2).

In contrast to hematopoietic malignancies, solid tumors have been less responsive to RAIT. Because the efficacy of RAIT depends on several factors, including cumulative radiation dose delivered, dose rate, penetration, and tumor radiosensitivity, one major reason for the diminished success of RAIT in solid tumors may be the suboptimal tumor uptake of the targeting antibody. At an accretion of 0.001–0.01% ID of the radiolabeled antibody/gram tumor, a cumulative tumor dose of <1500 cGy is usually delivered, which falls short of the typical >5000 cGy needed to achieve therapeutic responses in most neoplasms, based on external beam irradiation of adenocarcinomas (3). When the accretion is limited, doses needed to achieve therapeutic response are restricted because of other normal organ dose limitations. For RAIT, bone marrow toxicity often determines the maximum tolerated dose that can be administered.

The challenge of treating solid tumors by RAIT has stimulated a number of approaches to improve the radiation dose delivered and to achieve a more uniform distribution of ionizing radiation, with the ultimate goal being the delivery of tumoricidal doses while sparing normal tissues. One of these methods is pretargeting, a strategy conceived in the 1980s initially for improving the selective delivery of radionuclides by antibodies to tumors and later extended to include nonradioactive agents and nonantibody delivery systems. This minireview addresses the various refinements of the pretargeting strategy, with emphasis on those involving the use of bsAb and radioactive agents in the therapy of cancer. The progress of pretargeting research in the realm of nuclear medicine has received periodic appraisals over the years through excellent editorials (4–8) and reviews (9–15).

Received 12/20/01; revised 2/25/02; accepted 3/8/02.
[1] Supported in part by USPHS Grant R01-CA-84379 from the NIH and Department of Energy Grant DE-FG01-00NE22941 (both to R. M. S.).
[2] To whom requests for reprints should be addressed, at Garden State Cancer Center, 520 Belleville Avenue, Belleville, NJ 07109-0023. Phone: (973) 844-7000; Fax: (973) 844-7020; E-mail: dmg.gscancer@worldnet.att.net.

[3] The abbreviations used are: RAID, radioimmunodetection; RAIT, radioimmunotherapy; ID, injected dose; AES, affinity enhancement system; NCAM, neural cell adhesion molecule; HSG, histamine-succinyl-glycine; DTPA, diethylenetriaminepentaacetic acid; CEA, carcinoembryonic antigen; DOTA, 1,4,7,10-tetra-azacylododecane-*N,N′,N″,N‴*-tetraacetic acid; TETA, 1,4,8,11-tetraazacyclotetradecane-*N,N′,N″,N‴*-tetraacetic acid; CHX-A-DTPA, stereoisomers of cyclohexyl-DTPA; PEG, polyethyleneglycol; bsAb, bispecific antibody.

Approaches Taken to Improve Target:Nontarget Ratios

The inherent incompatibility between a tumor-targeting antibody and the radiation carried by it became evident from early imaging studies in experimental animals and in patients. Intact immunoglobulins directly labeled with a radioisotope were found to clear very slowly from the circulation, resulting in high background activity, thereby limiting the efficacy for both the detection and treatment of tumors (4). The use of antibody fragments, such as Fab or $F(ab')_2$, which clear from the blood more rapidly than whole IgG, improves target:background ratios but at the expense of lower uptake of the radiolabeled antibody in the tumor, when compared with whole IgG (16). Other approaches to reduce background radioactivity while maintaining tumor uptake of a directly radiolabeled antibody agent have been explored and met with variable degrees of success, *e.g.,* administration of antiantibodies (such as an anti-idiotype antibody) can remove radiolabeled antibodies from blood faster than from tumor and is considered particularly applicable to radioiodinated antibodies (17). For antibodies labeled with a radiometal, the insertion of a metabolizable linker between the chelate and protein is also effective in improving tumor:background ratios, despite a modest drop in tumor concentrations (18). The fact that an antibody can be facilely labeled *in vivo* with a radioactive hapten to which it binds with a high affinity is fundamental to the development of pretargeting strategies, which successfully overcome the problem of low target:background ratios by temporal separation of the slow antibody-targeting step from the delivery of the radionuclide.

Principles, Strategies, and Key Components of Pretargeting

The essence of pretargeting involves the use of a macromolecule that is capable of binding with a high affinity to a radioactive agent of low molecular weight (usually M_r $<$10,000) and can also selectively target a tumor antigen. The macromolecule is administered first, and the radioactive agent ("the effector") is given at a later time, ideally when the concentration of the macromolecule in the tumor is greater than in other tissues. Favorable tumor:normal tissue ratios of radioactivity are thus achievable, because the small size of the nontargeted radioactive agent permits its rapid elimination from the body. This sequence of events is commonly referred to as the two-step protocol (Fig. 1). The key for the success of the two-step method is that the macromolecule must be cleared sufficiently from the blood and normal tissues, otherwise the radioactive effector molecule will be retained wherever the macromolecule is distributed. An alternative to the two-step protocol is the three-step protocol, in which a specific molecule ("the chaser") is injected to remove the residual macromolecule from the bloodstream before giving the radioactive agent, so that further enhancement in the tumor:blood ratios of radioactivity can be obtained. To date, the potential value of applying pretargeting strategies to cancer imaging and therapy has been demonstrated in animal models, as well as in clinical trials for many macromolecule/effector systems, with or without the chase step, using radioactive or nonradioactive effectors (12, 14, 19).

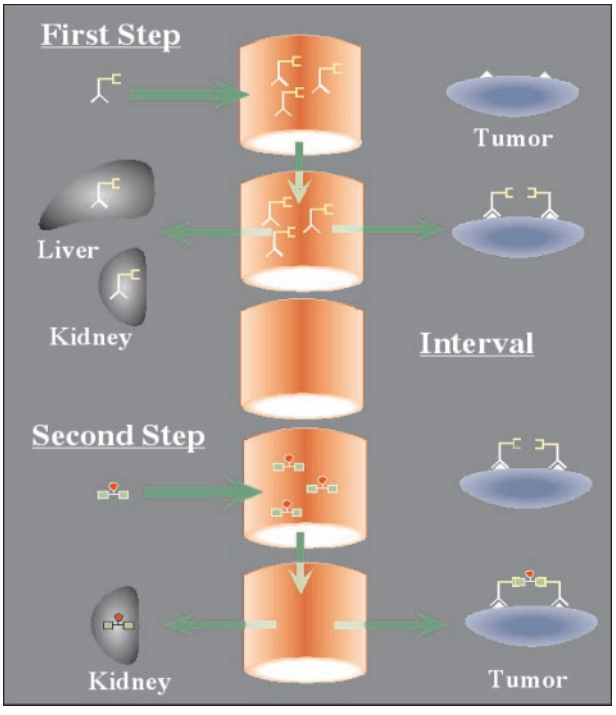

Fig. 1. The two-step pretargeting protocol illustrated with a bsAb and a bivalent hapten.

To be useful for pretargeting applications, the macromolecule can be just an antihapten monoclonal antibody that recognizes a structural component (hapten) of an effector (20) or even merely a biotin-binding protein like streptavidin (21–23). These molecules can be used because tumors or inflammatory/infectious foci, because of their leaky capillaries, can accumulate macromolecules nonspecifically through passive diffusion, leading to a preferential localization of the macromolecule at the target. In most cases studied, however, the macromolecule of interest consists of a modified antitumor antibody carrying a secondary recognition moiety, in addition to its primary target-binding function. Representative examples of such modified antibodies can be distinguished into five types: (*a*) bsAb (24) constructed to contain one hapten-binding site and one or two target-binding sites; (*b*) antibodies conjugated to streptavidin (25) or avidin (26) to enable the binding of biotin; (*c*) biotinylated antibodies capable of complexing with avidin (27) or streptavidin (28, 29); (*d*) antibodies conjugated to DNA (30) to promote binding to complementary nucleotide sequences; and (*e*) antibodies conjugated to enzymes (19) to activate a prodrug at the target site. In general, for each targeting macromolecule selected, a series of effectors may be devised and evaluated under pretargeting conditions for their respective uptake in tumor relative to normal tissues. These effectors, while having the common hapten or structural component for binding to the macromolecule, could differ in other attributes, such as hapten valency (31, 32), lipophilicity (33), dissociation rate

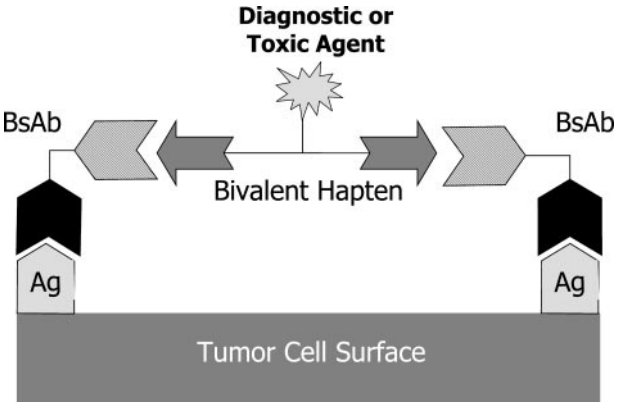

Fig. 2. The AES showing the cross-linking of 2 bsAb on the targeted tumor surface with one bivalent hapten. The bivalent hapten can carry a diagnostic or toxic agent.

(34), *in vivo* stability (35), binding affinity (36), and optical property (37), each of which may affect the distribution of the effectors in target as well as in normal organs.

A particular attribute that has proved to be influential on the tumor uptake of an effector is hapten valency. Effectors that contain a bivalent hapten are better tumor localizers than their monovalent analogs. The enhancement is believed to be because of the ability of the bivalent hapten to cross-link the pretargeted macromolecule at the tumor site, resulting in the formation of a more stable complex (Fig. 2) and, therefore, a longer tumor residence time. Bivalent haptens pretargeted with bsAb have been patented as the AES (38). As shown in the autoradiographs of Fig. 3, the clear tumor-to-nontumor contrast provided by the AES pretargeting method in an animal model with human small cell lung carcinoma expressing NCAM with a bispecific anti-NCAM × anti-HSG conjugate (NK1NBl1 × 679, Fab′ × Fab′) and a radioiodinated (^{125}I) di-HSG hapten is striking, compared with that obtained from a directly radiolabeled anti-NCAM antibody (39). Excellent images were also obtained (40) in nude mice bearing human renal cell carcinoma xenografts, by pretargeting with a bispecific antirenal cell carcinoma × anti-indium-DTPA conjugate (G250 × 734, Fab′ × Fab′) for 72 h followed by ^{111}In-labeled di-DTPA hapten (Fig. 4). Clinical trials (41) using the AES pretargeting method with a bispecific anti-CEA × anti-indium-DTPA conjugate (hMN14 × 734, Fab′ × Fab′) and a radioiodinated (^{131}I) di-indium-DTPA hapten (Pentacea; IBC Pharmaceuticals, Morris Plains, NJ) also produced impressive images of tumor uptake with high target-to-background contrast, as shown in Figs. 5 and 6 for two patients with CEA-expressing cancers.

The different pretargeting systems that have been investigated are grouped into nine categories in Table 1 according to the constituent of the targeting macromolecule. Within each assigned category, they may be further differentiated with respect to the following parameters: molecular size of the macromolecule, molecular form of the antibody component comprising the macromolecule, method of preparing the macromolecule or source of the macromolecule, valency of the macromolecule to the target, valency of the macromolecule to the hapten or biotin, hapten valency of the effector to the macromolecule, and other functional groups carried by the effector. The search for an effective pretargeting system worthy of commercialization necessitates continuous optimization of several or all of these parameters.

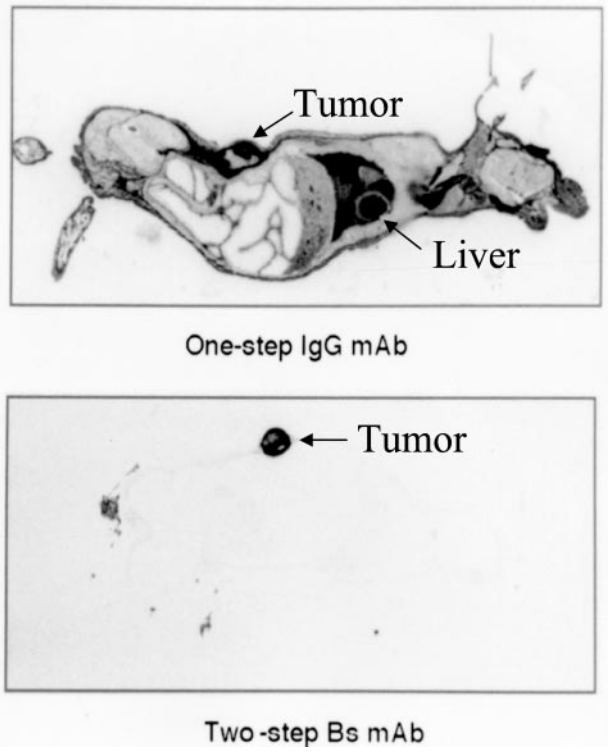

Fig. 3. Autoradiographs of mice bearing National Cancer Institute-H69 tumor obtained with a directly radiolabeled (^{125}I) N1KNBL1 (*top panel*) or a radiolabeled (^{125}I) di-HSG hapten pretargeted with bispecific NK1NBL1 × 679 for 48 h (*bottom panel*). Both autoradiographs were taken at 48 h after injection of the respective radiolabeled agents (from Ref. 39, with permission).

Early Demonstration of Pretargeting Concept

Before the term "pretargeted immunoscintigraphy" first appeared in publication (44) in 1987, the feasibility of using a bsAb or an antihapten antibody to increase the tumor concentration of diagnostic imaging agents had been assessed in several seminal studies with encouraging results (45–47). These earlier achievements were facilitated by the availability of a murine monoclonal antibody, CHA255, having a high binding affinity (Kd = 10^{-9} to 10^{-10} M) for indium benzyl EDTA (48) and a synthetic bsAb, ZCE × CHA (49), prepared by linking the Fab′ of an anti-CEA murine monoclonal antibody (ZCE025) with the Fab′ of CHA 255. Coadministration of ^{111}In benzyl EDTA or derivatives of ^{111}In benzyl EDTA containing cobalt-bleomycin (BLEDTA II or BLEDTA IV) with a sufficient amount of CHA255 was found to alter profoundly the pharmacokinetics of these small molecules in tumor-bearing mice (45). Specifically, with CHA255 acting as an *in vivo* carrier, the biological half-life of these small molecules was increased from minutes to days, and the concentrations

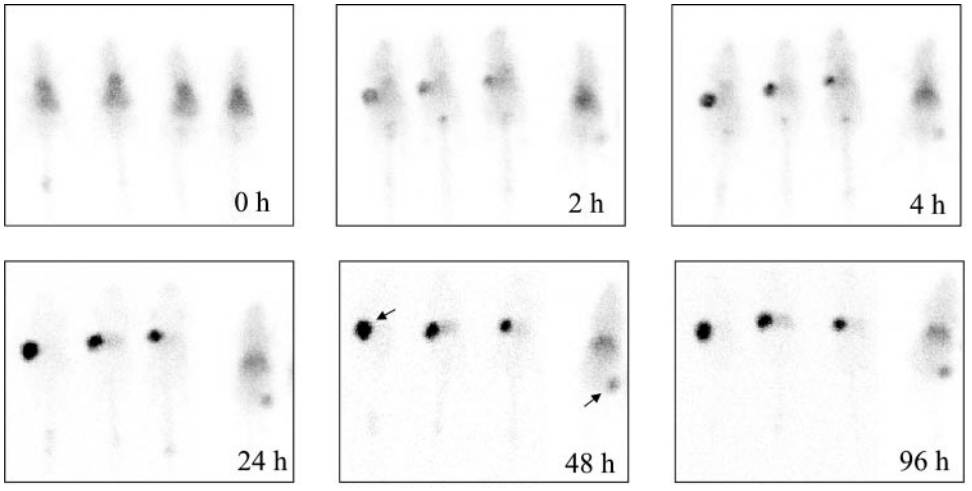

Fig. 4. Whole body images of NU-12 tumor-bearing mice targeted with a directly radiolabeled (^{111}In) monoclonal antibody G250 or a radiolabeled (^{111}In) di-DTPA hapten administered 3 days after the bsAb G250 × DTIn1. Each frame shows one mouse (*far right image*) using the one-step approach and three mice (the remaining three images) using the two-step pretargeting approach, at the indicated time points. The location of tumor was indicated with an *arrow* in the 48-h frame (from Ref. 40, with permission).

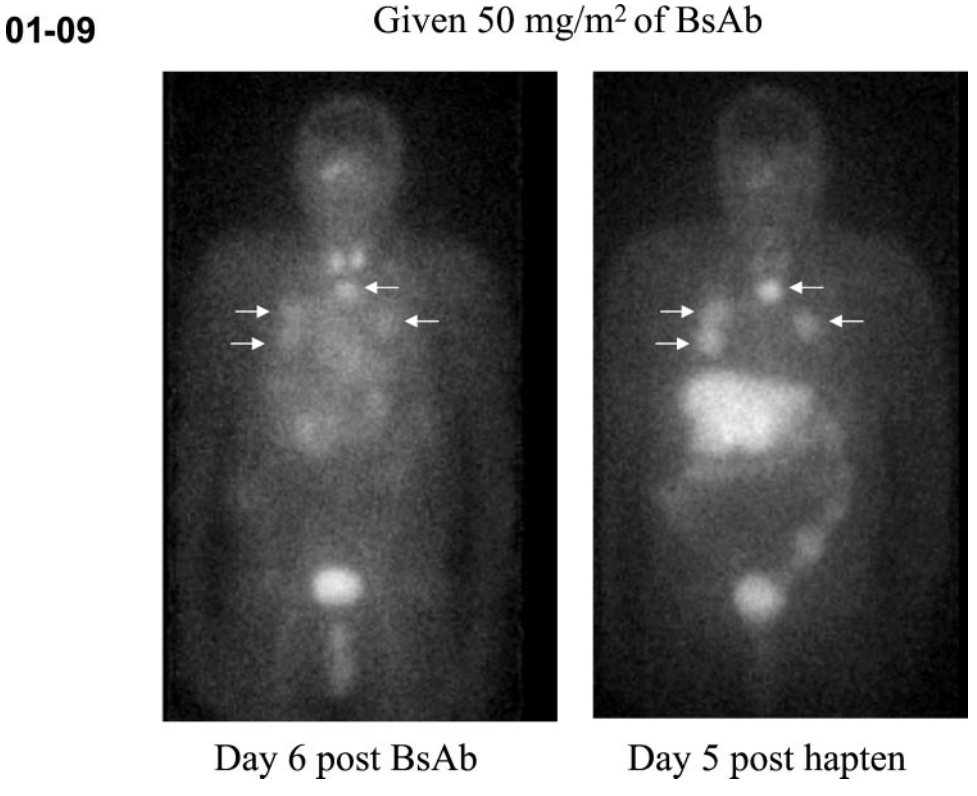

Fig. 5. Anterior views of a patient (*01–09*) with rectum carcinoma on day-6 postinjection of ^{131}I-labeled hMN14 × 734 (50 mg/m^2, 10 mCi, *left panel*) and on day-5 postinjection of ^{131}I-labeled Pentacea (100 mCi, 70 μg, *right panel*). The hapten was injected 7 days after the bsAb. The malignant lesions, as documented by computed tomography of the chest, lungs, and liver, were revealed in both images (indicated by *arrows* for those in the chest and lungs), but the contrast was much greater with the hapten.

of radioactivity in all organs, most notably tumor and blood, were markedly higher, when measured at 24 h. Furthermore, the injection of a "flushing" dose of unlabeled indium benzyl EDTA was effective at reducing more radioactivity in blood than in tumor, resulting in as much as a 50-fold increase in the tumor:blood ratio (from 0.8 to 47.1 for ^{111}In-benzyl EDTA) within 3 h. Comparable improvements (46, 47) in tumor:background ratios were observed in tumor-bearing mice for ZCE × CHA, using ^{111}In benzyl EDTA or ^{111}In BLEDTA IV premixed with a finite amount of ZCE × CHA as a carrier to ensure a higher tumor uptake. In an optimized protocol in which 14 μg of the synthetic bispecific Fab′ × Fab′ conjugate were allowed to prelocalize for 24 h before injecting 10μCi of ^{111}In BLEDTA IV premixed with 3 μg of ZCE × CHA, the concentration of radioactivity in tumor at 24 h was 7% ID/gram (50), with the tumor:blood ratio being ~5. Presumably, transferring ^{111}In BLEDTA IV from circulating ZCE × CHA to prelocalized ZCE × CHA at the tumor had occurred efficiently to account for the observed *in vivo* targeting. Additional biodistribution studies (33, 50) undertaken to compare BLEDTA IV with a series of benzyl EDTA analogs concluded that ^{111}In hydroxyethylthiourea-benzyl-EDTA, while

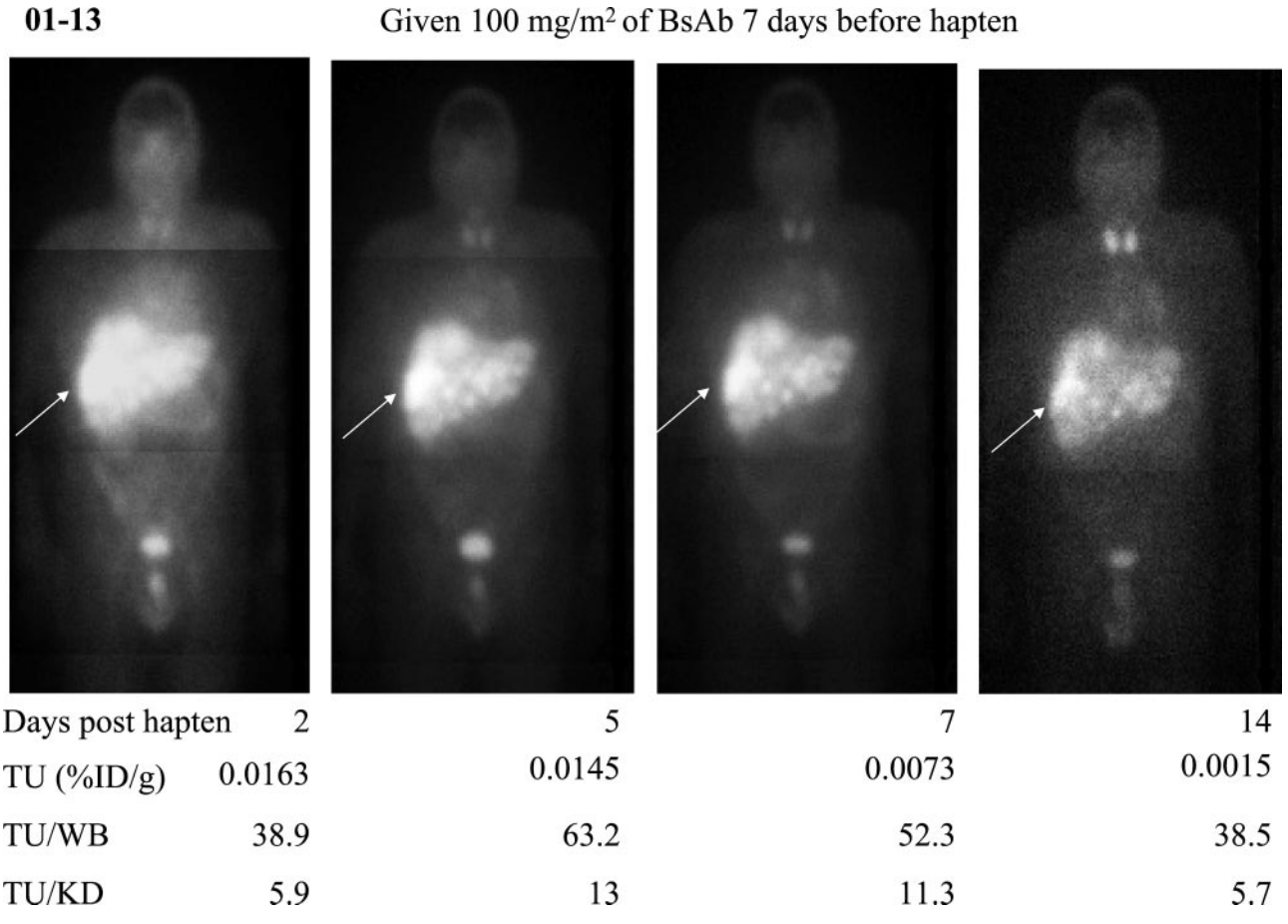

Fig. 6. Anterior views of a patient (*01–13*) with small cell lung carcinoma on days 2, 5, 7, and 14 postinjection of ^{131}I-labeled Pentacea (100 mCi, 70 μg). The hapten was injected 7 days after the bsAb (100 mg/m^2). The multiple metastases, as documented by computed tomography in the liver, were remarkably shown in all images. The %ID/gram in the tumor (*TU*), the ratio of tumor:whole body (*TU:WB*), and the ratio of tumor:kidney (*TU:KD*) were measured for the hottest and largest tumor in the external part of the right lobe (*arrow*). The calculated values were shown for each time point under the respective panels.

showing similar uptake in normal tissues as ^{111}In BLEDTA IV, had a remarkable increase in tumor concentration, topping 18% ID/gram at 24 h (50).

Critical Factors of Pretargeting

As the general principles of pretargeting were being demonstrated, a number of key factors that could affect the tumor-targeting potential of a hapten-antibody delivery system was also elucidated. Subsequent efforts to address these factors to the advantage of pretargeting applications have led to various refinements of the original approach exemplified by ZCE $\times$ CHA and ^{111}In BLEDTA IV (42).

Binding Constant. A higher affinity of hapten for antibody is important to pretargeting, because it would enhance the noncovalent association between antibody and hapten, thus reducing the amount of antibody required for capturing hapten at the target site. Because binding constants of antibodies rarely exceed 10^{-10} M, additional improvements coming from binding constants would have to rely on other existing noncovalent systems that display affinities much higher than 10^{-10} M. Accordingly, the very high affinity of biotin for avidin or streptavidin ($K_d = 10^{-15}$ M, about six to seven orders of magnitude above the binding constants usually achievable with antibodies) was distinguished immediately, and the suitability of biotin-avidin or biotin-streptavidin for pretargeting applications was quickly established (26). Pretargeting systems based on the binding of biotin to avidin or streptavidin have been developed, and some tested extensively in the clinic (27, 51, 52). The simplest system involves only streptavidin and a labeled biotin-chelate conjugate. Pretargeting with streptavidin followed by ^{111}In labeled EDTA-hydrazino-biotin was shown to be useful for imaging infectious lesions in a mouse model (21). Other systems are more elaborate and may consist of one of the following: (*a*) pretargeting with a streptavidin-antibody conjugate followed by a radiolabeled biotin-chelate (25); (*b*) pretargeting with a streptavidin-antibody conjugate followed by a clearing agent and then a radiolabeled biotin-chelate (51–55); (*c*) pretargeting with an avidin-antibody conjugate followed by a radiolabeled biotin-chelate (26); (*d*) pretargeting with a biotin-antibody conjugate followed by a radiolabeled streptavidin (29); (*e*) pretargeting with a biotin-antibody conjugate followed by avidin and then

Table 1 Pretargeting systems investigated[a]

Category	Targeting macromolecule					Effector			Representative references
	Constituent	Source or production method	Molecular size	Molecular form of Ab	Valency toward target	Valency toward effector	Constituent	Valency of effector	Function group other than chelate or biotin	
I	Ab	Hybridoma	M_r 160,000	IgG	0	2	Hapten-chelate	1		20
					0	2		2		32
II	SA	Commercial	M_r 54,000		0	4	Biotin-chelate	1		21
III	BsAb	Chemical conjugation	M_r 150,000	$F(ab')_2 \times Fab'$	2	1	Hapten-chelate	1		31
					2	1		2		31
			M_r 100,000	$Fab' \times Fab'$	1	1		1	Cobalt-bleomycin	42
					1	1		2		39
		Recombinant	M_r 54,000	Diabody	1	1		2		43
IV	Ab-SA	Chemical conjugation	M_r 215,000	IgG	2	4	Biotin-chelate	1		25
V	Ab-A	Chemical conjugation	M_r 225,000	IgG	2	4	Biotin-chelate	1		26
VI	Ab-B	Chemical conjugation	M_r 160,000	IgG	2	Multiple	Biotin-chelate	1		27
				IgG	2	Multiple	SA	4		29
			M_r ~50,000	Fab′	1	Multiple	rSA	4		28
VII	Ab-DNA	Chemical conjugation	M_r ~170,000	IgG	2	Multiple	DNA-tyrosine			30
VIII	SA/B-PNA	Complex formation	?		2	Multiple	PNA-chelate			23
IX	Ab-E	Chemical conjugation or recombinant	M_r 74–250,000	IgG; $F(ab')_2$; Fab′, scFv	2 or 1		Prodrug			19

[a] Ab, antibody; SA, streptavidin; rSA, recombinant SA; Ab-SA, antibody-streptavidin conjugate; Ab-A, antibody-avidin conjugate; Ab-B, biotinylated antibody; Ab-DNA, antibody-DNA conjugate; SA/B-PNA, complex of streptavidin and biotinylated PNA; Ab-E, antibody-enzyme conjugate.

a radiolabeled biotin-chelate (56); and (*f*) pretargeting with a biotin-antibody conjugate followed by avidin, then streptavidin, and finally a radiolabeled biotin-chelate (57). Thus, procedures can consist of two, three, or more steps, all intended to increase tumor:nontumor ratios. However, both endogenous biotin and the immunogenicity of avidin and streptavidin can affect the results (54, 58). In addition to (strep)avidin–biotin, other recognition systems conferring high specificity and affinity, either based on complementary oligonucleotide binding (30) or enzyme-substrate interaction (19), have also been adapted for pretargeting applications. The preference of higher affinity also applies to the binding of antibody to target antigen. For a bsAb (monovalent Fab′ × monovalent Fab′) with a K_d for target antigen already in the 10^{-9} to 10^{-10} M range, additional improvements may still be possible by incurring bivalent binding with a construct like $F(ab')_2 \times Fab'$ or an engineered bispecific protein preserving the bivalency for the target antigen.

Hapten Valency. Compared with their monovalent analogs, bivalent haptens have consistently shown higher tumor uptake, a phenomenon attributed to cross-linking antibodies that are specifically bound to tumor surface or nonspecifically accumulated in tumor. Bivalent haptens are used today in almost all pretargeting systems, including those involving biotin/(strept)avidin. The binding between a DNA antibody and its antisense oligonucleotides can also be regarded as multivalent. A variant of bivalent hapten is the so-called asymmetric bivalent hapten (59), which contains two different haptens on the same molecule, with each hapten suitable for binding to a different antihapten antibody. Asymmetric bivalent haptens were designed to increase the targeting specificity when used in conjunction with two distinct bsAb, which differ from each other in antigen, as well as hapten specificity (60). This triple-component system for pretargeting would ensure that only cells expressing double antigens could have accrued both bsAb, which in turn could be cross-linked only by a relevant asymmetric hapten. The advantage of bivalency also applies to the binding of antibodies to target antigen. A modified antibody capable of binding bivalently to target antigen is likely to show an increase in target residence time, which may be further extended by using a relevant bivalent hapten.

Specific Activity. The amount of macromolecule available for capturing hapten at the target site is usually very small (in the order of nmol/gram tumor in human subjects). Consequently, the amount of hapten deliverable to the target is quite limited. With a hapten labeled at a higher specific activity, increased radiation dose can be imparted per unit mass to effect more damage or easier detection of the target, thereby improving pretargeting efficacy. Specific activities not <1 mCi/nmol was thought necessary for good pretargeting results (6). In clinical studies, biotin-chelates or hapten-chelates labeled to specific activity ranging from 0.05 to 1.5 mCi/nmol have been acceptable.

Two factors pursuant to specific activity are choice of radionuclide and chelate. Radionuclides that have been evaluated for pretargeting applications include ^{111}In and ^{99m}Tc for single photon imaging (20–22, 25–27, 31, 32, 42, 50);

Table 2 Radionuclides of current interest for RAIT

Isotope	Half-life (h)	Useful energy (keV)			Maximum particle range (mm)	Production mode[a]	Suitable chelate
		α_{max}	β_{max}	γ			
^{131}I	193		610	364	2.0	Fission product	
^{90}Y	64		2,280		12.0	^{90}Sr/^{90}Y generator	DOTA
^{177}Lu	161		496	113, 208	1.5	^{176}Lu (n, γ)	DOTA
^{67}Cu	62		577	184	1.8	^{67}Zn (n, p)	TETA[b]
^{186}Re	91		1,080	137	5.0	^{185}Re (n, γ)	N_2S_2 or MAG3
^{188}Re	17		2,120	155	11.0	^{188}W/^{188}Re generator	N_2S_2 or MAG3
^{212}Bi	1	8,780			0.09	^{224}Ra/^{212}Pb generator	DOTA[c]
^{213}Bi	0.77	>6,000			<0.1	^{225}Ac/^{213}Bi generator	CHX-A-DTPA
^{211}At	7.2	7,450			0.08	^{207}Bi (α, 2n)	

[a] Other modes of production may be available.
[b] TETA, 1,4,8,11-tetraazaryclotetradecane-*N,N′,N″,N‴*-tetraacetic acid.
[c] ^{212}Bi is generated *in situ* from DOTA-complexed ^{212}Pb.

Table 3 Dosimetry data obtained in animal models with pretargeting

	Mean Absorbed Dose (cGy/mCi)						
	^{131}I (Ref. 39)[a]	^{131}I (Ref. 64)[b]	^{131}I (Ref. 64)[c]	^{131}I (Ref. 67)[d]	^{188}Re (Ref. 67)[e]	^{188}Re (Ref. 66)[f]	^{90}Y (Ref. 68)[g]
Tumor	3420	3190	1282	2598	1726	1954	2350
Liver	240	310	181	161	228	155	730
Spleen		264	103	104	104	132	230
Kidney	420	456	262	307	250	269	870
Lungs	650					150	480
Blood	550	470	164	322	454	407	110
Stomach						621	
Small int.				52	753	383	750
Large int.						87	
Bone				53	122		130
Bone marrow	200						
Heart							500
Muscle							260
Skin							100

[a] BsAb (anti-NCAM × anti-HSG), 0.5 nmol; 48 h; ^{125}I-labeled di-HSG hapten, 10 pmol; nude mice bearing human small cell lung cancer (National Cancer Institute-H69). Absorbed doses calculated on the basis of the biodistribution data of ^{125}I.
[b] BsAb (anti-CEA × anti-DTPA), 1 nmol; 20 h; ^{131}I-labeled di-DTPA hapten, 0.5 nmol; nude mice bearing human colon cancer xenografts (LS174T).
[c] BsAb (anti-CEA × anti-DTPA), 5 nmol; 48 h; ^{131}I-labeled di-DTPA hapten, 2.5 nmol; nude mice bearing human colon cancer xenografts (LS174T).
[d] BsAb (anti-CEA × anti-HSG), 0.5 nmol; 24 h; ^{125}I-labeled di-HSG hapten, 0.25 nmol; nude mice bearing human colon cancer xenografts (LS174T). Absorbed doses calculated on the basis of the biodistribution data of ^{125}I.
[e] BsAb (anti-CEA × anti-HSG), 0.5 nmol; 24 h; ^{188}Re-labeled di-HSG hapten, 0.25 nmol; nude mice bearing human colon cancer xenografts (LS174T).
[f] BsAb (anti-CEA × anti-DTPA), 0.15 nmol; 24 h; ^{188}Re-labeled di-DTPA hapten, 16 pmol; nude mice bearing human colon cancer xenografts (GW-39).
[g] Anti-yttrium-DOTA mouse monoclonal antibody (2D12.5), 033–0.67 nmol, 20 h; chase, 1 h; ^{88}Y-labeled di-DOTA hapten, 0.484 nmol; BALB/c mice bearing KHJJ mouse breast adenocarcinoma. Absorbed doses calculated on the basis of the biodistribution data of ^{88}Y.

^{68}Ga for PET imaging (37, 61, 62); ^{131}I, ^{90}Y, and ^{188}Re for β-particle therapy (36, 51–53, 63–68); and ^{212}Bi for α-particle therapy (13). Table 2 lists several physical features (half-life, energy of emission, type of emission, maximum particle range, and production mode) of radionuclides that are of current interest for RAIT. Additionally listed in Table 2 are characteristic chelating agents capable of forming stable *in vivo* complexes with each radiometal. For α-emitters, more work may be needed to develop better strategies or chelates for binding them in a stable manner. The subjects of therapeutic radionuclides and choices of their chelates have been reviewed elsewhere (69, 70).

Preferably, a therapeutic radionuclide selected for use with a particular pretargeting system should possess the following properties: (*a*) a half-life similar to that of the radiolabeled hapten-chelate at the target site; (*b*) the ability to discharge particles of higher energy for effective cell killing; (*c*) the availability of a suitable hapten-chelate for efficient labeling and the resultant formation of a stable *in vivo* complex that exhibits rapid diffusion, little uptake in nontarget tissues, and fast clearance; and (*d*) the feasibility of production with a generator after easy elution. The most prevalent radionuclide used for therapy in clinical trials today is still ^{131}I, despite its relatively long half-life (8 days), suboptimum energy of β-particle, and concomitant emission of γ rays, which requires isolation of patients in shielded rooms for several days but provides the benefit of imaging. Compared with ^{131}I, ^{90}Y is almost ideal for therapy with pretargeting approaches, because: (*a*) it has a half-life (67 h) similar to that of the radiolabeled effector after prelocalization of the targeting macromolecule; (*b*) it discharges only electrons of higher energy to cause deeper tissue penetration; (*c*) it can be labeled efficiently with DOTA-bearing haptens, forming a stable *in vivo* com-

Table 4 Biodistribution[a] of ^{111}In-IMP241 with pretargeted BS1.5 *vs.* hMN14 × 679

	BS1.5 (8 h)		hMN14 × 679 (24 h)		BS1.5 (8 h)		hMN14 × 679 (24 h)	
	3 h	T/NT	3 h	T/NT	24 h	T/NT	24 h	T/NT
	%ID/gram		%ID/gram		%ID/gram		%ID/gram	
Tumor	10.31 ± 2.70		11.28 ± 2.17		6.33 ± 2.21		6.87 ± 0.84	
Liver	0.15 ± 0.02	68	0.53 ± 0.14	22	0.13 ± 0.01	48	0.31 ± 0.05	22
Spleen	0.07 ± 0.01	157	0.42 ± 0.12	28	0.05 ± 0.01	126	0.40 ± 0.13	18
Kidney	3.58 ± 0.76	3	4.61 ± 0.71	2.5	2.29 ± 0.45	3	2.60 ± 0.43	2.7
Lung	0.21 ± 0.01	51	0.83 ± 0.23	14	0.05 ± 0.01	116	0.29 ± 0.05	24
Blood	0.06 ± 0.01	167	1.44 ± 0.33	8	0.01 ± 0.00	631	0.43 ± 0.10	16
Stomach	0.02 ± 0.01	488	0.11 ± 0.02	103	0.02 ± 0.00	270	0.06 ± 0.01	117
Small intestine	0.13 ± 0.10	116	0.23 ± 0.08	53	0.04 ± 0.01	166	0.10 ± 0.02	67
Large intestine	0.19 ± 0.05	59	0.31 ± 0.07	37	0.06 ± 0.02	113	0.11 ± 0.03	66

[a] The biodistribution data shown were obtained with pretargeted BS1.5 or hMN14 × 679 in nude mice bearing human colon cancer xenografts (GW-39). BS1.5 and hMN14 × 679 were allowed to prelocalize for 8 and 24 h, respectively, before injecting ^{111}In-IMP241. The animals were sacrificed at 3 and 24 h postinjection of ^{111}In-IMP241.

plex that exhibits satisfactory pharmacokinetics; and (*d*) it can be produced by a generator and available commercially with acceptable purity. Other therapeutic radionuclides with one or more favorable physical properties than ^{131}I are ^{188}Re and several α-emitters under active development. Like ^{90}Y, ^{188}Re is available carrier free and, except for a shorter half-life (17 h), has three of the four attributes characteristic of ^{90}Y as a promising therapeutic radionuclide. It is noted that short-lived radionuclides ($t_{1/2}$ about hours), α-emitters in particular, are necessarily restricted to rapid targeting processes, which are feasible with pretargeting approaches because the radiolabel resides on a fast-diffusing, small molecule. Radioisotopes emitting α-particles offer several advantages over β-emitters for therapy: (*a*) discharged α-particles with a mass 8,000 times greater than β-particles exhibit high linear energy transfer (100 KeV/μm) and are therefore very cytotoxic, requiring as few as 6 or 7 disintegrations for internalized α-emitters and ~25 disintegrations for surface-bound α-emitters to kill a cell; (*b*) the extremely short path length (40–80 μm) of α-particles greatly reduces the nonspecific irradiation of normal tissue around the target cell; (*c*) the DNA damage caused by α-particles is not easily repaired by the cell; and (*d*) the cytotoxicity of α-particles is not affected by oxygen, and, therefore, cell killing is effective under hypoxic conditions usually existing in established tumors. However, the lack of suitable chemistry or effective chelates for preparing stably attached α-emitters has hampered their further development as agents for pretargeting applications. Table 3 compares the dosimetry data obtained from animal studies with ^{131}I, ^{90}Y, and ^{188}Re under specified pretargeting conditions, as reported in a limited number of publications (39, 64, 66–68). In each case, the tumor was shown to receive a radiation dose considerably higher than any of the normal organs.

In addition to those discussed above, numerous other factors have also been considered as important for improving pretargeting efficacy, *e.g.,* issues like molecular size, immunogenicity, target specificity, antigen density, and antigen modulation should be carefully considered in the design and construction of ultimate targeting macromolecules. As for the hapten-chelates or biotin-chelates, molecular size, immunogenicity, clearance rate, elimination route, and *in vivo* stability deserve proper attention. Pretargeting systems using a chase step may further face optimization issues involving the chase molecule, such as molecular size, valency, immunogenicity, clearance rate (71), and elimination route.

Recent Progress and Future Directions

The success of receptor targeting by peptides, as demonstrated by the regulatory approvals of peptide imaging agents, such as ^{111}In-DTPA-pentatreotide for human neuroendocrine tumors, has prompted the adaptation of peptides for pretargeting approaches. One possible system described by Goodwin and Meares (5) involves the conjugation of receptor-specific peptides to long-circulating PEG, which is also derived with an avidin or streptavidin for the recognition by a radiolabeled DOTA-biotin dimer. By using PEG with branched structures, multiple peptides and other optional agents can be attached, along with avidin or streptavidin, thereby yielding a targeting macromolecule with many of the desirable features, including multivalency for the target (through multiple peptide), multivalency for the effector (through multiple binding sites on avidin/streptavidin for biotin), reduced immunogenicity (via PEG), prolonged circulation to reach high target concentration (via PEG of suitable molecular size), and the option of rapid clearance with the use of a biotin-PEG conjugate, which should be nonimmunogenic.

The trend at using smaller antibody fragments for *in vivo* applications has spurred the development of bispecific diabody or bispecific tetrabodies suitable for pretargeting approaches. We have produced two bispecific diabodies (BS1.5 and BS1.5H) for pretargeted delivery of radiolabeled bivalent haptens to tumors expressing CEA (43). BS1.5 (M_r ~54,000) consists of two heterologous polypeptide chains associated noncovalently to form one binding site for CEA from the variable domains of hMN14 (a humanized anti-CEA antibody from Immunomedics, Inc., Morris Plains, NJ) and one binding site for HSG from the variable domains of 679 (a murine monoclonal antibody specific for HSG). In

BS1.5H, humanized 679 V_H and V_K domains were used. The V domains were engineered into a dicistronic *Escherichia coli* expression vector for directing the synthesis of the two polypeptides, 679V_H-GGGGS-hMN14V_K−6His and hMN14V_H-GGGGS-679V_K−6His, in the periplasmic space of *E. coli*. The diabody was purified from the soluble fraction of isopropyl-1-thio-β-D-galactopyranoside-induced *E. coli* culture by Immobilized Metal Ion Affinity Chromatography and ion exchange chromatography. Kinetic analyses on BIAcore using HSG-immobilized sensor chips showed that the binding affinity for HSG was similar for BS1.5, BS1.5H, and chemically linked hMN14 × 679 (M_r ~100,000, Fab′ × Fab′). The bispecificity of BS1.5 or BS1.5H was demonstrated on BIAcore by measuring the additional increase in response units on successive injections of the bispecific diabody followed by CEA. The utility of BS1.5 for tumor pretargeting was evaluated in CEA human tumor-bearing mice using a bivalent HSG hapten (IMP-241) labeled with ^{111}In, and the results obtained were compared with those of hMN14 × 679. For BS1.5 injected 8 h before giving the hapten, tumor uptake of ^{111}In was 10.3 and 6.3% ID/gram at 3 and 24 h, respectively, with the tumor:blood ratios being 167 at 3 h and 631 at 24 h. For hMN14 × 679 injected 24 h before giving the hapten, the tumor uptakes of ^{111}In were 11.3 and 6.9% ID/gram at 3 and 24 h, respectively, with the tumor:blood ratios being 8.1 at 3 h and 16.4 at 24 h. Thus, the concentrations of hapten in the tumor were similar between BS1.5 prelocalized for 8 h and hMN14 × 679 prelocalized for 24 h. However, because the radioactivity in all nontumor organs was higher for hMN14 × 679, the tumor:nontumor ratios were superior for BS1.5. These results, as summarized in Table 4, indicate that BS1.5 is an attractive candidate for use in a variety of pretargeting applications.

The potential of using a radiosensitizer to augment the therapeutic efficacy of RAIT delivered by the AES pretargeting method has also been demonstrated. In one study (72), paclitaxel, but not doxorubicin, was shown to improve the antitumor response in animals given an anti-CEA, anti-indium-DTPA bsAb (F6 × 734), followed by ^{131}I-labeled bivalent hapten and the chemotherapeutic drugs. A synergistic effect was observed without increasing toxicity.

These promising developments seem to indicate that the future evolution of pretargeting technology will be directed to at least three potential approaches. The first is to use peptides instead of antibodies for specific targeting. The second is to replace chemically linked bsAb conjugates with recombinant fusion proteins of multispecificity and multivalency. The third is to investigate whether a certain chemotherapeutic agent, which is also a known radiosensitizer, can produce synergistic effects when combined with a pretargeting system for treating a particular cancer. In addition, the potential of a hapten or hapten-chelate that also contains a receptor-binding moiety for enhancing the specific uptake in receptor-positive target cells is worth exploring. Needless to say, the urgent need to establish, for each pretargeting system in carefully designed clinical trials, the optimal amount of the targeting macromolecule, the best timing of the second injection with the effector ("two-step protocol") or the chaser ("three-step protocol"), the maximum tolerated dose, and the therapeutic efficacy for a certain indication continues to exist and should serve as a basis of future experimentation.

References

1. Goldenberg, D. M. Radioimmunotherapy. *In:* L. M. Freeman (ed.), Nuclear Medicine Annual 2001, pp. 167–206. Philadelphia: Lippincott Williams & Wilkins, 2001.

2. Goldenberg, D. M. The role of radiolabeled antibodies in the treatment of non-Hodgkin's lymphoma: the coming of age of radioimmunotherapy. Crit. Rev. Oncol. Hematol., *39:* 195–201, 2001.

3. Govindan, S. V., Goldenberg, D. M., Hansen, H. J., and Griffiths, G. L. Advances in the use of monoclonal antibodies in cancer radiotherapy. Pharm. Sci. Techn. Today, *3:* 90–98, 2000.

4. Goodwin, D. A. Pharmacokinetics and antibodies. J. Nucl. Med., *28:* 1358–1362, 1987.

5. Goodwin, D. A. New methods for localizing infection: a role for avidin-biotin? J. Nucl. Med., *33:* 1816–1818, 1992.

6. Goodwin, D. A. Tumor pretargeting: almost the bottom line. J. Nucl. Med., *36:* 876–879, 1995.

7. Lovqvist, A., and Divgi, C. Bullets to magic bullets–and miles to go before we sleep. J. Nucl. Med., *39:* 1776–1777, 1998.

8. Goodwin, D. A., and Meares, C. F. Pretargeted peptide imaging and therapy. Cancer Biother. Radiopharm., *14:* 145–152, 1999.

9. Le Doussal, J. M., Barbet, J., and Delaage, M. Bispecific-antibody-mediated targeting of radiolabeled bivalent haptens: theoretical, experimental, and clinical results. Int. J. Cancer (Suppl.), *7:* 58–62, 1992.

10. Chatal, J-F., Faivre-Chauvet, A., Bardies, M., Peltier, P., Gautherot, E., and Barbet, J. Bifunctional antibodies for radioimmunotherapy. Hybridoma, *14:* 125–128, 1995.

11. Rosebrough, S. F. Two-step immunological approaches for imaging and therapy. Q. J. Nucl. Med., *40:* 234–251, 1996.

12. Goodwin, D. A., and Meares, C. F. Pretargeting: general principles. Cancer (Suppl.), *80:* 2675–2680, 1997.

13. Fritzberg, A. R. Antibody pretargeted radiotherapy: a new approach and a second chance. J. Nucl. Med., *39:* 20N, 1998.

14. Barbet, J., Kraeber-Bodéré, F., Vuillez, J-P., Gautherot, E., Rouvier, E., and Chatal, J-F. Pretargeting with the affinity enhancement system for radioimmunotherapy. Cancer Biother. Radiopharm., *14:* 153–166, 1999.

15. Gruaz-Guyon, A., Janevik-Ivanovska, E., Raguin, O., De Labriolle-Vaylet, C., and Barbet, J. Radiolabeled bivalent haptens for tumor immunodetection and radioimmunotherapy. Q. J. Nucl. Med., *45:* 201–206, 2001.

16. Wahl, R. L., Parker, C. W., and Philpott, G. W. Improved radioimaging and tumor localization with monoclonal F(ab′)$_2$. J. Nucl. Med., *24:* 316–325, 1983.

17. Blumenthal, R. D., Sharkey, R. M., and Goldenberg, D. M. Overcoming dose-limiting, radioantibody-induced myelotoxicity. *In:* D. M. Goldenberg (ed.), Cancer Therapy with Radiolabeled Antibodies, pp. 295–314. Boca Raton, FL: CRC Press, 1995.

18. Haseman, M. K., Goodwin, D. A., Meares, C. F., Kaminski, M. S., Wensel, T. G., McCall, M. J., and Levy, R. Metabolizable In-111 chelate conjugated anti-idiotype monoclonal antibody for radioimmunodetection of lymphoma in mice. Eur. J. Nucl. Med., *12:* 455–460, 1986.

19. Bagshawe, K. D., Sharma, S. K., Burke, P. J., Melton, R. G., and Knox, R. J. Developments with targeted enzymes in cancer therapy. Curr. Opin. Immunol., *11:* 579–583, 1999.

20. Goodwin, D. A., Meares, C. F., McCall, M. J., McTigue, M., and Chaovapong, W. Pre-targeted immunoscintigraphy of murine tumors with indium-111-labeled bifunctional haptens. J. Nucl. Med., *29:* 226–234, 1988.

21. Rusckowski, M., Fritz, B., and Hnatowich, D. J. Localization of infection using straptavidin and biotin: an alternative to nonspecific polyclonal immunoglobulin. J. Nucl. Med., *33:* 1810–1815, 1992.

22. Hnatowich, D. J., Fritz, B., Virzi, F., Mardirossian, G., and Rusckowski, M. Improved tumor localization with (strept)avidin and labeled biotin as a substitute for antibody. Nucl. Med. Biol., *20:* 189–193, 1993.

23. Rusckowski, M., Qu, T., Chang, F., and Hnatowich, D. J. Pretargeting using peptide nucleic acid. Cancer (Suppl.), *80:* 2699–2705, 1997.

24. Cao, Y., and Suresh, M. Bispecific antibodies as novel bioconjugates. Bioconjug. Chem., *9:* 635–644, 1998.

25. Kalofonos, H. P., Rusckowski, M., Siebecker, D. A., Sivolapenko, G. B., Snook, D., Lavender, J. P., Epenetos, A. A., and Hnatowich, D. J. Imaging of tumor in patients with indium-111-labeled biotin and streptavidin-conjugated antibodies: preliminary communication. J. Nucl. Med., *31:* 1791–1796, 1990.

26. Hnatowich, D. J., Virzi, F., and Rusckowski, M. Investigations of avidin and biotin for imaging applications. J. Nucl. Med., *28:* 1294–1302, 1987.

27. Paganelli, G., Magnani, P., Zito, F., Villa, E., Sudati, F., Lopalco, L., Rossetti, C., Malcovati, M., Chiolerio, F., Seccamani, E., Siccardi, A. G., and Fazio, F. Three-step monoclonal antibody tumor targeting in carcinoembryonic antigen-positive patients. Cancer Res., *51:* 5960–5966, 1991.

28. Wilbur, D. S., Hamlin, D. K., Vessella, R. L., Stray, J. E., Buhler, K. R., Stayton, P. S., Klumb, L. A., Pathare, P. M., and Weerawarna, S. A. Antibody fragments in tumor pretargeting. Evaluation of biotinylated Fab′ colocalization with recombinant streptavidin and avidin. Bioconjug. Chem., *7:* 689–702, 1996.

29. Saga, T., Weinstein, J. N., Jeong, J. M., Heya, T., Le, J. T., Le, N., Paik, C. H., Sung, C., and Neumann, R. Two-step targeting of experimental lung metastases with biotinylated sntibody and radiolabeled streptavidin. Cancer Res., *54:* 2160–2165, 1994.

30. Kuijpers, W. H. A., Bos, E. S., Kaspersen, F. M., Veeneman, G. H., and van Boeckel, C. A. A. Specific recognition of antibody-oligonucleotide conjugates by radiolabeled antisense nucleotides: a novel approach for two-step radioimmunotherapy of cancer. Bioconjug. Chem., *4:* 94–102, 1993.

31. Le Doussal, J-M., Martin, M., Gautherot, E., Delaage, M., and Barbet. J. *In vitro* and *in vivo* targeting of radiolabeled monovalent and divalent haptens with dual specificity monoclonal antibody conjugates: enhanced divalent hapten affinity for cell-bound antibody conjugate. J. Nucl. Med., *30:* 1358–1366, 1989.

32. Goodwin, D. A., Meares, C. F., McTigue, M., Chaovapong, W., Diamanti, C. I., Ransone, C. H., and McCall, M. J. Pretargeted immunoscintigraphy: effect of hapten valency on murine tumor uptake. J. Nucl. Med., *33:* 2006–2013, 1992.

33. Anderson, L. D., Meyer, D. L., Battersby, T. R., Frincke, J. M., Mackensen, D., Lowe, S., and Connolly, P. Optimization of ligand structure for imaging with a bifunctional antibody. J. Nucl. Med., *29:* 835–836, 1988.

34. Meyer, D. L., Fineman, M., Unger, B. W., and Frincke, J. M. Kinetics of the dissociation of indium-(p-Substituted-benzyl)ethylenediaminetetraacetic acid hapten analogues from the monoclonal anti-hapten antibody CHA255. Bioconjug. Chem., *1:* 278–284, 1990.

35. Gruaz-Guyon, A., Gras-Masse, H., and Le Doussal, J. M. Radiolabeled hapten derivatized peptides for tumor imaging with bispecific antibody conjugates. *In:* E. Giraldt and D. Andreu (eds.), Peptides 1990, pp. 822–825. The Netherlands: ESCOM, Leiden, 1991.

36. Janevik-Ivanovska, E., Gautherot, E., de Boisferon, M. H., Cohen, M., Milhaud, G., Tartar, A., Rostene, W., Barbet, J., and Gruaz-Guyon, A. Bivalent hapten-bearing peptides designed for iodine-131 pretargeted radioimmunotherapy. Bioconjug. Chem., *8:* 526–533, 1997.

37. Klivényi, G., Schuhmacher, J., Patzelt, E., Hauser, H., Matys, R., Moock, M., Regiert, T., and Maier-Borst, W. Gallium-68 chelate imaging of human colon carcinoma xenografts pretargeted with bispecific anti-CD44V6/anti-gallium chelate antibodies. J. Nucl. Med., *39:* 1769–1776, 1998.

38. Barbet, J., Delaage, M., and Le Doussal, J-M. Affinity enhancement immunological reagents for *in vivo* detection and killing of specific target cells. U. S. Patent Number: 5,256,395. U. S. Patent Office, Washington, DC., 1993.

39. Hosono, M., Hosono, M. N., Kraeber-Bodéré, F., Devys, A., Thédrez, P., Faivre-Chauvet, A., Gautherot, E., Barbet, J., and Chatal, J-F. Two-step targeting and dosimetry for small cell lung cancer xenograft with anti-NACM/antihistamine bispecific antibody and radioiodinated bivalent hapten. J. Nucl. Med., *40:* 1216–1221, 1999.

40. Boerman, O. C., Kranenborg, M. H. G. C., Oosterwijk, E., Griffiths, G. L., McBride, W. J., Oyen, W. J. G., de Weijert, M., Oosterwijk-Wakka, J., Hansen, H. J., and Corstens, F. H. M. Pretargeting of renal cell carcinoma: improved tumor targeting with a bivalent chelate. Cancer Res., *59:* 4400–4405, 1999.

41. Kraeber-Bodéré, F., Faivre-Chauvet, A., Cerato, E., Devillers, A., Sharkey, R. M., Chang, K., Goldenberg, D. M., Barbet, J., and Chatal, J-F. Clinical optimization of two-step radioimmunotherapy (RAIT) using an ^{131}I-labeled anti-CEA/anti-DTPA bispecific antibody and an ^{131}I-labeled bivalent DTPA hapten in patients with CEA-expressing tumor. J. Nucl. Med. (Suppl.), *42:* 123P, 2001.

42. Stickney, D. R., Slater, J. B., Kirk, G. A., Ahlem, C., Chang, C-H., and Frincke, J. M. Bifunctional antibody: ZCE/CHA ^{111}Indium BLEDTA-IV clinical imaging in colorectal carcinoma. Antibody Immunoconjug. Radiopharm., *2:* 1–13, 1989.

43. Sharkey, R. M., Rossi, E., Karacay, H., McBride, W., Chang, K., Zeng, L., Qu, T., Griffiths, G., Hansen, H. J., and Goldenberg, D. M. A novel recombinant bispecific antibody pretargeting system for cancer radioimmunotherapy. Clin. Cancer Res. (Suppl.), *7:* 3786s, 2001.

44. Goodwin, D. A., Meares, C. F., McCall, M. J., McTigue, M., and Chaovapong, W. Pre-targeted immunoscintigraphy with chimeric antibodies. J. Nucl. Med., *28:* 561, 1987.

45. Goodwin, D. A., Meares, C. F., David, G. S., McTique, M., McCall, M. J., Frincke, J. M., Stone, M. R., Bartholomew, R. M., and Leung, J. P. Monoclonal antibodies as reversible equilibrium carriers of radiopharmaceuticals. Nucl. Med. Biol. *13:* 383–391, 1986.

46. Frincke, J. M., Chang, C-H., Ahlem, C. N., David, G. S., Bartholomew, R. M., Anderson, L. D., Hagen, P. L., Halpern, S. E., and Carlo, D. J. Pharmacokinetics of bifunctional antibody delivered In-111 benzyl EDTA to colon tumors in nude mice. J. Nucl. Med., *27:* 1042, 1986.

47. Frincke, J. M., Halpern, S. E., Chang, C.-H., Ahlem, C., Anderson, L. D., Bartholomew, R. M., Poggenburg, J. K., Hagan, P. L., and Carlo, D. J. Radioimmunodetection approach using an ^{111}In hapten monoclonal antibody: studies in the nude mouse-human colon tumor model. J. Nucl. Med., *28:* 711, 1987.

48. Reardan, D. T., Meares, C. F., Goodwin, D. A., McTigue, M., David, G. S., Stone, M. R., Leung, J. P., Bartholomew, R. M., and Frincke, J. M. Antibodies against metal chelates. Nature (Lond.), *316:* 265–268, 1985.

49. Chang, C-H., Ahlem, C. N., Wolfert, B., Hochschwender, S. M., Jue, R., Frincke, J. M., and Carlo, D. J. Preparation and characterization of bifunctional antibodies with reactivity to carcinoembryonic antigen and indium benzyl EDTA. J. Nucl. Med., *27:* 1041, 1986.

50. Stickney, D. R., Anderson, L. D., Slater, J. B., Ahlem, C. N., Kirk, G. A., Schweighardt, S. A., and Frincke, J. M. Bifunctional antibody: a binary radiopharmaceutical delivery system for imaging colorectal carcinoma. Cancer Res., *51:* 6650–6655, 1991.

51. Knox, S. J., Goris, M. L., Tempero, M., Weiden, P. L., Gentner, L., Breitz, H., Adams, G. P., Axworthy, D., Gaffigan, S., Bryan, K., Fisher, D. R., Colcher, D., Horak, I. D., and Weiner, L. M. Phase II trial of yttrium-90-DOTA-biotin pretargeted by NR-LU-10 antibody/streptavidin in patients with metastatic colon cancer. Clin. Cancer Res., *6:* 406–414, 2000.

52. Breitz, H. B., Weiden, P. L., Beaumier, P. L., Axworthy, D. B., Seiler, C., Su, F-M., Graves, S., Bryan, K., and Reno, J. Clinical optimization of pretargeted radioimmunotherapy with antibody-strepavidin conjugate and ^{90}Y-DOTA-biotin. J. Nucl. Med., *41:* 131–140, 2000.

53. Axworthy, D. B., Reno, J. M., Hylarides, M. D., Mallett, R. W., Theodore, L. J., Gustavson, L. M., Su, F-M., Hobson, L. J., Beaumier, P. L., and Fritzberg, A. R. Cure of human carcinoma xenografts by a single dose of pretargeted yttrium-90 with negligible toxicity. Proc. Natl. Acad. Sci. USA, *97:* 1802–1807, 2000.

54. Sharkey, R. M., Karacay, H., Griffiths, G. L., Behr, T. M., Blumenthal, R. D., Mattes, M. J., Hansen, H. J., and Goldenberg, D. M. Development of a streptavidin-anti-carcinoembryonic antigen antibody, radiolabeled biotin pretargeting method for radioimmunotherapy of colorectal cancer. Studies in a human colon cancer xenograft model. Bioconjug. Chem., *8:* 595–604, 1997.

55. Press, O. W., Corcoran, M., Subbian, K., Hamlin, D. K., Wilbur, D. S., Johnson, T., Theodore, L., Yau, E., Mallett, R., Meyer, D. L., and Axworthy, D. A comparative evaluation of conventional and pretargeted radioimmunotherapy of CD20-expressing lymphoma xenografts. Blood, *98:* 2535–2543, 2001.

56. Magnani, P., Paganelli, G., Modorati, G., Zito, F., Songini, C., Sudati, F., Koch, P., Maecke, H. R., Brancato, R., Siccardi, A. G., and Fazio, F. Quantitative comparison of direct antibody labeling and tumor pretargeting in uveal melanoma. J. Nucl. Med., *37:* 967–971, 1996.

57. Casalini, P., Luison, E., Ménard, S., Colnaghi, M I., Paganelli, G., and Canevari, S. Tumor pretargeting: role of avidin/streptavidin on monoclonal antibody internalization. J. Nucl. Med., *38:* 1378–1381, 1997.

58. Rusckowski, M., Fogarasi, M., Fritz, B., and Hnatowich, D. J. Effect of endogenous biotin on the application of streptavidin and biotin in mice. Nucl. Med. Biol., *29:* 263–268, 1997.

59. LeDoussal, J-M., Gautherot, E., Martin, M.,. Barbet., J., and Delaage, M. Enhanced *in vivo* targeting of an asymmetric bivalent hapten to double-antigen-positive mouse B cells with monoclonal antibody conjugate cocktails. J. Immunol., *146:* 169–175, 1991.

60. De Boisferon, M. H., Raguin, O., Dussaillant, M., Rostène, W., Barbet, J., and Gruaz-Guyon, A. Enhanced targeting specificity to tumor cells by simultaneous recognition of two antigens. Bioconjug. Chem., *11:* 452–460, 2000.

61. Schuhmacher, J., Klivényi, G., Matys, R., Stadler, M., Regiert, T., Hauser, H., Doll, J., Maier-Borst, W., and Zöller, M. Multistep tumor targeting in nude mice using bispecific antibodies and a galium chelate suitable for immunoscintigraphy with positron emission tomography. Cancer Res., *55:* 115–123, 1995.

62. Schuhmacher, J., Kaul, S., Klivényi, G., Junkermann, H., Magener, A., Henze, M., Doll, J., Haberkorn, U., Amelung, F., and Bastert, G. Immunoscintigraphy with positron emission tomography: gallium-68 chelate imaging of breast cancer pretargeted with bispecific anti-MUC1/anti-Ga chelate antibodies. Cancer Res., *61:* 3712–3717, 2001.

63. Gautherot, E., Bouhou, J., Le Doussal, J-M., Manetti, C., Martin, M., Rouvier, E., and Barbet, J. Therapy for colon carcinoma xenografts with bispecific antibody-targeted, iodine-131-labeled bivalent hapten. Cancer (Suppl.), *80:* 2618–2623, 1997.

64. Gautherot, E., LeDoussal, J-M., Bouhou, J., Manetti, C., Martin, M., Rouvier, E., and Barbet, J. Delivery of therapeutic doses of radioiodine using bispecific antibody-targeted bivalent haptens. J. Nucl. Med., *39:* 1937–1943, 1998.

65. Gautherot, E., Rouvier, E., Daniel, L., Loucif, E., Bouhou, J., Manetti, C., Martin, M., LeDoussal, J-M., and Barbet, J. Pretargeted radioimmunotherapy of human colorectal xenografts with bispecific antibody and ^{131}I-labeled bivalent hapten. J. Nucl. Med., *41:* 480–487, 2000.

66. Karacay, H., McBride, W. J., Griffiths, G. L., Sharkey, R. M., Barbet, J., Hansen, H. J., and Goldenberg, D. M. Experimental pretargeting studies of cancer with a humanized anti-CEA x murine anti-[In-DTPA] bispecific antibody construct and a ^{99m}Tc/^{188}Re-labeled peptide. Bioconjug. Chem., *11:* 842–854, 2000.

67. Gestin, J. F., Loussouarn, A., Bardiès, M., Gautherot, E., Gruaz-Guyon, A., Saï-Maurel, C., Barbet, J., Curtet, C., Chatal, J. F., and Faivre-Chauvet, A. Two-step targeting of xenografted colon carcinoma using a bispecific antibody and ^{188}Re-labeled bivalent hapten: biodistribution and dosimetry studies. J. Nucl. Med., *42:* 146–153, 2001.

68. Lubic, S. P., Goodwin, D. A., Meares, C. F., Song, C., Osen, M., and Hays, M. Biodistribution and dosimetry of pretargeted monoclonal antibody 2D12.5 and Y-Janus-DOTA in BALB/c mice with KHJJ mouse adenocarcinoma. J. Nucl. Med., *42:* 670–678, 2001.

69. Volkert, W. A., Goeckeler, W. F., Ehrhardt, G. J., and Ketring, A. R. Therapeutic radionuclides: production and decay property considerations. J. Nucl. Med., *32:* 174–185, 1991.

70. Schubiger, P. A., Alberto, R., and Smith, A. Vehicles, chelators, and radionuclides: choosing the "building blocks" of an effective therapeutic radioimmunoconjugate. Bioconjug. Chem., *7:* 165–179, 1996.

71. Wilbur, D. S., Hamlin, D. K., Quinn, J, and Vessella, R. L. Development of an improved biotinylated clearing agent for pretargeting applications. J. Nucl. Med. (Suppl.), *42:* 148P, 2001.

72. Kraeber-Bodéré, F., Saï-Maurel, C., Campion, L., Faivre-Chauvet, A., Mirallie, E., Chérel, M., LeBoterff, J., Barbet, J., Chatal, J-F, and Thédrez, P. Late synergistic effect of paclitaxel associated with two-step radioimmunotherapy in medullary thyroid cancer xenograft. Mol. Cancer Ther., *1:* 267–274, 2002.