Biotinylated Iodo-Polylysine for Pretargeted Radiation Delivery

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Efforts to achieve rapid specific targeting of radioisotopes to disease processes using antibodies conjugated with avidin or streptavidin for pretargeting and radiobiotin derivatives for isotope delivery are attracting substantial interest. At present, these approaches appear to be limited by low delivery of radiotracer to the target. As an alternate radiobiotin tracer, biotinylated/iodinated polylysine (BIP) was prepared by conjugating poly-L-lysine (MW ≈10,200) with biotin succinimide esters and the Bolton-Hunter reagent. This reagent was then radioiodinated with ^{125}I via the Iodogen method. BIP was characterized by radio-HPLC and its in vitro binding to streptavidin. The in vivo localization of BIP was evaluated in a rat model in which streptavidin agarose beads were physically localized to precapillary arterioles in the lungs. Biodistribution and blocking studies performed at 4 and 24 hr after BIP injection indicated specific binding and localization of the radiolabeled peptide to the lungs (lung-to-blood ratio ≈8 at 4 hr postinjection). Comparative studies of BIP and ^{111}In chelated to biotin showed BIP to have two-fold higher lung targeting and lower splenic and hepatic uptake than the ^{111}In biotin derivative. Our study demonstrates: (1) the feasibility of using a small peptide as a biotin carrier for pretargeting (and for solubilizing organic tracers which may otherwise be difficult to administer in vivo) and (2) that BIP and BIP-like compounds may be suitable and simple alternatives to radiometal-labeled biotin for pretargeting and may offer improved targeting to prelocalized streptavidin.

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Low target-to-background ratios and low absolute tracer delivery to tumors represent the major limitations for radiolabeled monoclonal antibody imaging. Improvements in target-to-background ratios for radiolabeled monoclonal antibody targeting of tumors can, in many cases, be achieved through the use of smaller immunoconjugates such as F(ab')$_2$ and Fab fragments. It is also possible that a pretargeted approach using a lower-molecular weight radiolabeled complex as a secondary tracer which binds to pretargeted (by antibody) avidin and streptavidin with high affinity may also improve tracer localization (*1,2*). Examples of antibody pretargeting include the use of heterobifunctional IgG conjugates and biotinylated IgG or streptavidin-IgG conjugates followed with radio-metal chelates, radiostreptavidin and biotin (*3,4*). Most studies in the use of radiolabeled biotin have been limited to the use of [^{111}In]DTPA-biotin, which has been shown to bind with high affinity to avidin or streptavidin (*1,2*). Through use of somewhat lengthy radiosyntheses, iodophenylester and ^{18}F radiolabeled biotin analogues have been reported (*5,6*).

In conjunction with our work on sulfhydryl site-specific attachment of radiolabels to monoclonal antibodies, we sought to prepare an easily-constructed small peptide with multiple biotins which could be readily radiolabeled. Such a multi-biotinylated peptide may exhibit better in vivo targeting and have the potential of delivering higher quantities of radioactivity than biotin alone, particularly if it is retained in the blood longer than biotin, thus allowing greater opportunity for binding interaction. Thus, we developed a multi-valent biotinylated peptide for in vivo localization to pretargeted streptavidin and evaluated it in vitro and in an in vivo model system in which beads coupled to streptavidin were prelocalized to the lungs.

EXPERIMENTAL DESIGN AND METHODS

Preparation of BIP

BIP was prepared by dissolving 3-(4-hydroxyphenyl)propionic acid, succinimidyl ester (Bolton Hunter ester, 9.4 mg, 0.04 mmole, Molecular Probes, Inc., Eugene, OR), 6-((-6-((biotinoyl)aminohexanoyl)amino)hexanoic acid, succinimidyl ester (22 mg, 0.04 mmole) and 19 mg poly-L-lysine (mol wt 10,200 cut, Sigma Chemical Co., St. Louis, MO) in 75, 75 and 100 μl DMSO (Sigma Chemical Co.), respectively. Twenty-five microliters of the Bolton Hunter ester and 25 μl of the biotin derivative were added to 25 μl of the polylysine solution. The mixture was vortexed thoroughly, allowed to stand for 5–7 hr and was then treated with 0.5 ml of 0.1 *M* sodium phosphate buffer (pH ≈7.1). An approximately 50-fold molar excess of the Bolton-Hunter ester and the biotin derivative were reacted with the polylysine. The resulting slurry was allowed to stand at 4°C overnight. The mixture was then centrifuged and the supernatant separated from the insoluble organic material. The supernatant was subsequently chromatographed and desalted using Sephadex G-25 (size exclusion chromatography). The conjugated Bolton-Hunter residue bound to the peptide was then labeled with ^{125}I via the Iodogen method (*7*) to give a radioiodinated product with a specific activity of at least 1 μCi/μg of polylysine. We refer to the biotinylated Bolton-Hunter iodinated polylysine as BIP. Incorporation of hydroxyphenyl and biotinoyl moieties were confirmed by radioiodination of phenolic

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residues (7), binding of the radiolabeled peptide to immobilized streptavidin and subsequent radio-HPLC and in vivo experiments in rats (vide infra). Specific binding to streptavidin was confirmed using unblocked and biotin-blocked streptavidin agarose beads (Pierce Chemical Co., Rockford, IL) described in a subsequent section.

Preparation of ^{111}In Chelated Biotin

Radiolabeled chelated ^{111}In (2) was prepared by complexation of ^{111}InCl (New England Nuclear, N. Billerica, MA) with diethylenetriamine-pentaacetic acid, α,ω-bis(biocytinamide) (Sigma Chemical Co.). Indium-111-chloride (≈1.2 mCi) was mixed with a solution of the indium-biotinylated chelate (≈3.0 mg/ml) in sodium phosphate buffer and incubated at ambient temperature for 10 min. Removal of uncomplexed indium was accomplished by cation-exchange chromatography using Sep-PAK CM columns (Waters-Millipore Corp., Milford, MA) to give the desired biotinylated indium chelate conjugate (≈0.3 μCi/μg). Binding to streptavidin of the radiolabeled chelate was confirmed using immobilized streptavidin (Pierce Chemical Co.) as previously reported (2).

Animal Studies

Three groups of five female Sprague Dawley rats (≈250 g each) were administered 1–3 μCi of ^{125}I BIP via tail vein injection. In two of the groups, this administration was performed 1 hr following the injection of a slurry of unblocked or biotin-blocked immobilized streptavidin agarose beads (≈250 μl) which contained no more than 20 μl of agarose gel (Pierce Chemical Co., 64–165 μm diameter). Doses >20 μl of beads were commonly lethal. These beads had 1–2 μg streptavidin/μl of beads. The biotin-blocked streptavidin beads were prepared by saturation of the biotin sites with d-biotin (Sigma Chemical Co.) (133 mg biotin/5 ml gel, >24 hr) prior to use. The remaining group of rats received no beads. In a preceding separate experiment, the exceedingly high pulmonary uptake of the immobilized streptavidin agarose beads was verified by injection of a nonlethal dose of unlabeled beads containing a tracer quantity of ≈1–3 μCi of ^{131}I radiolabeled streptavidin beads (7). The three groups of rats were sacrificed at 4 hr postinjection and assayed for normal tissue activity in liver, kidney, spleen, heart, lung, muscle, intestines, bone and blood. A similar biodistribution study was performed at 24 hr postinjection. For comparative studies using ^{111}In chelate, two groups of rats (≈250 g each) were also given ≈3 μCi of ^{111}In chelated biotinylated conjugate intravenously following injection of the unblocked or biotin-blocked immobilized streptavidin agarose beads.

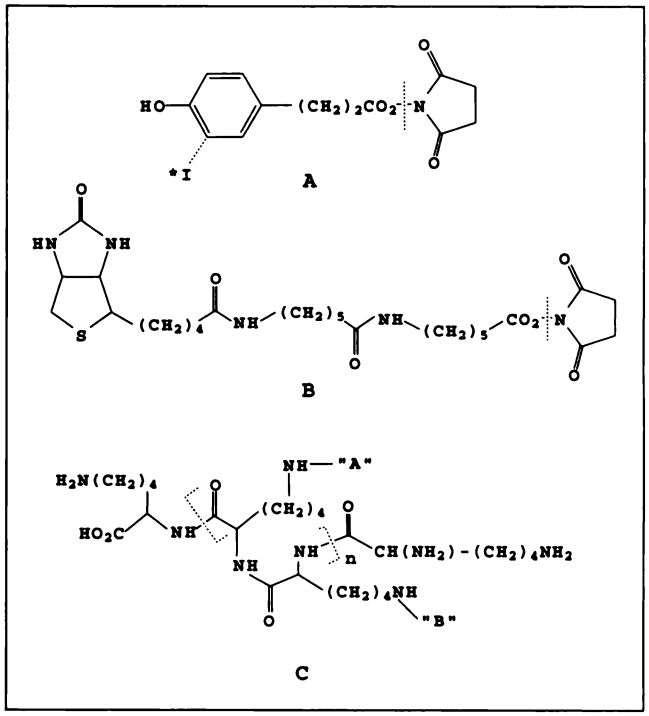

FIGURE 1. Chemical depiction of (A) 3-(4-hydoxyphenyl)propionic acid, succinimidyl ester (Bolton Hunter ester), (B) 6-((-6-((biotinoyl)aminohexanoyl)amino)hexanoic acid, succinimidyl ester and (C) BIP.

Radio-HPLC Studies

Size-exclusion radio-HPLC studies were carried out using Pharmacia Superose 12 columns and a Pharmacia P-500 pump with 0.1 *M* sodium phosphate buffer as eluant. Plots of activity fractions were obtained by counting sample fractions with a Packard gamma counter. Calibration of molecular weights on this type of column have been described previously (8). Serum samples were passed through 0.22-μm filters prior to injection onto the HPLC system.

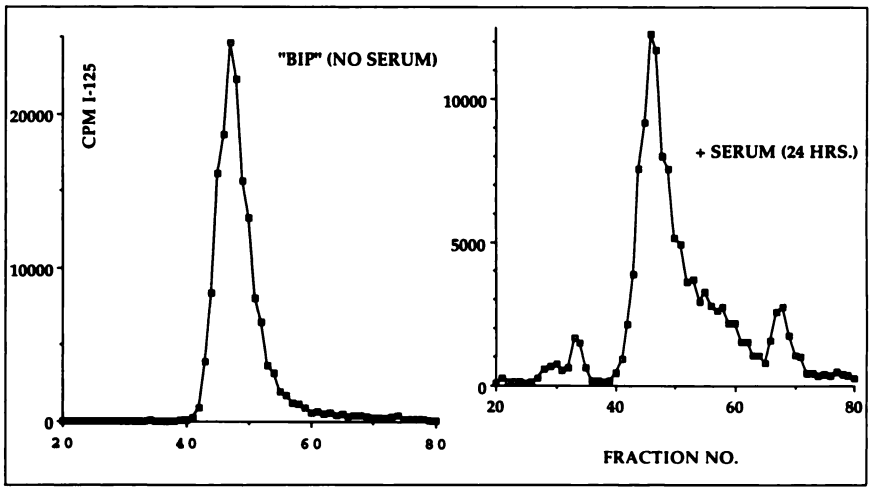

FIGURE 2. Size-exclusion radio-HPLC profiles of BIP and serum stability (24 hr).

RESULTS

Medium to high molecular weight polylysines have been exploited by several groups as carriers of radioactive isotopes, boron and as probes which bind to antibodies and other proteins (*9,10*). The large number of nucleophilic primary ε-amino functions facilitate crosslinking and N-alkylation with electrophilic chelates (*11*). We believed that a smaller biotinylated polylysine could also be radiolabeled by coattachment of a suitable precursor molecule (e.g., phenolic residue). To this end, we synthesized the peptide depicted in Figure 1 as described in the Methods section.

The radioiodinated peptide exhibited strong binding to excess streptavidin immobilized on agarose beads (Pierce Chemical Co.). In pilot in vitro experiments, a 1:1 starting molar ratio in biotin/Bolton-Hunter active ester gave moderate BIP specific binding (≈40%). An increase in the biotin derivative-to-Bolton-Hunter molar ratio (≈3:1) gave radiolabeled poly-L-lysine conjugates with better (>80%) specific binding. This is probably due to a reduction in the formation of iodinated poly-L-lysine conjugates lacking biotin. The radiolabeled peptide was further characterized by size-exclusion radio-HPLC (Pharmacia Superose 12). BIP revealed a single broad radioactive peak (Fig. 2) in the 10,000–20,000 molecular mass range which showed a modest degree of aggregation and degradation upon standing in serum for 24 hr (Fig. 2). The appearance of a broad peak is in full agreement with polylysine, which is a heterogeneous mixture. Figure 3 demonstrates the in vitro binding of BIP to SA beads. Indium-111-labeled biotin also had approximately 80% specific binding.

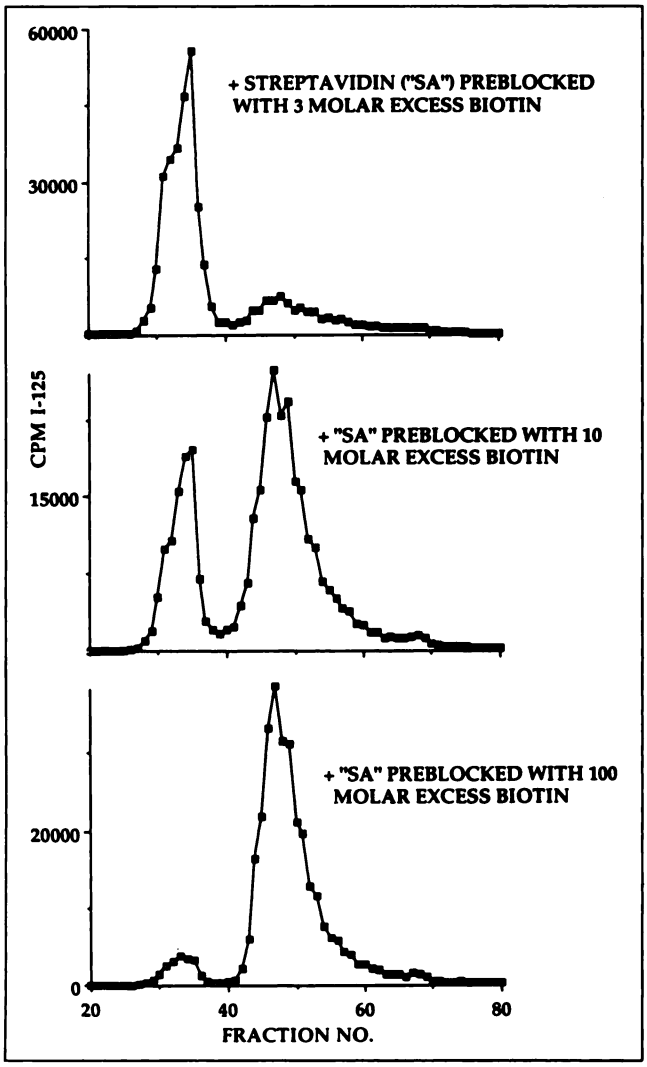

FIGURE 3. Radio-HPLC behavior of BIP in the presence of streptavidin. Streptavidin was preincubated with increasing amounts of biotin.

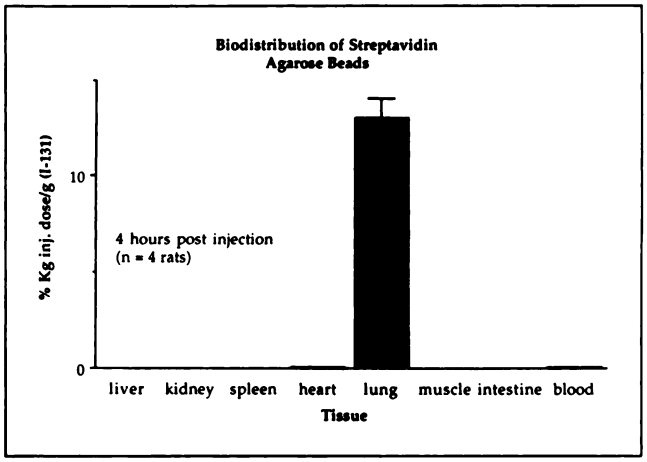

FIGURE 4. Biodistribution of streptavidin agarose beads (spiked with ^{131}I-labeled beads) at 4 hr in rats (n = 4) following intravenous administration. (Values are expressed in mean %kg ID/g of tissue ±s.e.m.)

Following the rationale of an earlier method (*12*), two groups of rats were intravenously injected with a suspension of streptavidin beads (Pierce Chemical Company, 64–165 μm in diameter), which were either unblocked or preincubated with excess biotin (nonspecific binding control group). Within seconds after intravenous injection, these beads localized in precapillary lung blood vessels, thus serving to approximate a disease model in which a biotin plus a streptavidin complex is localized to a site of disease. This pulmonary localization was verified by spiking the injected slurry of streptavidin agarose with streptavidin beads directly labeled with ^{131}I (*7*). Figure 4 shows the extensive lung localization of this mixture of cold and radiostreptavidin beads upon injection into rats at 4 hr postinjection.

Addition of streptavidin (mol wt ≈60,000) to BIP shifted the molecular mass of the radioactive BIP peak (Figs. 2 and 3) to a major species with a molecular mass in the range of 130–150 KD (*8*). This shift to a higher molecular weight is consistent with univalent binding of the streptavidin to the BIP. The presence of multiple biotinylated species was apparent from the radio-HPLC profiles (Fig. 3) obtained upon the reaction of peptide with streptavidin, which was first preincubated with either 3, 10 and 100 molar equivalents of biotin. Partial saturation of streptavidin sites under these conditions should lead to the enrichment of streptavidin species with less than four biotin binding sites, which

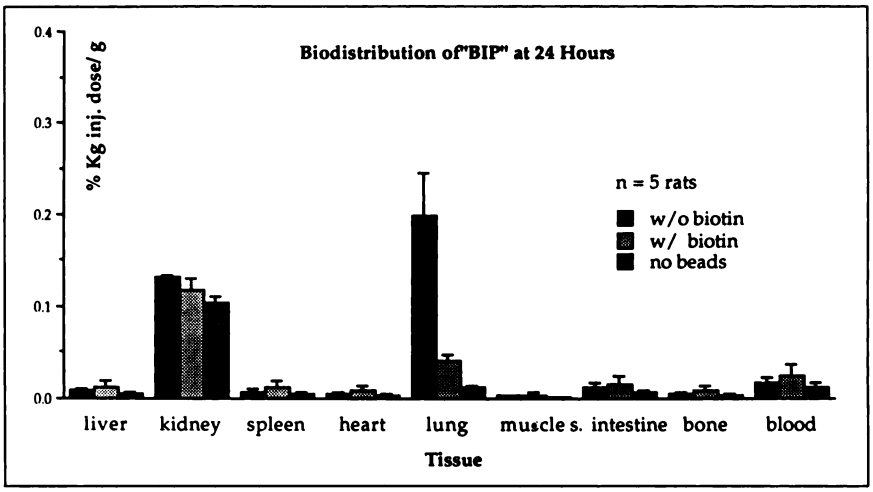

FIGURE 5. Biodistribution of BIP at 24 hr (n = 5 rats). BIP was given 1 hr after beads (solid = unblocked beads, light shaded = blocked and hatched = no beads added). (Values are expressed in mean %kg ID/g of tissue ±s.e.m.)

also minimizes peptide crosslinking. Again radio-HPLC shows a predominant complex in the 100,000–150,000 molecular weight range (consistent with roughly one peptide plus two streptavidins). The absence of polymeric species with higher molecular weights in all the radio-HPLC tracers is striking and suggests that the small size of the peptide compared to that of streptavidin (approximately one-sixth the mass of streptavidin) may be the limiting factor for the

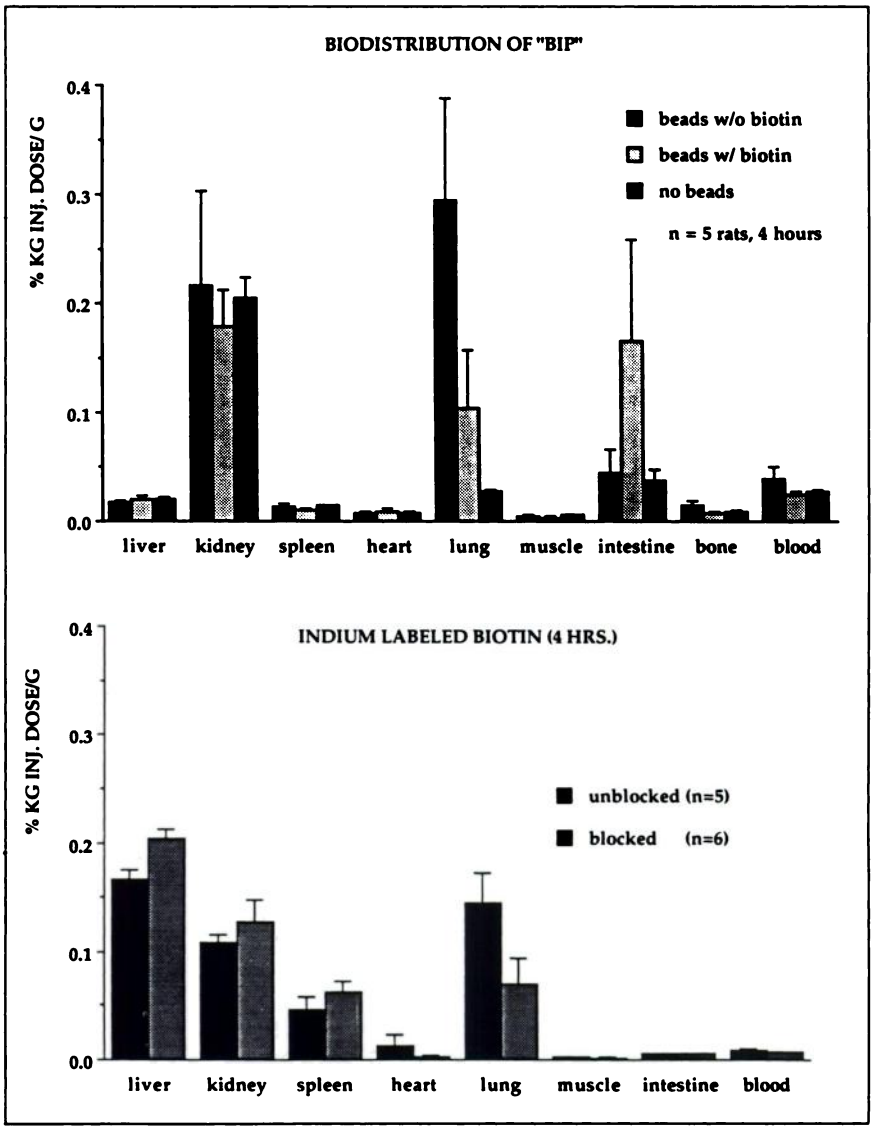

FIGURE 6. Comparative biodistribution of BIP and indium-chelated biotin at 4 hr postinjection in the same animal model. Values are expressed in mean %kg ID/g of tissue ±s.e.m.

maximum number of streptavidin complexes. In addition, our synthesis probably resulted in a BIP reagent of relatively low biotin valency.

In vivo localization properties of the radiolabeled peptide was evaluated by examining its biodistribution in three groups of rats (n = 5) which initially received no streptavidin, unblocked streptavidin and blocked streptavidin beads. The biodistribution of the BIP (>80% specific binding) at 4 and 24 hr postinjection in the three groups of rats is shown in Figures 5 and 6. For rats that did not initially receive streptavidin beads, substantial accumulation of BIP activity was notable only in the kidneys. At 4 and 24 hr after BIP injection, kidney BIP accumulation remained high for all groups of rats. Specific localization of the peptide in the lungs was apparent even at 4 hr after BIP injection, when comparing the group that received unblocked streptavidin beads versus those receiving biotin-saturated (blocked) streptavidin beads. It should also be noted that the radioiodine uptake in the intestines after 4 hr appears moderately high for the control group given blocked beads, although these data are quite variable. BIP renal targeting parallels that reported by Hnatowich et al. for ^{111}In-labeled biotin (*2*), who showed that the improvement in target-to-nontarget ratios in vivo was least apparent in the kidneys when using ^{111}In-labeled chelates of biotin. The high target- (lung) to-liver, target-to-spleen and target-to-blood ratios seen with BIP are especially encouraging, since relatively high accumulation into these organs is commonly associated with the use of higher molecular weight protein radioactivity carriers such as radioantibodies (*13*).

By using the same animal model, the biodistribution of radio-indium-chelated biotin was undertaken for comparison (Fig. 6). At 4 hr, specific BIP uptake was seen with ^{111}In-biotin in the lungs, although the radiolabeled conjugate displayed significantly higher uptake in the spleen and liver than BIP. The high ^{111}In uptake in the liver is probably the result of ^{111}In catabolism and transfer mechanisms which have previously been reported for indium-labeled antibodies (*14*). As is apparent from Figure 6, the lower liver and spleen uptake of BIP may be a useful advantage over the use of ^{111}In-DTPA-labeled biotin (*13*). In addition, BIP delivery to the lung target was nearly twice that of ^{111}In-labeled biotin (Fig. 6). This apparently higher targeting may be due to a longer period of circulation for the BIP versus the ^{111}In-labeled biotin or to multivalence of the biotinylated polylysine. This phenomenon needs additional study.

DISCUSSION

The present work suggests that BIP and similar peptide conjugates may represent useful alternatives to existing radio-biotin tracers. Our study also demonstrates that small peptides which serve both as carriers of target probes (e.g., biotin) and radioactivity can be synthesized with reasonable simplicity. This study further demonstrates the concept of using a fairly small peptide for targeting and with it solubilizing organic derivatives which are otherwise difficult to deliver in vivo. The BIP peptide and overall methodology described herein could be relatively easily applied in the synthesis of other biotinylated probes containing other isotopes such as ^{123}I, technetium or ^{18}F (*6, 15*).

The approach we outlined may allow for specific tailoring of the secondary radiotracer to the delivery situation in question. A change in the character of the polypeptide carrier may alter serum clearance rate, normal organ targeting and, thus, total targeting. We believe that this approach complements others in the literature and adds to the repertoire of agents available for biotin/streptavidin pretargeted delivery.

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