Pergamon

European Journal of Cancer Vol. 33, No. 2, pp. 186–192, 1997

Printed in Great Britain
0959-8049/97 $17.00+0.00

PII: S0959-8049(96)00477-7

Review

Pretargeting Strategies for Radio-immunoguided Tumour Localisation and Therapy

H.S. Stoldt,[1] F. Aftab,[1] M. Chinol,[2] G. Paganelli,[2] F. Luca,[1] A. Testori[1] and J.G. Geraghty[1]

[1]Department of General Surgical Oncology; and [2]Department of Nuclear Medicine, European Institute of Oncology, Via Ripamonti 435, 20141 Milan, Italy

The selective recognition of tumour cells by monoclonal antibodies, labelled with radioactive isotopes, for use in diagnosis and treatment, forms the basis of immunoscintigraphy, radio-immunoguided surgery and radio-immunotherapy. Research into the application of these systems has encountered multiple difficulties, most notably a low tumour to non-tumour ratio of radioactivity. The development of pretargeting systems, separating the individual steps of tumour cell targeting and the introduction of the radioactive label, have led to significant increments in tumour to non-tumour ratios and an improvement in diagnostic accuracy. Before pretargeting strategies are applied clinically, a thorough understanding of these systems is required and forms the backbone of this report. Clinical examples of early trials have already confirmed many of the theoretical advantages of pretargeting systems and new protocols are already being investigated. © **1997 Elsevier Science Ltd. All rights reserved.**

Key words: avidin–biotin, immunoscintigraphy, monoclonal antibody, pretargeting, radio-immunotherapy

Eur J Cancer, Vol. 33, No. 2, pp. 186–192, 1997

INTRODUCTION

THE POTENTIAL of selective recognition and killing of malignant cells for the purpose of diagnosis and therapy is enormous. This concept, which forms the basis of immunoscintigraphy (ISG), radio-immunoguided surgery (RIGS) and radio-immunotherapy (RIT) has been an active area of research in various fields of oncology [1–4]. Investigation has focused on several parts of this process, including tumour antigen expression; tumour targeting vehicles; pharmacokinetic aspects and immunogenicity of these recognition units; radioisotopes; and nuclear imaging technology [5–9]. Although several difficulties have been encountered, recent developments in these areas have led to a more thorough understanding of radio-immunodetection, and improvements have led to a more successful application of this strategy. In an attempt to overcome some of the drawbacks encountered, most notably a low tumour to non-tumour ratio of radioactivity, clearing agents have been introduced, and multistep pretargeting strategies have been developed. We describe here, the pretargeting systems that have so far been devised and their clinical application in radio-immunoguided tumour detection and therapy.

Correspondence to H.S. Stoldt.
Received 13 Mar. 1996; revised 3 Sep. 1996; accepted 13 Nov. 1996.

CURRENT PROBLEMS IN TUMOUR TARGETING

Intrinsic tumour characteristics

Various tumour antigens, present on tumour cells at a relatively high density and acting as receptors for the corresponding antibodies, have been described [5, 8]. Tumours often display intrinsic heterogeneity in antigen density. This factor, together with the non-uniformity of tumour vascularisation, capillary permeability, degree of tumour necrosis and difference of interstitial pressure, accounts for the heterogeneous distribution of antibodies in targeted tumors [9]. In addition, a binding site barrier, caused by the successful binding of antibodies to peripheral tumour antigens and causing a delayed penetration of further molecules into the tumour, has been described [10]. The first problem encountered in radio-immunodetection thus relates to adverse intrinsic tumour characteristics.

Monoclonal antibodies

The advent of hybridoma technology in 1975, allowed the production of different monoclonal antibodies (MAbs) with high specificity towards tumour-associated antigens [11]. Pharmacokinetic studies subsequently showed that only a very small dose, in the order of 0.015% of radiolabelled tumour-directed MAbs, reached their target [12]. Despite the fact that MAbs are superior to conventional polyclonal sera, these results are disappointing. Tumour to non-tumour ratios remain extremely low and a prolonged period of time of 5 to 12 days postinjection is required before ratios rise to a sufficient level at which these can be used for specific imaging techniques. A low tumour to background ratio remains the main problem of radio-immunodetection today. Larger doses of injected antibodies can increase the amount delivered to the target, but this raises the potentially serious problem of intrinsic immunogenicity of the monoclonal antibodies.

A number of factors are known to affect the biodistribution of MAbs. The advent of recombinant DNA and gene transfection techniques has allowed the production of genetically engineered 'designer' antibodies, including chimeric and humanised antibodies, antigen-binding peptide and protein molecules, and molecular recognition units [4, 6, 13]. Bivalent antibodies, divalent haptens and various related fragments, diabodies, F(ab')2 and Fab', have been developed as a result [14, 15]. Through the use of these agents, antigen-binding specificity and affinity can be optimised. Largely due to their size, the pharmacokinetic properties of these 'designer' molecules should excel those of whole antibodies, whose slow time of accumulation at target sites, together with slow blood clearance, limit their clinical utility for localisation. Unfortunately, the use of fragments displaying a faster blood clearance have not improved tumour localisation and, therefore, tumour to non-tumour ratios of radioactivity to a sufficient extent to allow their use in radio-immunotherapy.

Further investigation into solving the problem of high tumour to non-tumour ratios, by lowering the background radioactivity, led to the use of 'chasers' [16–19]. MAbs are biotinylated before targeting and subsequently cleared from the blood with the use of avidin or streptavidin. Although the radioactivity associated with the tumour is also reduced after the use of a 'chase', tumour to non-tumour ratios have been improved.

PRETARGETING SYSTEMS

In an attempt to overcome the low uptake of label by a tumour and improve the tumour to blood ratio, various groups have investigated the concept of tumour pretargeting based on the separate administration of MAbs and radiolabelled isotopes [20–27]. With this approach, short-lived isotopes can be used for imaging of tumours by recognising already localised MAbs. Their injection time can be delayed to a time when most of the primary MAb has been cleared from the blood and normal tissues, therefore decreasing non-tumour binding and achieving, with the use of this strategy, higher tumour to non-tumour ratios.

First conjugates

The first molecules to target a tumour are referred to as targeting vehicles or first conjugates, since a specific tag is carried by them. Four different types of tagged targeting vehicles for the use in pretargeting strategies have been described in the literature: (1) biotin-conjugated or biotinylated antibodies [23, 28, 29]; (2) streptavidin-conjugated or streptavidinylated antibodies [27]; (3) bifunctional MAbs (Bs-MAbs) [30]; and (4) monoclonal antibody–oligonucleotide conjugates [20]. These vehicles first require tagging of the Fc portion of the MAb to enable their recognition by a second molecule. The specific properties required for a successful tagging are outlined in Table 1.

The biotinylation of MAbs is easily accomplished, and two or three biotin molecules can be incorporated per antibody molecule without loss of immunoreactivity [16]. Biotinylation of MAbs increases the molecular weight only minimally, and has no effect on either the plasma kinetics or the rates of antibody permeation and diffusion [28]. In addition, the use of a long spacer arm between the protein-binding site and biotin reduces steric hindrance of the subsequent avidin–biotin reaction [16].

The conjugation of streptavidin to MAb results in a molecular weight 40% greater than that of native MAb and, therefore, shows slower pharmacokinetics than MAb alone [27, 31]. Streptavidin is made up of four identical subunits, only one of which is presumed to attach to the MAb. Reactivity of unbound subunits to endogenous biotin or other streptavidin binding compounds in plasma is assumed to be minimal due to the low concentrations (0.5–1.0 nM in humans) of these compounds [27]. Immunoreactivity of these conjugates seems to be preserved.

Bispecific antibodies with two different antigen-specific binding sites, one for the tumour-associated antigen and one for the radioactive effector compound, have been proposed by Goodwin and associates [21]. Unpredictable pharmacokinetic properties due to the many chemical manipulations required in the production of coupled Fab' fragments, as used by LeDoussal and colleagues [30], can partially be overcome by using Bs-MAbs produced by hybrid hybridomas [32]. However, because of univalency of Bs-MAbs for the tumour antigen, as well as mismatched association of parental chains, tumour-binding affinity is generally deficient.

Table 1. Targeting vehicles used in pretargeting systems and the required properties for successful tagging

Type of tagged targeting vehicle	Required properties for tagging of targeting vehicles
1. Biotin-conjugated antibodies (biotinylated)	Immune specificity must be preserved Antigen-binding affinity must be preserved without causing premature shedding or internalisation
2. Streptavidin-conjugated antibodies (streptavidinylated)	Steric hindrance to the binding of multiple antibodies should not occur
3. Bifunctional monoclonal antibodies (Bs-MAbs)	Biodistribution and pharmacokinetics should not be altered significantly
4. Monoclonal antibody–oligonucleotide conjugates	A selective and high affinity for the second recognition molecule needs to be displayed Synthesis of tagged bioconjugates must keep immunogenicity low

The attachment of oligonucleotides to MAbs, described by Bos and associates [20], typically binds three to five oligonucleotides per antibody molecule, and although it fully preserves their specificity, it reduces the immunoreactivity to an average of 50% normal due to steric hindrance or charge interference. Being DNA based conjugates, these targeting structures also need careful preservation and lose stability more rapidly than other MAb conjugates.

Second conjugates

Once the tagged MAbs have targeted the preselected tumour antigen, a second conjugate that will selectively recognise the tag on the first MAb is administered. Theoretically, this is best done at a time when the highest concentration of tagged MAbs are found on the tumour cells. This implies that MAbs must have permeated to the centre of the tumour, bound to the respective antigen, and have been minimally internalised. In addition, most of the tagged MAbs, which did not selectively bind to tumour antigens, must have been cleared from the normal tissues and blood and must have been excreted. The second molecule again needs to exhibit low immunogenicity. Its pharmacokinetic properties require a wide biodistribution and a high selectivity for the tagged MAb. Most importantly, this conjugate needs to be cleared rapidly from the normal tissues and blood after achieving its highest concentration on tumour-bound antibodies. This is best measured by the tumour to non-tumour ratio, and the higher this ratio, the more sensitive is the tumour localisation.

Second conjugates are most often radiolabelled avidin, streptavidin or biotin molecules directed at the biotinylated or avidinylated MAbs [33]. This pretargeting technique, thus takes advantage of the avidin–biotin system, which has long and widely been used for *in vitro* applications, such as immunocytochemistry, ELISA and molecular biology.

The avidin–biotin system

Briefly, avidins are small oligomeric proteins made up of four identical subunits, each bearing a single binding site for biotin. They can, therefore, bind up to four moles of biotin per mole of protein. Avidins are functionally defined by their ability to bind biotin with high affinity and specificity without recognising or binding any other physiological compound with any strength. The affinity of avidin for biotin is extremely high, with a dissociation constant of the avidin–biotin complex in the order of 10^{15} [34]. For practical purposes, their binding can be regarded as irreversible. Biotin itself is recognised by a functional 'head' region which binds avidin, and a functionally irrelevant carboxyl 'tail' end, which can be chemically altered with little or no effect on the molecule. It is here in the carboxyl group that a spacer arm can be introduced to facilitate binding of biotin to MAbs.

Radioisotope labelling

Radioisotope labelling of biotin, avidin or streptavidin is either done directly to the protein or by covalently linking a chelating agent, which in turn can bind a suitable radionuclide. In the case of biotin, isotope labelling occurs at the carboxyl group. The most commonly used chelating agents for indirect labelling are EDTA, DTPA and its derivatives. The molecular integrity of biotin or avidin is not altered significantly by radiolabelling, and reactivity of these proteins is preserved in 90–100% of the corresponding unlabelled molecules [35]. Although the choice of the chelating agent and radiolabel do alter the biodistribution kinetics, these compounds are rapidly cleared from the whole body after administration, making them highly suitable as second conjugates in pretargeting systems. The extremely rapid blood clearance and relatively low organ retention renders avidin a slightly more appropriate candidate for radiolabelling than biotin [36]. Further research into the choice of ligand and radiolabel for the use on avidins or biotin is needed.

PRETARGETING STRATEGIES

The use of MAbs in targeting tumours prior to the administration of radiolabelled conjugates has led to the development of pretargeting protocols of two- and three-steps.

Two-step protocols

The injection of radiolabelled streptavidin after pretargeting with biotinylated antibody, and the injection of labelled biotin after the use of streptavidinylated MAb, are the two-step protocols most often applied [23, 27, 29]. These two methods were compared in pharmacokinetic properties by Sung and associates [27] who showed that the maximum molar concentration of radiolabelled species attained in the tumours was comparable in both protocols, but was reached much faster in the radiolabelled biotin protocol. Khawli and colleagues [37] demonstrated that the treatment of tumour-bearing mice with biotinylated MAbs resulted in a 1.3–2.6-fold increase in radiolabel localisation in tumour compared with directly labelled antibodies. These results were confirmed by Sung and coworkers [31], who demonstrated that the two-step protocol of pretargeted biotinylated MAb followed by radiolabelled streptavidin yielded a 2–3-fold increase in tumour to blood ratio compared to the direct (one-step) method. In addition, Saga and associates [29] demonstrated that the concentration of streptavidin in metastatic lung nodules of ten guinea pigs was 5.6 times higher for the pretargeted group than for the non-pretargeted group and that the metastases to blood ratio increased from 1.2 to 2.4 with the use of the two-step protocol. Furthermore, a comparative study by Rosario and Wahl [38] of radiolabelled biotin in the form of a small peptide to which organic derivatives were solubilised (biotinylated iodo-polylysine-BIP) and In-111 chelated to biotin, showed that the small peptide (BIP) had a 2-fold higher lung targeting than the In-111 biotin derivative when targeted at prelocalised streptavidin.

Bispecific Fab′–Fab and F(ab′)2–F(ab)′ antibodies followed by radiolabelled bivalent haptens have been studied in both *in vitro* and *in vivo* models in two-step protocols by LeDoussal and colleagues [22, 30, 39]. Bs-MAb conjugate cocktails used together with asymmetric bivalent haptens to specifically pretarget tumour cells co-expressing two distinct antigens were used in the former study to raise the sensitivity of detection with positive results. Although both of these protocols show an increased selectivity of bivalent haptens for the Bs-MAbs, its major drawbacks are a decreased bioavailability of antibody fragments and the intrinsically low affinity of the Bs-MAbs. Despite these drawbacks, pretargeting with Bs-MAbs followed by radiolabelled haptens, convincingly demonstrates an improved tumour to normal tissue ratio [21, 22, 39–41].

In an alternative approach, Bos and associates [20] have described the use of MAb–DNA immunoconjugates followed by radiolabelled complementary oligonucleotides. A decrease in immunoreactivity, possibly due to the binding of DNA to the antibody, was noted with this protocol. Although non-specific binding was not shown, oligonucleotides could potentially bind and be taken up by other cell types and tissues. Early *in vitro* results indicate that DNA–DNA recognition can be a successful pretargeting concept, but further investigation towards the applicability of this protocol in clinical trials is needed.

Three-step protocols

Removal of the free, tumour-unbound MAbs from the circulation by the administration of a 'chase' molecule before giving the radiolabelled conjugate, forms the basis of the three-step pretargeting protocols. First postulated by Hnatowich and associates [42] using the avidin–biotin system, the three-step protocol was applied in an animal model using bifunctional haptens for targeting, chelate–transferrin conjugates as the 'chaser', and a bifunctional radiolabelled chelate as the third step by Goodwin and coworkers [21].

Avidin-induced blood clearance of biotinylated MAbs is extremely rapid (5–15 min) and produces up to a 10-fold increase in contrast between the blood and target tissues in animal models as shown by Kobayashi and associates [16]. The use of the avidin 'chase' as the second part of a three-step protocol was further explored using *in vivo* studies by the same author, who reported an additional improvement in the biodistribution of radiolabelled biotinylated MAbs and a further decrease in background radioactivity with the use of a second avidin 'chase' [43].

The use of the three-step pretargeting method based on the avidin–biotin system has been extensively researched by Paganelli and colleagues [23–25, 28]. The multiple advantages of this three-step pretargeting protocol are listed in Table 2.

The necessity of repeat injections, paying particular attention to both doses and timing, is essential for three-step protocols to work and constitutes one of its major disadvantages. Molar ratios between biotinylated MAbs and avidin are extremely important in order to obtain a good clearance of free circulating MAbs and subsequently optimise the 'avidinisation' of the tumour. The immunogenicity of avidin and streptavidin is an additional disadvantage of pretargeting techniques utilising these proteins, and Paganelli and associates [25] reported a 6% human anti-mouse response (HAMA) and a 21% anti-avidin immune response (HAAR) in a cohort of 66 patients. Alternative clearing agents, for example non-radioactive chelates coupled to high weight carrier proteins such as transferrin, injected prior to the radiolabel, have been studied. These agents have been found to suppress labelled-chelate binding in the blood and to raise tumour to normal tissue ratios. Schuhmacher and colleagues [26] describe the use of Bs-MAbs made from F(ab)2–F(ab') fragments, followed by a blocker of chelate coupled to transferrin, and a univalent radioactive gallium chelate in an animal model. This protocol increased the tumour to blood ratio by a factor of five and allowed the use of a short-lived positron-emitter such as Ga-66.

Table 2. Advantages of the three-step pretargeting method based on the avidin–biotin system

1. MAb immunoreactivity is not altered by biotin binding
2. Signal amplification can be achieved through binding of multiple avidin molecules to the pretargeted biotinylated MAbs as well as to multiple radioactive biotin conjugates
3. Significant background reduction is achieved through the removal of circulating MAbs by cold avidin. These complexes are taken up and metabolised by the reticulo-endothelial system of the liver
4. Fast label clearance occurs with the use of the small molecule avidin, further decreasing background radioactivity and also reducing transchelation of radioactive metal onto transferrin, which results in lower liver uptake and bone marrow exposure
5. Short half-life radionuclides like In-111 and Tc-99m can be used because of faster label clearance

MAb, monoclonal antibody.

CLINICAL APPLICATIONS

(I) Two-step clinical protocols

Clinical protocols that utilise a two-step approach have been reported for the detection of lung metastases as well as for intraperitoneal localisation of ovarian tumours, intrapleural detection of tumour deposits and intra-arterial or intraportal injection for liver metastases.

Lung cancer (diagnosis)

Clinical application of the two-step pretargeting method for imaging was reported by Kalofonos and colleagues [44] using a streptavidin conjugated MAb followed by In-11-DTPA-biotin. In a preliminary study, 10 patients with non-small cell carcinomas of the lung were investigated. Tumour could be localised by scintigraphy as early as 30 min after injection. Of note in this study was an increase in the mean tumour to normal lung ratio in 4 patients after the use of the pretargeting protocol when compared to the administration of biotin alone.

Ovarian cancer (diagnosis)

In patients with ovarian carcinoma, where the tumour is confined to the peritoneal cavity, the locoregional approach, utilising an intraperitoneal injection has shown increases in tumour to non-tumour ratio. In the work by Crippa and associates [45], intraperitoneal injection was compared to the intravenous route in 30 patients with ovarian cancer showing enhanced tumour uptake and a higher tumour to background ratio after intraperitoneal injection. A two-step approach, described by Paganelli and associates [46], for imaging ovarian cancer in 15 patients utilised biotinylated MAbs injected intraperitoneally and followed 3–5 days later by an In-111-labelled streptavidin dose. A SPECT study and planar spot views were acquired 1–3 h after the streptavidin injection and surgery was performed in all patients 1–8 days after scintigraphy. The mean tumour to blood ratio was 14:1 (range 4:1–301) and the injected dose per gram of tumour absorbed was 0.112 for recurrences and 0.05 for primary tumours. Tumour uptake was inversely proportional to tumour size and 14 of 15 patients had positive scans. Both per cent of injected dose per gram of tumour and tumour to blood ratio were, on average, greater than those obtained with directly labelled antibodies [47].

Table 3. Immunoscintigraphy with three-step pretargeting techniques in different tumour types utilising different MAbs

Tumour type	No. of patients	MAbs	ISG positive	ISG negative
Breast carcinoma	10	FO23C5	8	2
Cerebral glioma	28	BC2	22	6
Colorectal carcinoma	55	FO23C5	51	4
Lung carcinoma	15	FO23C5	12	3
Liver tumours	5	FO23C5	5	0
MTC	26	FO23C5	24	2
Neuroendocrine	21	A11	19	2
Ocular melanoma	19	225.28S	13	6
Pancreatic carcinoma	9	FO23C5	8	1
Pituitary neoplasms	10	A11	7	3
Total	198		169 (85%)	29 (15%)

MAbs, monoclonal antibodies; ISG, immunoscintigraphy; MTC, medullary thyroid carcinoma.

(II) Three-step clinical protocols

The clinical application of the avidin–biotin system in three-step protocols has been selectively investigated by Paganelli and colleagues [24, 25, 48] after the observation, in various models, that maximal uptake of MAbs occurs within 24–48 h after injection and well before clearance of unbound MAbs from the bloodstream is completed. These protocols use tumour pretargeting with biotinylated MAbs, followed 1–5 days later by two subsequent injections of unlabelled avidin. The first injection allows precipitation of free circulating biotinylated MAbs (1 mg cold avidin) while the second targets the biotinylated tumour-bound MAbs with an excess of cold avidin (4 mg injection). The third step labels the tumour with a fast-clearing radioactive biotin derivative (In-111-biotin or Tc-99m-biotin) 1–3 days later.

Currently, 198 patients have been studied utilising a three-step protocol based on the avidin–biotin labelling technique and MAbs specific for different tumours including gliomas, melanomas, apudomas and medullary thyroid carcinomas. Results of immunoscintigraphy in these patients are listed in Table 3.

CEA-positive tumours (diagnosis)

The first clinical application of the three-step protocol was carried out in patients bearing CEA-expressing tumours originating in the gastro-intestinal tract, breast, lung and thyroid gland [24]. The blood clearance of In-111-biotin showed an exponential decay with fast and slow components. Since blood clearance was fast, patients were studied 90 to 120 min after the administration of the radiolabel. The absolute amount of label delivered to the tumour was 0.012% of the injected dose, a figure in the range of the best results obtained with directly labelled MAbs.

Colorectal cancer (diagnosis)

Locoregional recurrence and liver metastases from colorectal cancer have been investigated in a study by Paganelli and associates [24]. Biotinylated MAbs against CEA were injected intravenously, followed 24 h later by two unlabelled avidin 'chases' and 48 h later by an injection of an In-111-DTPA-biotin conjugate. Of the 21 patients tested, none developed an antibody response against mouse immunoglobulins, while only 3 patients developed anti-avidin antibodies. Since blood clearance was fast, images were acquired 1–3 h after In-111-biotin injection with a tumour to blood ratio of 5.5 ± 3.2 and a tumour to liver ratio of 6.7 ± 3.9. The results revealed 22 positive and 8 negative cases giving a sensitivity of 86%, a specificity of 71% and an accuracy of 83%. Immunoscintigraphy revealed the presence of hepatic lesions in 3 patients and locoregional disease in 2 patients that were not revealed by other diagnostic modalities. These lesions were subsequently confirmed on follow-up by computed tomography and/or ultrasound.

Cerebral glioma (diagnosis and therapy)

The three-step pretargeting method has been recently applied clinically for the imaging of cerebral gliomas [25]. A new biotin derivative, propylenediamine dioxine-biotin (PnAO-biotin), was labelled with Tc-99m and injected systemically after pretargeting with anti-tenascin MAbs. Tumours were detected in 15 out of 18 patients and 2 patients with negative results had no evidence of tenascin staining on subsequent immunohistochemical analysis of the tumour. Excellent tumour to background ratios were achieved 1 h post-injection, at which time single-photon emission computed tomography (SPECT) imaging could be performed.

A therapeutic trial based on this pretargeting method has been conducted in 19 patients with histological diagnosis of grade III–IV malignant glioma and immunohistochemical evidence of tenascin on tissue sections. The therapy utilised the Y-90 radioisotope and was well tolerated by all patients. The mean dose delivered to the tumour was 1520 ± 870 cGy, while the liver absorbed 150 ± 100 cGy, the kidneys 270 ± 160 cGy, the brain 60 ± 30 cGy and the bone marrow 80 ± 50 cGy. Of 16 patients evaluated over a follow-up period of 2–12 months, one showed a complete response, 4 had a partial response and 6 cases were classified as having stable disease.

Ocular melanoma (diagnosis)

Most recently, Magnani and colleagues [49] have performed a comparative study of direct antibody labelling and tumour pretargeting in 15 patients with a diagnosis of uveal melanoma. A high resolution SPECT-ISG was performed in all patients with directly labelled MAb followed a week later by the three-step pretargeting technique. In all three-step ISGs, there was a reduction of non-specific background and the tumour to non-tumour ratio was improved to 3.1 ± 1.3 as compared to 1.5 ± 0.5 with conventional ISG. The percentage of injected dose on the tumour however was similar for the two methods.

CONCLUSION

Pretargeting strategies have been developed to increase the sensitivity of radio-immunodetection by decreasing tumour to non-tumour ratios. Two step pretargeting protocols seek to achieve specific tumour localisation of radioisotopes by utilising the pharmacokinetically slow, but antigen-specific MAbs as a first component, followed by the comparatively faster isotope-labelled conjugate as a second component. In three step protocols, a 'chase' molecule is administered after the first component, clearing it from the circulation before the radiolabelled conjugate is injected. The avidin–biotin system has been the most widely applied method in pretargeting protocols. Although much higher tumour to non-tumour ratios have been achieved by the use of these protocols, further development and testing of variables is required. These strategies will hopefully lead to higher specificity and greater clinical applicability of pretargeting protocols in radio-immunodetection and therapy.

1. Fazio F, Paganelli G. Antibody-guided scintigraphy: targeting of the "magic bullet". *Eur J Nucl Med* 1993, **20**, 1138–1140.
2. Kim JA, Triozzi PL, Martin EW. Radioimmunoguided surgery for colorectal cancer. *Oncology* 1993, **7**, 55–64.
3. Leichner PK, Koral KF, Jaszczak RJ, *et al.* An overview of immaging techniques and physical aspects of treatment planning in radioimmunotherapy. *Med Physics* 1993, **20**, 569–577.
4. Chetanneau A, Barbet J, Peltier P, *et al.* Pretargetted imaging of colorectal cancer recurrences using an In-111-labelled bivalent hapten and a bispecific antibody conjugate. *Nucl Med Commu* 1994, **15**, 972–980.
5. Hirai H. Use of tumor receptors for diagnostic imaging. *Acta Radiol* 1990, **374**, 57–64.
6. Hazra DK, Britton KE, Lahiri VL, *et al.* Immunotechnological trend in radioimmuno-targeting: from 'magic bullet' to 'smart bomb'. *Nucl Med Comm* 1995, **16**, 66–75.
7. Kuhn JA, Corbisiero RM, Buras RR, *et al.* Intraoperative gamma detection probe with presurgical antibody imaging in colon cancer. *Arch Surg* 1991, **126**, 1398–1403.
8. Schlom J. Basic principles and applications of monoclonal antibodies in the management of carcinomas: the Richard and Hinda Rosenthal Foundation Award Lecture. *Cancer Res* 1986, **46**, 3225–3238.
9. Strand SE, Zanzonico P, Johnson TK. Pharmacokinetic modeling. *Med Physics* 1993, **20**, 515–527.
10. Juweid M, Neumann R, Paik C, *et al.* Micropharmacology of monoclonal antibodies in solid tumors: direct experimental evidence for a binding site barrier. *Cancer Res* 1992, **52**, 5144–5153.
11. Kohler G, Milstein C. Continuous cultures of fused cells secreting antibodies of predefined specificity. *Nature* 1975, **256**, 495–497.
12. Epenetos AA, Snook D, Durbin H, *et al.* Limitations of radiolabeled monoclonal antibodies for localization of human neoplasms. *Cancer Res* 1986, **46**, 3183–3191.
13. Serafini AN. From monoclonal antibodies to peptides and molecular recognition units: an overview. *J Nucl Med* 1993, **34**, 533–536.
14. Haunschild J, Faro HP, Pack P, *et al.* Pharmacokinetic properties of bivalent miniantibodies and comparison to other immunoglobulin forms. *Antibody, Immunoconj Pharm* 1995, **8**, 111–128.
15. Holliger P, Prospero T, Winter G. "Diabodies": small bivalent and bispecific antibody fragments. *Proc Natl Acad Sci USA* 1993, **90**, 6444–6448.
16. Kobayashi H, Sakahara H, Hosono M, *et al.* Improved clearance of radiolabeled biotinylated monoclonal antibody following the infusion of avidin as a "chase" without decreased accummulation in the target tumor. *J Nucl Med* 1994, **35**, 1677–1684.
17. Marshall D, Pedley RB, Boden JA, *et al.* Clearance of circulating radio-antibodies using streptavidin or second antibodies in a xenograft model. *Br J Cancer* 1993, **69**, 502–507.
18. Marshall D, Pedley RB, Melton RG, *et al.* Galactosylated streptavidin for improved clearance of biotinylated intact and F(ab')2 fragment of an anti-tumour antibody. *Br J Cancer* 1995, **71**, 18–24.
19. Sinitsyn VV, Mamantova AG, Checkneva YY, *et al.* Rapid blood clearance of biotinylated IgG after infusion of avidin. *J Nucl Med* 1989, **30**, 66–69.
20. Bos ES, Kuijpers WHA, Meesters-Winters M, *et al.* In vitro evaluation of DNA–DNA hybridization as a two-step approach in radioimmunotherapy of cancer. *Cancer Res* 1994, **54**, 3479–3486.
21. Goodwin DA, Meares CF, McCall M, *et al.* Targeted immunoscintigraphy of murine tumors with indium-111-labeled bifunctional haptens. *J Nucl Med* 1988, **29**, 226–234.
22. LeDoussal JM, Martin M, Gautherot E, *et al.* 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* 1989, **30**, 1358–1366.
23. Paganelli G, Riva P, Deleide G, *et al.* In vivo labelling of biotinylated monoclonal antibodies by radioactive avidin: a strategy to increase tumor radiolocalization. *Int J Cancer* 1988, **2**, 121–125.
24. Paganelli G, Malcovati M, Fazio F. Monoclonal antibody pretargeting techniques for tumour localization: the avidin–biotin system. *Nucl Med Commu* 1991, **12**, 211–234.
25. Paganelli G, Magnani P, Siccardi AG, *et al.* Clinical application of the avidin–biotin system for tumor targeting. In Goldenberg DM, ed. *Cancer Therapy with Radiolabeled Antibodies*. CRC Press, 1995, 239–254.
26. Schuhmacher J, Klivenyi G, Matys R, *et al.* Multistep tumor targeting in nude mice using bispecific antibodies and a gallium chelate suitable for immunoscintigraphy with positron emission tomography. *Cancer Res* 1995, **55**, 115–123.
27. Sung C, Osdol WW. Pharamcokinetic comparison of direct antibody targeting with pretargeting protocols based on streptavidin–biotin binding. *J Nucl Med* 1995, **36**, 867–876.
28. Paganelli G, Pervez S, Siccardi A, *et al.* Intraperitoneal radiolocalization of tumors pretargeted by biotinylated monoclonal antibodies. *Int J Cancer* 1990, **45**, 1184–1189.
29. Saga T, Weinstein JN, Jeong JM, *et al.* Two-step targeting of experimental lung metastases with biotinylated antibody and radiolabeled streptavidin. *Cancer Res* 1994, **54**, 2160–2165.
30. LeDoussal JM, Barbet J, Delaage M. Bispecific-antibody-mediated targeting of radiolabeled bivalent haptens: theoretical, experimental and clinical results. *Int J Cancer* 1992, **7**, 58–62.
31. Sung C, van Osdol WW, Saga T, *et al.* Streptavidin distribution in metastatic tumors pretargeted with a biotinylated monoclonal antibody: theoretical and experimental pharmacokinetics. *Cancer Res* 1994, **54**, 2166–2175.
32. Suresh MR, Cuello AC, Milstein C. Bispecific monoclonal antibodies from hybrid hybridomas. *Methods Enzymol* 1986, **121**, 210.
33. Stella M, DeNardi P, Paganelli G, *et al.* Avidin–biotin system in radioimmunoguided surgery for colorectal cancer. *Dis Colon Rectum* 1994, **37**, 335–343.
34. Green NM. Avidin-1. The use of [14C] biotin for kinetic studies and for assay. *Biochem J* 1963, **89**, 585.
35. Gitlin G, Bayer EA, Wilchek M. Studies on the biotin-binding sites of avidin and streptavidin. *Biochem J* 1990, **269**, 527–530.
36. Schechter B, Silberman R, Arnon R, *et al.* Tissue distribution of avidin and streptavidin injected to mice. *Eur J Biochem* 1990, **89**, 327–331.
37. Khwali LA, Alauddin MM, Miller GK, *et al.* Improved immunotargeting of tumors with biotinylated monoclonal antibodies and radiolabeled streptavidin. *Antibody, Immunoconj Radiopharmaceuticals* 1993, **6**, 13–19.
38. Rosario RB, Wahl RL. Biotinylated iodo-polylysine for pretargeted radiation delivery. *J Nucl Med* 1993, **34**, 1147–1151.
39. LeDoussal JM, Gautherot E, Martin M, *et al.* Enhanced in-vivo targeting of an asymmetric bivalent hapten to double-antigen-positive mouse B cells with monoclonal antibody conjugate cocktails. *J Immun* 1991, **146**, 169–175.

40. Somasundaram C, Matzku S, Schumacher J, *et al.* Development of a bispecific monoclonal antibody against a gallium-67 chelate and the human melanoma-associated antigen p97 for potential use in pretargeted immunoscintigraphy. *Cancer Immu Immunother* 1993, **36**, 337–345.
41. Chetanneau A, Barbet J, Peltier P, *et al.* Pretargetted imaging of colorectal cancer recurrences using an In-111-labelled bivalent hapten and a bispecific antibody conjugate. *Nucl Med Commu* 1994, **15**, 972–980.
42. Hnatowich DN, Virzi F, Rusckowski M. Investigations of avidin and biotin for imaging applications. *J Nucl Med* 1987, **28**, 1294–1302.
43. Kobayashi H, Sakahara H, Endo K, *et al.* Repeating the avidin "chase" markedly improved the biodistribution of radiolabelled biotinylated antibodies and promoted the excretion of additional background radioactivity. *Eur J Cancer* 1995, **31A**, 1689–1696.
44. Kalofonos HP, Rusckowsk M, Siebecker DA, *et al.* Imaging of tumor in patients with indium-111-labeled biotin and streptavidin-conjugated antibodies: preliminary communication. *J Nucl Med* 1990, **31**, 1791–1796.
45. Crippa F, Buraggi GL, Di Re E, *et al.* Radioimmuno-scintigraphy of ovarian cancer with the MOv18 monoclonal antibody. *Eur J Cancer* 1991, **27**, 724–729.
46. Paganelli G, Magnani P, Zito F, *et al.* Three-step monoclonal antibody tumor targeting in carcinoembryonic antigen-positive patients. *Cancer Res* 1992, **51**, 5960–5966.
47. Delaloye AB, Delaloye B. Tumor imaging with monoclonal antibodies. *Semin Nucl Med* 1995, **25**, 144–164.
48. Paganelli G, Stella M, Zito F, *et al.* Radioimmunoguided surgery using iodine-125-labeled biotinylated monoclonal antibodies and cold avidin. *J Nucl Med* 1994, **35**, 1970–1975.
49. Magnani P, Paganelli G, Modorati G, *et al.* Immunoscintigraphy of uveal melanoma: a quantitative comparison of direct antibody labeling and tumor pretargeting. *J Nucl Med*, in press.