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(54) **Title:** PRETARGETING METHODS AND COMPOUNDS

(57) **Abstract**

Methods, compounds, compositions and kits that relate to pretargeted delivery of diagnostic and therapeutic agents are disclosed. In particular, methods for radiometal labeling of biotin, as well as related compounds, are described. Clearing agents and clearance mechanisms are also discussed.

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PRETARGETING METHODS AND COMPOUNDS

Technical Field

The present invention relates to methods, compounds, compositions and kits useful for delivering to a target site a targeting moiety that is conjugated to one member of a ligand/anti-ligand pair. After localization and clearance of the targeting moiety conjugate, direct or indirect binding of a diagnostic or therapeutic agent conjugate at the target site occurs. Methods for radiometal labeling of biotin or other small molecules, as well as the related compounds, are also disclosed. Clearing agents and clearance mechanisms are discussed, which agents or mechanisms facilitate a decrease in the serum half-life of targeting moiety-ligand or targeting moiety-anti-ligand conjugates.

Background of the Invention

Conventional cancer therapy is plagued by two problems. The generally attainable targeting ratio (ratio of administered dose localizing to tumor versus administered dose circulating in blood or ratio of administered dose localizing to tumor versus administered dose migrating to bone marrow) is low.

Also, the absolute dose of radiation or therapeutic agent delivered to the tumor is insufficient in many cases to elicit a significant tumor response. Improvement in targeting ratio or absolute dose to tumor is sought.

Summary of the Invention

The present invention is directed to diagnostic and therapeutic pretargeting methods, moieties useful therein and methods of making those moieties. Such pretargeting methods are characterized by an improved targeting ratio or increased absolute dose to the target cell sites in comparison to conventional cancer therapy.

The present invention provides clearing agents that incorporate ligand derivatives or anti-ligand derivatives, wherein such derivatives exhibit a lower affinity for the complementary ligand/anti-ligand pair member than the native form of the compound. In embodiments of the present invention employing a biotin-avidin or biotin-streptavidin ligand/anti-ligand pair, preferred clearing agents incorporate either a biotin derivative exhibiting a lower affinity for avidin or streptavidin than biotin or an avidin or a streptavidin derivative exhibiting a lower affinity for biotin than avidin or streptavidin. Preferred biotin derivatives for use in the practice of the present invention are 2'-thiobiotin, desthiobiotin, 1-oxy-biotin, 1-oxy-2'-thiobiotin, 1-sulfoxide-biotin, 1-sulfoxide-2'-thiobiotin, 1-sulfone-biotin, 1-sulfone-2'-thiobiotin, lipoic acid imminobiotin and the like.

The present invention further provides methods of increasing active agent localization at a target cell site of a mammalian recipient, which methods include:

administering to the recipient a first conjugate comprising a targeting moiety and a member of a ligand-anti-ligand binding pair;

thereafter administering to the recipient a clearing agent capable of directing the clearance of circulating first conjugate via hepatocyte receptors of the recipient, wherein the clearing agent does not incorporate a member of the ligand-anti-ligand binding pair or a lower binding affinity derivative thereof; or

thereafter administering to the recipient a clearing agent capable of directing the clearance of circulating first conjugate via hepatocyte receptors of the recipient, wherein the clearing agent incorporates a lower binding affinity derivative of a ligand/anti-ligand binding pair member, wherein the second conjugate binding pair member is complementary to that of the first conjugate; and

subsequently administering to the recipient a second conjugate comprising an active agent and a ligand/anti-ligand binding pair member, wherein the second conjugate binding pair member is complementary to that of the first conjugate.

In addition, the present invention provides methods of increasing active agent localization at a target cell site of a mammalian recipient, which methods include:

administering to the recipient a receptor blocking agent in an amount sufficient to substantially block a subpopulation of hepatocyte receptors;

administering to the recipient a first conjugate comprising a targeting moiety, a hepatocyte receptor recognizing agent, and a member of a ligand-anti-ligand binding pair; and

subsequently administering to the recipient a second conjugate comprising an active agent and a ligand/anti-ligand binding pair member, wherein the

second conjugate binding pair member is complementary to that of the first conjugate.

For this embodiment of the present invention, preferred receptor blocking agents include galactose-IgG conjugate, asialorosomucoid galactosylated biotins and other small molecule clearing agents and the like. The receptor blocking agents are preferably administered in multiple doses over time to facilitate substantially continuous blockage of a substantial portion of the relevant hepatocyte receptors. The receptor becomes deblocked through receptor-based clearance of the blocking agent and cessation of administration of such blocking agent. Preferably, the cessation/clearance events occur after a time sufficient to permit localization of the targeting moiety to target sites. In addition, the second conjugate is preferably administered after a time sufficient to permit receptor-based clearance of circulating first conjugate.

Brief Description of the Drawings

Figure 1 illustrates blood clearance of biotinylated antibody following intravenous administration of avidin.

Figure 2 depicts radiorhenium tumor uptake in a three-step pretargeting protocol, as compared to administration of radiolabeled antibody (conventional means involving antibody that is covalently linked to chelated radiorhenium).

Figure 3 depicts the tumor uptake profile of NR-LU-10-streptavidin conjugate (LU-10-StrAv) in comparison to a control profile of native NR-LU-10 whole antibody.

Figure 4 depicts the tumor uptake and blood clearance profiles of NR-LU-10-streptavidin conjugate.

Figure 5 depicts the rapid clearance from the blood of asialoorosomucoid in comparison with

orosomucoid in terms of percent injected dose of I-125-labeled protein.

Figure 6 depicts the 5 minute limited biodistribution of asialoorosomucoid in comparison with orosomucoid in terms of percent injected dose of I-125-labeled protein.

Figure 7 depicts NR-LU-10-streptavidin conjugate blood clearance upon administration of three controls (o, ●, ■) and two doses of a clearing agent (□, ◇) at 25 hours post-conjugate administration.

Figure 8 shows limited biodistribution data for LU-10-StrAv conjugate upon administration of three controls (Groups 1, 2 and 5) and two doses of clearing agent (Groups 3 and 4) at two hours post-clearing agent administration.

Figure 9 depicts NR-LU-10-streptavidin conjugate serum biotin binding capability at 2 hours post-clearing agent administration.

Figure 10 depicts NR-LU-10-streptavidin conjugate blood clearance over time upon administration of a control (o) and three doses of a clearing agent (▽, △, □) at 24 hours post-conjugate administration.

Figure 11A depicts the blood clearance of LU-10-StrAv conjugate upon administration of a control (PBS) and three doses (50, 20 and 10 μg) of clearing agent at two hours post-clearing agent administration.

Figure 11B depicts LU-10-StrAv conjugate serum biotin binding capability upon administration of a control (PBS) and three doses (50, 20 and 10 μg) of clearing agent at two hours post-clearing agent administration.

Figure 12 depicts the blood clearance of PIP-125 LU-10/SA with and without 50:1 biotin-sc-galactose in BALB/c mice.

Figure 13 depicts the blood clearance of PIP-LU-10/SA with and without galactose-biotin analogs in BALB/c mice.

Figure 14 depicts the blood clearance of PIP-125 LU-10/SA pre-complexed with galactose-biotin analogs.

Figure 15 also depicts the blood clearance of PIP-125 LU-10/SA pre-complexed with galactose-biotin analogs.

Figure 16 depicts blood clearance in BALB/c mice of I-125 LU-10/SA following administration of 100 μg of biotin-galactose analogs.

Figure 17 depicts blood clearance in BALB/c mice of I-125 LU-10/SA following administration of 100 μg of biotin-galactose analogs.

Figure 18 depicts the structure of a preferred biotin-galactose analog, $(gal)_{16}$-Bt.

Figure 19 depicts the blood clearance of PIP-125 LU-10/SA in BALB/c mice alone or precomplexed with $(gal)_{16}$-biotin.

Figure 20 depicts the blood clearance of PIP-125 following intravenous injection of $(gal)_{16}$-biotin at ratios of 100, 50 or 10:1 to circulating conjugate.

Figure 21 depicts blood clearance of PIP-125 following intravenous injection of $(gal)_{16}$-biotin at ratios of 100, 50 or 10:1 to circulating conjugate.

Figure 22a depicts blood clearance of PIP-125 LU-10/SA with and without gal-HSA-Bt or $(gal)_{16}$-Bt clearing agents.

Figure 22b contains the blood clearance data corresponding to the results depicted in Figure 22a.

Figure 23 depicts the biodistribution after two hours of Y-90-DOTA-biotin following LU-10/SA and either 220 μg gal-HSA-Bt or 46 μg of $(gal)_{16}$-Bt in SW-1222 tumored mice and SHT-1 tumored mice.

Figure 24 depicts biodistribution of Y-90-DOTA-biotin two hours after administration with 3 hour interval for $(gal)_{16}$-Bt or gal-HSA-Bt clearing agent.

Figure 25 depicts molar ratio of DOTA-biotin to LU-10/SA two hours after administration of DOTA-biotin.

Detailed Description of the Invention

Prior to setting forth the invention, it may be helpful to set forth definitions of certain terms to be used within the disclosure.

Targeting moiety: A molecule that binds to a defined population of cells. The targeting moiety may bind a receptor, an oligonucleotide, an enzymatic substrate, an antigenic determinant, or other binding site present on or in the target cell population. Antibody is used throughout the specification as a prototypical example of a targeting moiety. Tumor is used as a prototypical example of a target in describing the present invention.

Ligand/anti-ligand pair: A complementary/anti-complementary set of molecules that demonstrate specific binding, generally of relatively high affinity. Exemplary ligand/anti-ligand pairs include zinc finger protein/dsDNA fragment, enzyme/inhibitor, hapten/antibody, lectin/carbohydrate, ligand/receptor, and biotin/avidin. Biotin/avidin is used throughout the specification as a prototypical example of a ligand/anti-ligand pair.

Anti-ligand: As defined herein, an "anti-ligand" demonstrates high affinity, and preferably, multivalent binding of the complementary ligand. Preferably, the anti-ligand is large enough to avoid rapid renal clearance, and contains sufficient multivalency to accomplish crosslinking and aggregation of targeting moiety-ligand conjugates. Univalent anti-ligands are also contemplated by the present invention. Anti-ligands of the present invention may exhibit or be derivitized to exhibit structural features that direct the uptake thereof, e.g., galactose residues that direct liver uptake. Avidin and streptavidin are used herein as prototypical anti-ligands.

Avidin: As defined herein, "avidin" includes avidin, streptavidin and derivatives and analogs thereof that are capable of high affinity, multivalent or univalent binding of biotin.

Ligand: As defined herein, a "ligand" is a relatively small, soluble molecule that exhibits rapid serum, blood and/or whole body clearance when administered intravenously in an animal or human. Biotin is used as the prototypical ligand.

Lower Affinity Ligand or Lower Affinity Anti-Ligand: A ligand or anti-ligand that binds to its complementary ligand-anti-ligand pair member with an affinity that is less than the affinity with which native ligand or anti-ligand binds the complementary member. Preferably, lower affinity ligands and anti-ligands exhibit between from about 10^{-6} to 10^{-10} M binding affinity for the native form of the complementary anti-ligand or ligand. Lower affinity ligands and anti-ligands may be employed in clearing agents or in active agent-containing conjugates of the present invention.

Active Agent: A diagnostic or therapeutic agent ("the payload"), including radionuclides, drugs, anti-tumor agents, toxins and the like. Radionuclide therapeutic agents are used as prototypical active agents.

N_xS_y Chelates: As defined herein, the term "N_xS_y chelates" includes bifunctional chelators that are capable of (i) coordinately binding a metal or radiometal and (ii) covalently attaching to a targeting moiety, ligand or anti-ligand. Particularly preferred N_xS_y chelates have N_2S_2 and N_3S cores. Exemplary N_xS_y chelates are described in Fritzberg et al., Proc. Natl. Acad. Sci. USA 85:4024-29, 1988; in Weber et al., Bioconj. Chem. 1:431-37, 1990; and in the references cited therein, for instance.

Pretargeting: As defined herein, pretargeting involves target site localization of a targeting moiety that is conjugated with one member of a ligand/anti-ligand pair; after a time period sufficient for optimal target-to-non-target accumulation of this targeting moiety conjugate, active agent conjugated to the opposite member of the ligand/anti-ligand pair is administered and is bound (directly or indirectly) to the targeting moiety conjugate at the target site (two-step pretargeting). Three-step and other related methods described herein are also encompassed.

Clearing Agent: An agent capable of binding, complexing or otherwise associating with an administered moiety (e.g., targeting moiety-ligand, targeting moiety-anti-ligand or anti-ligand alone) present in the recipient's circulation, thereby facilitating circulating moiety clearance from the recipient's body, removal from blood circulation, or inactivation thereof in circulation. The clearing agent is preferably characterized by physical properties, such as size, charge, configuration or a combination thereof, that limit clearing agent access to the population of target cells recognized by a targeting moiety used in the same treatment protocol as the clearing agent.

Conjugate: A conjugate encompasses chemical conjugates (covalently or non-covalently bound), fusion proteins and the like.

A recognized disadvantage associated with *in vivo* administration of targeting moiety-radioisotopic conjugates for imaging or therapy is localization of the attached radioactive agent at both non-target and target sites. Until the administered radiolabeled conjugate clears from the circulation, normal organs and tissues are transitorily exposed to the attached

radioactive agent. For instance, radiolabeled whole antibodies that are administered *in vivo* exhibit relatively slow blood clearance; maximum target site localization generally occurs 1-3 days post-administration. Generally, the longer the clearance time of the conjugate from the circulation, the greater the radioexposure of non-target organs.

These characteristics are particularly problematic with human radioimmunotherapy. In human clinical trials, the long circulating half-life of radioisotope bound to whole antibody causes relatively large doses of radiation to be delivered to the whole body. In particular, the bone marrow, which is very radiosensitive, is the dose-limiting organ of non-specific toxicity.

In order to decrease radioisotope exposure of non-target tissue, potential targeting moieties generally have been screened to identify those that display minimal non-target reactivity, while retaining target specificity and reactivity. By reducing non-target exposure (and adverse non-target localization and/or toxicity), increased doses of a radiotherapeutic conjugate may be administered; moreover, decreased non-target accumulation of a radiodiagnostic conjugate leads to improved contrast between background and target.

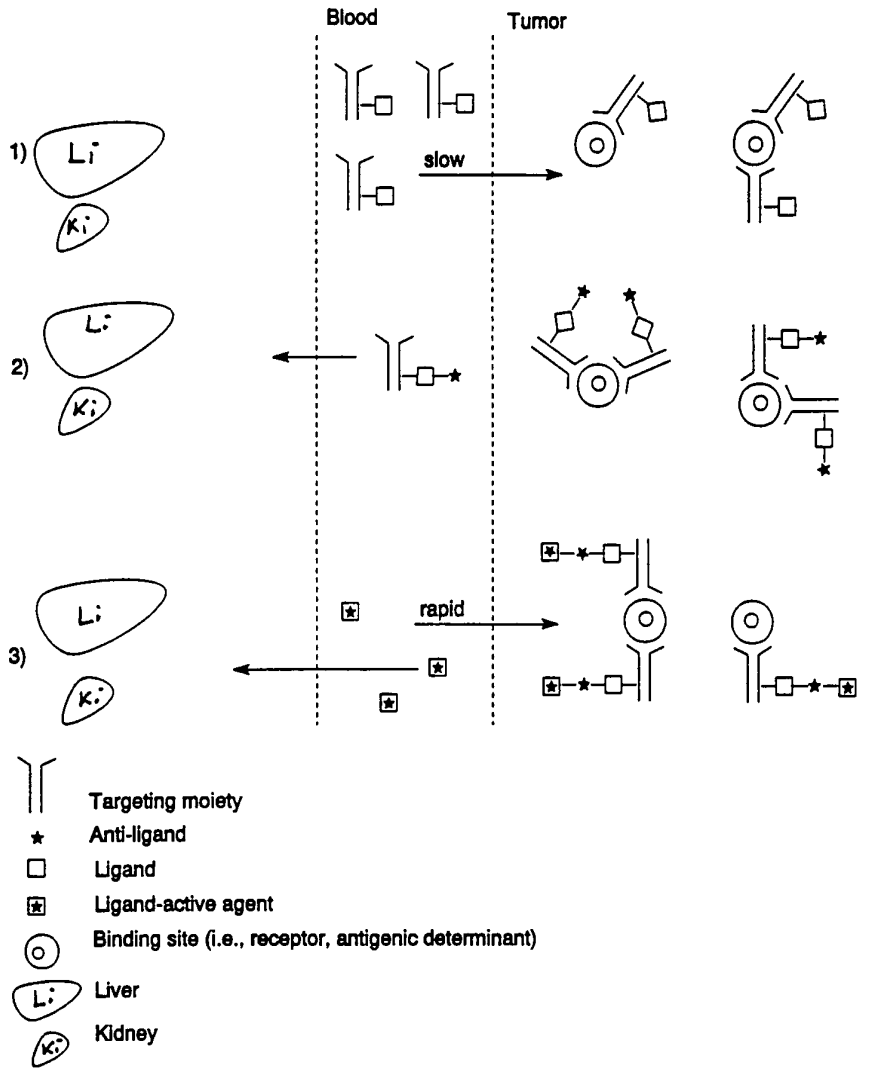
Therapeutic drugs, administered alone or as targeted conjugates, are accompanied by similar disadvantages. Again, the goal is administration of the highest possible concentration of drug (to maximize exposure of target tissue), while remaining below the threshold of unacceptable normal organ toxicity (due to non-target tissue exposure). Unlike radioisotopes, however, therapeutic drugs need to be taken into a target cell to exert a cytotoxic effect. In the case of targeting moiety-therapeutic drug conjugates, it would be advantageous to combine the

relative target specificity of a targeting moiety with a means for enhanced target cell internalization of the targeting moiety-drug conjugate.

In contrast, enhanced target cell internalization is disadvantageous if one administers diagnostic agent-targeting moiety conjugates. Internalization of diagnostic conjugates results in cellular catabolism and degradation of the conjugate. Upon degradation, small adducts of the diagnostic agent or the diagnostic agent per se may be released from the cell, thus eliminating the ability to detect the conjugate in a target-specific manner.

One method for reducing non-target tissue exposure to a diagnostic or therapeutic agent involves "pretargeting" the targeting moiety at a target site, and then subsequently administering a rapidly clearing diagnostic or therapeutic agent conjugate that is capable of binding to the "pretargeted" targeting moiety at the target site. A description of some embodiments of the pretargeting technique may be found in US Patent No. 4,863,713 (Goodwin et al.).

A typical pretargeting approach ("three-step") is schematically depicted below.



Briefly, this three-step pretargeting protocol features administration of an antibody-ligand conjugate, which is allowed to localize at a target site and to dilute in the circulation. Subsequently administered anti-ligand binds to the antibody-ligand conjugate and clears unbound antibody-ligand conjugate from the blood. Preferred anti-ligands are large and contain sufficient multivalency to accomplish crosslinking and aggregation of circulating antibody-ligand conjugates. The clearing by anti-ligand is

probably attributable to anti-ligand crosslinking and/or aggregation of antibody-ligand conjugates that are circulating in the blood, which leads to complex/aggregate clearance by the recipient's RES (reticuloendothelial system). Anti-ligand clearance of this type is preferably accomplished with a multivalent molecule; however, a univalent molecule of sufficient size to be cleared by the RES on its own could also be employed. Alternatively, receptor-based clearance mechanisms, e.g., Ashwell receptor or hexose residue, such as galactose or mannose residue, recognition mechanisms, may be responsible for anti-ligand clearance. Such clearance mechanisms are less dependent upon the valency of the anti-ligand with respect to the ligand than the RES complex/aggregate clearance mechanisms. It is preferred that the ligand-anti-ligand pair displays relatively high affinity binding.

A diagnostic or therapeutic agent-ligand conjugate that exhibits rapid whole body clearance is then administered. When the circulation brings the active agent-ligand conjugate in proximity to the target cell-bound antibody-ligand-anti-ligand complex, anti-ligand binds the circulating active agent-ligand conjugate and produces an antibody-ligand : anti-ligand : ligand-active agent "sandwich" at the target site. Because the diagnostic or therapeutic agent is attached to a rapidly clearing ligand (rather than antibody, antibody fragment or other slowly clearing targeting moiety), this technique promises decreased non-target exposure to the active agent.

Alternate pretargeting methods eliminate the step of parenterally administering an anti-ligand clearing agent. These "two-step" procedures feature targeting moiety-ligand or targeting moiety-anti-ligand administration, followed by administration of active agent conjugated to the opposite member of the ligand-

anti-ligand pair. As an optional step "1.5" in the two-step pretargeting methods of the present invention, a clearing agent (preferably other than ligand or anti-ligand alone) is administered to facilitate the clearance of circulating targeting moiety-containing conjugate.

In the two-step pretargeting approach, the clearing agent preferably does not become bound to the target cell population, either directly or through the previously administered and target cell bound targeting moiety-anti-ligand or targeting moiety-ligand conjugate. An example of two-step pretargeting involves the use of biotinylated human transferrin as a clearing agent for avidin-targeting moiety conjugate, wherein the size of the clearing agent results in liver clearance of transferrin-biotin-circulating avidin-targeting moiety complexes and substantially precludes association with the avidin-targeting moiety conjugates bound at target cell sites. (See, Goodwin, D.A., Antibod. Immunoconj. Radiopharm., 4: 427-34, 1991).

The two-step pretargeting approach overcomes certain disadvantages associated with the use of a clearing agent in a three-step pretargeted protocol. More specifically, data obtained in animal models demonstrate that *in vivo* anti-ligand binding to a pretargeted targeting moiety-ligand conjugate (i.e., the cell-bound conjugate) removes the targeting moiety-ligand conjugate from the target cell. One explanation for the observed phenomenon is that the multivalent anti-ligand crosslinks targeting moiety-ligand conjugates on the cell surface, thereby initiating or facilitating internalization of the resultant complex. The apparent loss of targeting moiety-ligand from the cell might result from internal degradation of the conjugate and/or release of active agent from the conjugate (either at the cell surface

or intracellularly). An alternative explanation for the observed phenomenon is that permeability changes in the target cell's membrane allow increased passive diffusion of any molecule into the target cell. Also, some loss of targeting moiety-ligand may result from alteration in the affinity by subsequent binding of another moiety to the targeting moiety-ligand, e.g., anti-idiotype monoclonal antibody binding causes removal of tumor bound monoclonal antibody.

The present invention recognizes that this phenomenon (apparent loss of the targeting moiety-ligand from the target cell) may be used to advantage with regard to *in vivo* delivery of therapeutic agents generally, or to drug delivery in particular. For instance, a targeting moiety may be covalently linked to both ligand and therapeutic agent and administered to a recipient. Subsequent administration of anti-ligand crosslinks targeting moiety-ligand-therapeutic agent tripartite conjugates bound at the surface, inducing internalization of the tripartite conjugate (and thus the active agent). Alternatively, targeting moiety-ligand may be delivered to the target cell surface, followed by administration of anti-ligand-therapeutic agent.

In one aspect of the present invention, a targeting moiety-anti-ligand conjugate is administered *in vivo*; upon target localization of the targeting moiety-anti-ligand conjugate (i.e., and clearance of this conjugate from the circulation), an active agent-ligand conjugate is parenterally administered. This method enhances retention of the targeting moiety-anti-ligand : ligand-active agent complex at the target cell (as compared with targeting moiety-ligand : anti-ligand : ligand-active agent complexes and targeting moiety-ligand : anti-ligand-active agent complexes). Although a variety of ligand/anti-ligand pairs may be suitable for use within the claimed

invention, a preferred ligand/anti-ligand pair is biotin/avidin.

In a second aspect of the invention, radioiodinated biotin and related methods are disclosed. Previously, radioiodinated biotin derivatives were of high molecular weight and were difficult to characterize. The radioiodinated biotin described herein is a low molecular weight compound that has been easily and well characterized.

In a third aspect of the invention, a targeting moiety-ligand conjugate is administered *in vivo*; upon target localization of the targeting moiety-ligand conjugate (i.e., and clearance of this conjugate from the circulation), a drug-anti-ligand conjugate is parenterally administered. This two-step method not only provides pretargeting of the targeting moiety conjugate, but also induces internalization of the subsequent targeting moiety-ligand-anti-ligand-drug complex within the target cell. Alternatively, another embodiment provides a three-step protocol that produces a targeting moiety-ligand : anti-ligand : ligand-drug complex at the surface, wherein the ligand-drug conjugate is administered simultaneously or within a short period of time after administration of anti-ligand (i.e., before the targeting moiety-ligand-anti-ligand complex has been removed from the target cell surface).

In a fourth aspect of the invention, methods for radiolabeling biotin with technetium-99m, rhenium-186 and rhenium-188 are disclosed. Previously, biotin derivatives were radiolabeled with indium-111 for use in pretargeted immunoscintigraphy (for instance, Virzi et al., Nucl. Med. Biol. 18:719-26, 1991; Kalofonos et al., J. Nucl. Med. 31: 1791-96, 1990; Paganelli et al., Canc. Res. 51:5960-66, 1991). However, ^{99m}Tc is a particularly preferred radionuclide for immunoscintigraphy due to (i) low cost, (ii)

convenient supply and (iii) favorable nuclear properties. Rhenium-186 displays chelating chemistry very similar to ^{99m}Tc, and is considered to be an excellent therapeutic radionuclide (i.e., a 3.7 day half-life and 1.07 MeV maximum particle that is similar to ^{131}I). Therefore, the claimed methods for technetium and rhenium radiolabeling of biotin provide numerous advantages.

The "targeting moiety" of the present invention binds to a defined target cell population, such as tumor cells or a thrombus site. Preferred targeting moieties useful in this regard include antibody and antibody fragments, peptides, and hormones. Proteins corresponding to known cell surface receptors (including low density lipoproteins, transferrin and insulin), fibrinolytic enzymes, anti-HER2, platelet binding proteins such as annexins, and biological response modifiers (including interleukin, interferon, erythropoietin and colony-stimulating factor) are also preferred targeting moieties. Also, anti-EGF receptor antibodies, which internalize following binding to the receptor and traffic to the nucleus to an extent, are preferred targeting moieties for use in the present invention to facilitate delivery of Auger emitters and nucleus binding drugs to target cell nuclei. Oligonucleotides, e.g., antisense oligonucleotides that are complementary to portions of target cell nucleic acids (DNA or RNA), are also useful as targeting moieties in the practice of the present invention. Oligonucleotides binding to cell surfaces are also useful. Analogs of the above-listed targeting moieties that retain the capacity to bind to a defined target cell population may also be used within the claimed invention. In addition, synthetic targeting moieties may be designed.

Functional equivalents of the aforementioned molecules are also useful as targeting moieties of the

present invention. One targeting moiety functional equivalent is a "mimetic" compound, an organic chemical construct designed to mimic the proper configuration and/or orientation for targeting moiety-target cell binding. Another targeting moiety functional equivalent is a short polypeptide designated as a "minimal" polypeptide, constructed using computer-assisted molecular modeling and mutants having altered binding affinity, which minimal polypeptides exhibit the binding affinity of the targeting moiety.

Preferred targeting moieties of the present invention are antibodies (polyclonal or monoclonal), peptides, oligonucleotides or the like. Polyclonal antibodies useful in the practice of the present invention are polyclonal (Vial and Callahan, Univ. Mich. Med. Bull., 20: 284-6, 1956), affinity-purified polyclonal or fragments thereof (Chao et al., Res. Comm. in Chem. Path. & Pharm., 9: 749-61, 1974).

Monoclonal antibodies useful in the practice of the present invention include whole antibody and fragments thereof. Such monoclonal antibodies and fragments are producible in accordance with conventional techniques, such as hybridoma synthesis, recombinant DNA techniques and protein synthesis. Useful monoclonal antibodies and fragments may be derived from any species (including humans) or may be formed as chimeric proteins which employ sequences from more than one species. See, generally, Kohler and Milstein, Nature, 256: 495-97, 1975; Eur. J. Immunol., 6: 511-19, 1976.

Human monoclonal antibodies or "humanized" murine antibody are also useful as targeting moieties in accordance with the present invention. For example, murine monoclonal antibody may be "humanized" by genetically recombining the nucleotide sequence encoding the murine Fv region (i.e., containing the

antigen binding sites) or the complementarity determining regions thereof with the nucleotide sequence encoding a human constant domain region and an Fc region, e.g., in a manner similar to that disclosed in European Patent Application No. 0,411,893 A2. Some murine residues may also be retained within the human variable region framework domains to ensure proper target site binding characteristics. Humanized targeting moieties are recognized to decrease the immunoreactivity of the antibody or polypeptide in the host recipient, permitting an increase in the half-life and a reduction in the possibility of adverse immune reactions. Also, single chain antibodies, FV's and dimers thereof are useful targeting moieties. Still further bispecific antibodies are suitable targeting moieties.

Types of active agents (diagnostic or therapeutic) useful herein include toxins, anti-tumor agents, drugs and radionuclides. Several of the potent toxins useful within the present invention consist of an A and a B chain. The A chain is the cytotoxic portion and the B chain is the receptor-binding portion of the intact toxin molecule (holotoxin). Because toxin B chain may mediate non-target cell binding, it is often advantageous to conjugate only the toxin A chain to a targeting protein. However, while elimination of the toxin B chain decreases non-specific cytotoxicity, it also generally leads to decreased potency of the toxin A chain-targeting protein conjugate, as compared to the corresponding holotoxin-targeting protein conjugate.

Preferred toxins in this regard include holotoxins, such as abrin, ricin, modeccin, Pseudomonas exotoxin A, Diphtheria toxin, pertussis toxin and Shiga toxin; and A chain or "A chain-like" molecules, such as ricin A chain, abrin A chain,

modeccin A chain, the enzymatic portion of Pseudomonas exotoxin A, Diphtheria toxin A chain, the enzymatic portion of pertussis toxin, the enzymatic portion of Shiga toxin, gelonin, pokeweed antiviral protein, saporin, tritin, barley toxin and snake venom peptides. Ribosomal inactivating proteins (RIPs), naturally occurring protein synthesis inhibitors that lack translocating and cell-binding ability, are also suitable for use herein. Extremely highly toxic toxins, such as palytoxin and the like, are also contemplated for use in the practice of the present invention.

Preferred drugs suitable for use herein include conventional chemotherapeutics, such as vinblastine, doxorubicin, bleomycin, methotrexate, 5-fluorouracil, 6-thioguanine, cytarabine, cyclophosphamide and cis-platinum, as well as other conventional chemotherapeutics as described in Cancer: Principles and Practice of Oncology, 2d ed., V.T. DeVita, Jr., S. Hellman, S.A. Rosenberg, J.B. Lippincott Co., Philadelphia, PA, 1985, Chapter 14. A particularly preferred drug within the present invention is a trichothecene.

Trichothecenes are drugs produced by soil fungi of the class *Fungi imperfecti* or isolated from *Baccharus megapotamica* (Bamburg, J.R. Proc. Molec. Subcell. Biol. 8:41-110, 1983; Jarvis & Mazzola, Acc. Chem. Res. 15:338-395, 1982). They appear to be the most toxic molecules that contain only carbon, hydrogen and oxygen (Tamm, C. Fortschr. Chem. Org. Naturst. 31:61-117, 1974). They are all reported to act at the level of the ribosome as inhibitors of protein synthesis at the initiation, elongation, or termination phases.

There are two broad classes of trichothecenes: those that have only a central sesquiterpenoid structure and those that have an additional

macrocyclic ring (simple and macrocyclic trichothecenes, respectively). The simple trichothecenes may be subdivided into three groups (i.e., Group A, B, and C) as described in U.S. Patent Nos. 4,744,981 and 4,906,452 (incorporated herein by reference). Representative examples of Group A simple trichothecenes include: Scirpene, Roridin C, dihydrotrichothecene, Scirpen-4, 8-diol, Verrucarol, Scirpentriol, T-2 tetraol, pentahydroxyscirpene, 4-deacetylneosolaniol, trichodermin, deacetylcalonectrin, calonectrin, diacetylverrucarol, 4-monoacetoxyscirpenol, 4,15-diacetoxyscirpenol, 7-hydroxydiacetoxyscirpenol, 8-hydroxydiacetoxy-scirpenol (Neosolaniol), 7,8-dihydroxydiacetoxyscirpenol, 7-hydroxy-8-acetyldiacetoxyscirpenol, 8-acetylneosolaniol, NT-1, NT-2, HT-2, T-2, and acetyl T-2 toxin. Representative examples of Group B simple trichothecenes include: Trichothecolone, Trichothecin, deoxynivalenol, 3-acetyldeoxynivalenol, 5-acetyldeoxynivalenol, 3,15-diacetyldeoxynivalenol, Nivalenol, 4-acetylnivalenol (Fusarenon-X), 4,15-idacetylnivalenol, 4,7,15-triacetylnivalenol, and tetra-acetylnivalenol. Representative examples of Group C simple trichothecenes include: Crotocol and Crotocin. Representative macrocyclic trichothecenes include Verrucarin A, Verrucarin B, Verrucarin J (Satratoxin C), Roridin A, Roridin D, Roridin E (Satratoxin D), Roridin H, Satratoxin F, Satratoxin G, Satratoxin H, Vertisporin, Mytoxin A, Mytoxin C, Mytoxin B, Myrotoxin A, Myrotoxin B, Myrotoxin C, Myrotoxin D, Roritoxin A, Roritoxin B, and Roritoxin D. In addition, the general "trichothecene" sesquiterpenoid ring structure is also present in compounds termed "baccharins" isolated from the higher plant *Baccharis megapotamica*, and these are described in the literature, for instance as disclosed by

Jarvis et al. (Chemistry of Alleopathy, ACS Symposium Series No. 268: ed. A.C. Thompson, 1984, pp. 149-159).

Experimental drugs, such as mercaptopurine, N-methylformamide, 2-amino-1,3,4-thiadiazole, melphalan, hexamethylmelamine, gallium nitrate, 3% thymidine, dichloromethotrexate, mitoguazone, suramin, bromodeoxyuridine, iododeoxyuridine, semustine, 1-(2-chloroethyl)-3-(2,6-dioxo-3-piperidyl)-1-nitrosourea, N,N'-hexamethylene-bis-acetamide, azacitidine, dibromodulcitol, Erwinia asparaginase, ifosfamide, 2-mercaptoethane sulfonate, teniposide, taxol, 3-deazauridine, soluble Baker's antifol, homoharringtonine, cyclocytidine, acivicin, ICRF-187, spiromustine, levamisole, chlorozotocin, aziridinyl benzoquinone, spirogermanium, aclarubicin, pentostatin, PALA, carboplatin, amsacrine, caracemide, iproplatin, misonidazole, dihydro-5-azacytidine, 4'-deoxy-doxorubicin, menogaril, triciribine phosphate, fazarabine, tiazofurin, teroxirone, ethiofos, N-(2-hydroxyethyl)-2-nitro-1H-imidazole-1-acetamide, mitoxantrone, acodazole, amonafide, fludarabine phosphate, pibenzimol, didemnin B, merbarone, dihydrolenperone, flavone-8-acetic acid, oxantrazole, ipomeanol, trimetrexate, deoxyspergualin, echinomycin, and dideoxycytidine (see NCI Investigational Drugs, Pharmaceutical Data 1987, NIH Publication No. 88-2141, Revised November 1987) are also preferred.

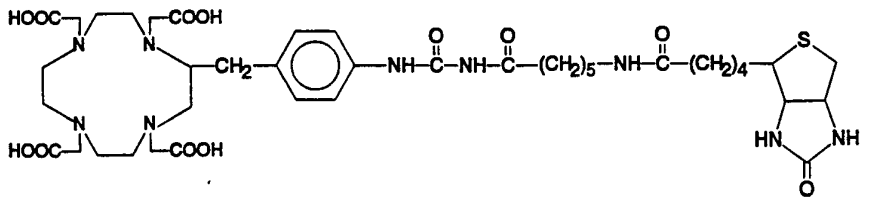
Radionuclides useful within the present invention include gamma-emitters, positron-emitters, Auger electron-emitters, X-ray emitters and fluorescence-emitters, with beta- or alpha-emitters preferred for therapeutic use. Radionuclides are well-known in the art and include ^{123}I, ^{125}I, ^{130}I, ^{131}I, ^{133}I, ^{135}I, ^{47}Sc, ^{72}As, ^{72}Se, ^{90}Y, ^{88}Y, ^{97}Ru, ^{100}Pd, ^{101m}Rh, ^{119}Sb, ^{128}Ba, ^{197}Hg, ^{211}At, ^{212}Bi, ^{153}Sm, ^{169}Eu, ^{212}Pb, ^{109}Pd, ^{111}In, ^{67}Ga, ^{68}Ga, ^{64}Cu, ^{67}Cu, ^{75}Br, ^{76}Br, ^{77}Br, ^{99m}Tc, ^{11}C, ^{13}N, ^{15}O, ^{166}Ho and ^{18}F. Preferred therapeutic

radionuclides include ^{188}Re, ^{186}Re, ^{203}Pb, ^{212}Pb, ^{212}Bi, ^{109}Pd, ^{64}Cu, ^{67}Cu, ^{90}Y, ^{125}I, ^{131}I, ^{77}Br, ^{211}At, ^{97}Ru, ^{105}Rh, ^{198}Au and ^{199}Ag, ^{166}Ho or ^{177}Lu.

Other anti-tumor agents,e.g., agents active against proliferating cells, are administrable in accordance with the present invention. Exemplary anti-tumor agents include cytokines, such as IL-2, tumor necrosis factor or the like, lectin inflammatory response promoters (selectins), such as L-selectin, E-selectin, P-selectin or the like, and like molecules.

Ligands suitable for use within the present invention include biotin, S-peptide, head activator peptide (HA-peptide), haptens, lectins, epitopes, dsDNA fragments, enzyme inhibitors and analogs and derivatives thereof. Useful complementary anti-ligands include avidin (for biotin), carbohydrates (for lectins) and antibody, fragments or analogs thereof, including mimetics (for haptens and epitopes) and zinc finger proteins (for dsDNA fragments) and enzymes (for enzyme inhibitors). Preferred ligands and anti-ligands bind to each other with an affinity of at least about $k_D \geq 10^9$ M.

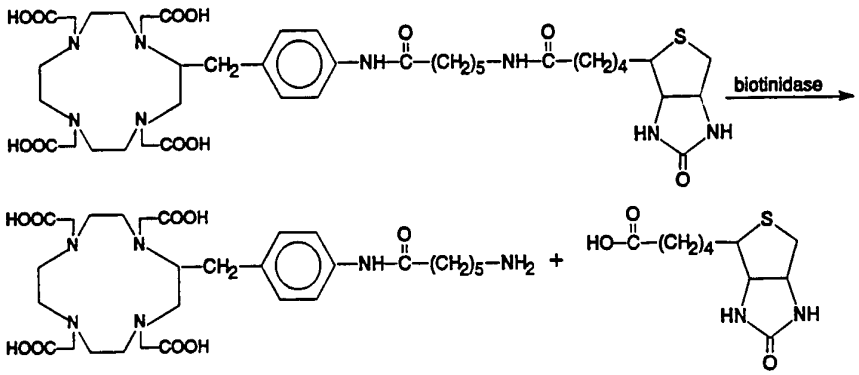
The 1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetra acetic acid (DOTA)-biotin conjugate (DOTA-LC-biotin) depicted below has been reported to have desirable *in vivo* biodistribution and is cleared primarily by renal excretion.



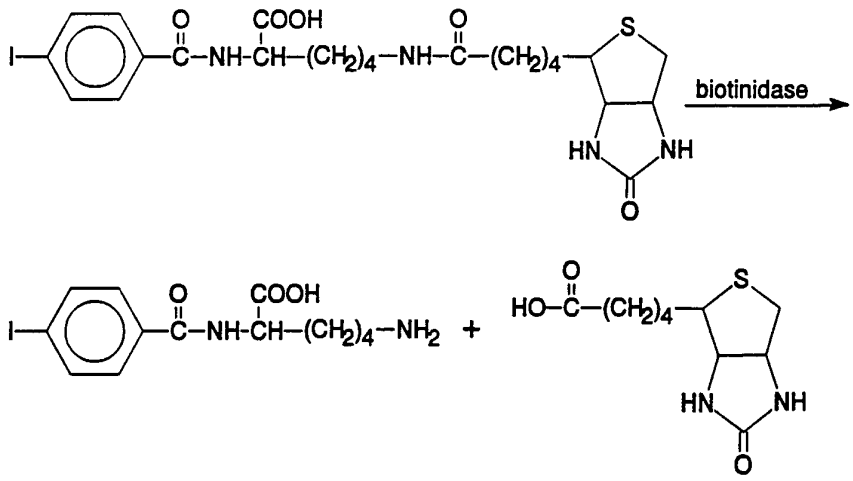
DOTA may also be conjugated to other ligands or to anti-ligands in the practice of the present invention.

Because DOTA strongly binds Y-90 and other radionuclides, it has been proposed for use in radioimmunotherapy. For therapy, it is very important that the radionuclide be stably bound within the DOTA chelate and that the DOTA chelate be stably attached to a ligand or anti-ligand. For illustrative purposes, DOTA-biotin conjugates are described. Only radiolabeled DOTA-biotin conjugates exhibiting those two characteristics are useful to deliver radionuclides to the targets. Release of the radionuclide from the DOTA chelate or cleavage of the biotin and DOTA conjugate components in serum or at non-target sites renders the conjugate unsuitable for use in therapy.

Serum stability of DOTA-LC-biotin (where LC refers to the "long chain" linker, including an aminocaproyl spacer between the biotin and the DOTA conjugate components) shown above, while reported in the literature to be good, has proven to be problematic. Experimentation has revealed that DOTA-LC-biotin is rapidly cleared from the blood and excreted into the urine as fragments, wherein the biotinamide bond rather than the DOTA-amide bond has been cleaved, as shown below.



Additional experimentation employing PIP-biocytin conjugates produced parallel results as shown below.



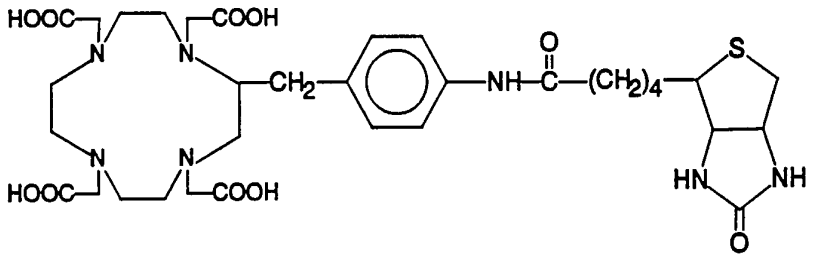
Cleavage of the benzamide was not observed as evidenced by the absence of detectable quantities of iodobenzoic acid in the serum.

It appears that the cleavage results from the action of serum biotinidase. Biotinidase is a hydrolytic enzyme that catalyzes the cleavage of biotin from biotinyl peptides. See, for example, Evangelatos, et al., "Biotinidase Radioassay Using an I-125-Biotin Derivative, Avidin, and Polyethylene Glycol Reagents," Analytical Biochemistry, 196: 385-89, 1991.

Drug-biotin conjugates which structurally resemble biotinyl peptides are potential substrates for cleavage by plasma biotinidase. Poor *in vivo* stability therefore limits the use of drug-biotin conjugates in therapeutic applications. The use of peptide surrogates to overcome poor stability of peptide therapeutic agents has been an area of intense research effort. See, for example, Spatola, Peptide Backbone Modification: A Structure-Activity Analysis of Peptide Containing Amide Bond Surrogates, "Chemistry and Biochemistry of Amino Acids, Peptides and Proteins," vol. 7, Weinstein, ed., Marcel Dekker, New York, 1983; and Kim et al., "A New Peptide Bond

Surrogate: 2-Isoxazoline in Pseudodipeptide Chemistry," Tetrahedron Letters, 45: 6811-14, 1991.

Elimination of the aminocaproyl spacer of DOTA-LC-biotin gives DOTA-SC-biotin (where the SC indicates the "short chain" linker between the DOTA and biotin conjugate components), which molecule is shown below:



DOTA-SC-biotin exhibits significantly improved serum stability in comparison to DOTA-LC-biotin. This result does not appear to be explainable on the basis of biotinidase activity alone. The experimentation leading to this conclusion is summarized in the Table set forth below.

Time Dependent Cleavage of DOTA-Biotin Conjugates

Time at 37°C	% Avidin Binding		
	PIP-Biocytin	Y-90-LC DOTA-Biotin	Y-90-SC DOTA-Biotin
5 Minutes	75%	50%	-
15 Minutes	57%	14%	-
30 Minutes	31%	12%	-
60 Minutes	-	0%	98%
20 Hours	-	0%	60%

where "-" indicates that the value was not measured.

The difference in serum stability between DOTA-LC-biotin and DOTA-SC-biotin might be explained by the fact that the SC derivative contains an aromatic amide linkage in contrast to the aliphatic amide linkage of

the LC derivative, with the aliphatic amide linkage being more readily recognized by enzymes as a substrate therefor. This argument cannot apply to biotinidase, however, because biotinidase very efficiently cleaves aromatic amides. In fact, it is recognized that the simplest and most commonly employed biotinidase activity measuring method uses N-(d-biotinyl)-4-aminobenzoate (BPABA) as a substrate, with the hydrolysis of BPABA resulting in the liberation of biotin and 4-aminobenzoate (PABA). See, for example, B. Wolf, et al., "Methods in Enzymology," pp. 103-111, Academic Press Inc., 1990. Consequently, one would predict that DOTA-SC-biotin, like its LC counterpart, would be a biotinidase substrate. Since DOTA-SC-biotin exhibits serum stability, biotinidase activity alone does not adequately explain why some conjugates are serum stable while others are not. A series of DOTA-biotin conjugates was therefore synthesized by the present inventors to determine which structural features conferred serum stability to the conjugates.

Some general strategies for improving serum stability of peptides with respect to enzymatic action are the following: incorporation of D-amino acids, N-methyl amino acids and alpha-substituted amino acids.

in vivo stable biotin-DOTA conjugates are useful within the practice of the present invention. *in vivo* stability imparts the following advantages:

1) increased tumor uptake in that more of the radioisotope will be targeted to the previously localized targeting moiety-streptavidin; and

2) increased tumor retention, if biotin is more stably bound to the radioisotope.

In addition, the linkage between DOTA and biotin may also have a significant impact on biodistribution (including normal organ uptake, target uptake and the like) and pharmacokinetics.

The strategy for design of the DOTA-containing molecules and conjugates of the present invention involved three primary considerations:

1) *in vivo* stability (including biotinidase and general peptidase activity resistance), with an initial acceptance criterion of 100% stability for 1 hour;

2) renal excretion; and

3) ease of synthesis.

The DOTA-biotin conjugates of the present invention reflect the implementation of one or more of the following strategies:

1) substitution of the carbon adjacent to the cleavage susceptible amide nitrogen;

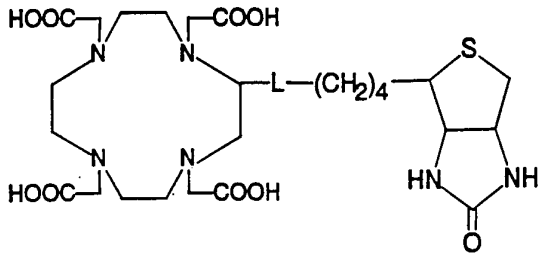
2) alkylation of the cleavage susceptible amide nitrogen;

3) substitution of the amide carbonyl with an alkyl amino group;

4) incorporation of D-amino acids as well as analogs or derivatives thereof; or

5) incorporation of thiourea linkages.

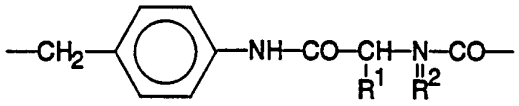
DOTA-biotin conjugates in accordance with the present invention may be generally characterized as follows: conjugates that retain the biotin carboxy group in the structure thereof and those that do not (i.e., the terminal carboxy group of biotin has been reduced or otherwise chemically modified. Structures of such conjugates represented by the following general formula have been devised:



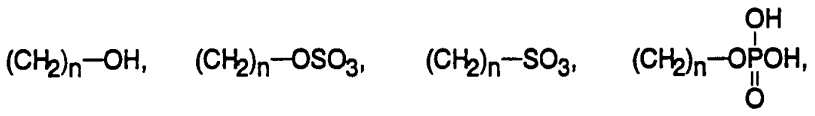
wherein L may alternatively be substituted in one of the following ways on one of the $-CH_2-COOH$ branches of the DOTA structure: $-CH(L)-COOH$ or $-CH_2COOL$ or $-CH_2COL$). When these alternative structures are employed, the portion of the linker bearing the functional group for binding with the DOTA conjugate component is selected for the capability to interact with either the carbon or the carboxy in the branch portions of the DOTA structure, with the serum stability conferring portion of the linker structure being selected as described below.

In the case where the linkage is formed on the core of the DOTA structure as shown above, L is selected according to the following principles, with the portion of the linker designed to bind to the DOTA conjugate component selected for the capability to bind to an amine.

A. One embodiment of the present invention includes linkers incorporating a D-amino acid spacer between a DOTA aniline amine and the biotin carboxy group shown above. Substituted amino acids are preferred for these embodiments of the present invention, because alpha-substitution also confers enzymatic cleavage resistance. Exemplary L moieties of this embodiment of the present invention may be represented as follows:



where R^1 is selected from lower alkyl, lower alkyl substituted with hydrophilic groups (preferably,



where n is 1 or 2), glucuronide-substituted amino acids or other glucuronide derivatives; and

R^2 is selected from hydrogen, lower alkyl, substituted lower alkyl (e.g., hydroxy, sulfate, phosphonate or a hydrophilic moiety (preferably OH).

For the purposes of the present disclosure, the term "lower alkyl" indicates an alkyl group with from one to five carbon atoms. Also, the term "substituted" includes one or several substituent groups, with a single substituent group preferred.

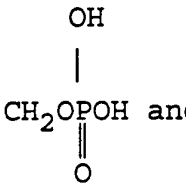
Preferred L groups of this embodiment of the present invention include the following:

R^1 = CH_3 and R^2 = H (a D-alanine derivative, with a synthetic scheme therefor shown in Example XV);

R^1 = CH_3 and R^2 = CH_3 (an N-methyl-D-alanine derivative);

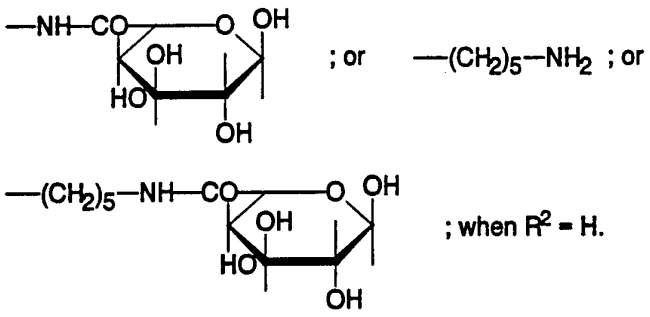
R^1 = CH_2-OH and R^2 = H (a D-serine derivative);

R^1 = CH_2OSO_3 and R^2 = H (a D-serine-O-sulfate-derivative); and

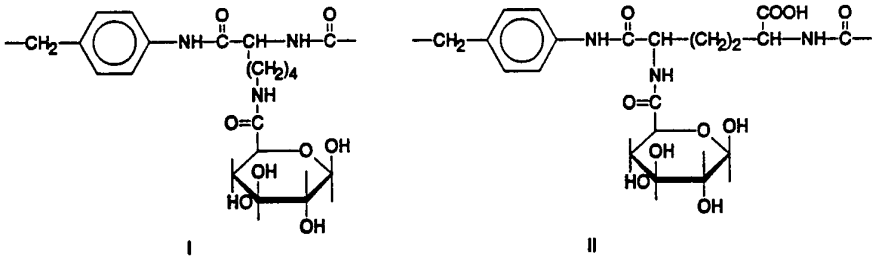
R^1 =  and R^2 = H (a D-serine-O-phosphonate-derivative);

Other preferred moieties of this embodiment of the present invention include molecules wherein R^1 is hydrogen and R^2 = $-(CH_2)_nOH$ or a sulfate or

phosphonate derivative thereof and n is 1 or 2 as well as molecules wherein R^1 is



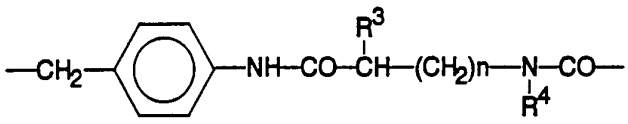
Preferred moieties incorporating the glucuronide of D-lysine and the glucuronide of amino pimelate are shown below as I and II, respectively.



A particularly preferred linker of this embodiment of the present invention is the D-alanine derivative set forth above.

B. Linkers incorporating alkyl substitution on one or more amide nitrogen atoms are also encompassed by the present invention, with some embodiments of such linkers preparable from L-amino acids. Amide bonds having a substituted amine moiety are less

susceptible to enzymatic cleavage. Such linkers exhibit the following general formula:



where R^4 is selected from hydrogen, lower alkyl, lower alkyl substituted with hydroxy, sulfate, phosphonate or the like and $-(CH_2)_{n+1}-CO-NH-$$-CH_2-DOTA$;

R_3 is selected from hydrogen; an amine; lower alkyl; an amino- or a hydroxy-, sulfate- or phosphonate-substituted lower alkyl; a glucuronide or a glucuronide-derivatized amino groups; and

n ranges from 0-4.

Preferred linkers of this embodiment of the present invention include:

R^3 = H and R^4 = CH_3 when n = 4, synthesizable as discussed in Example XV;

R^3 = H and R^4 = CH_3 when n = 0, synthesizable from N-methyl-glycine (having a trivial name of sarcosine) as described in Example XV;

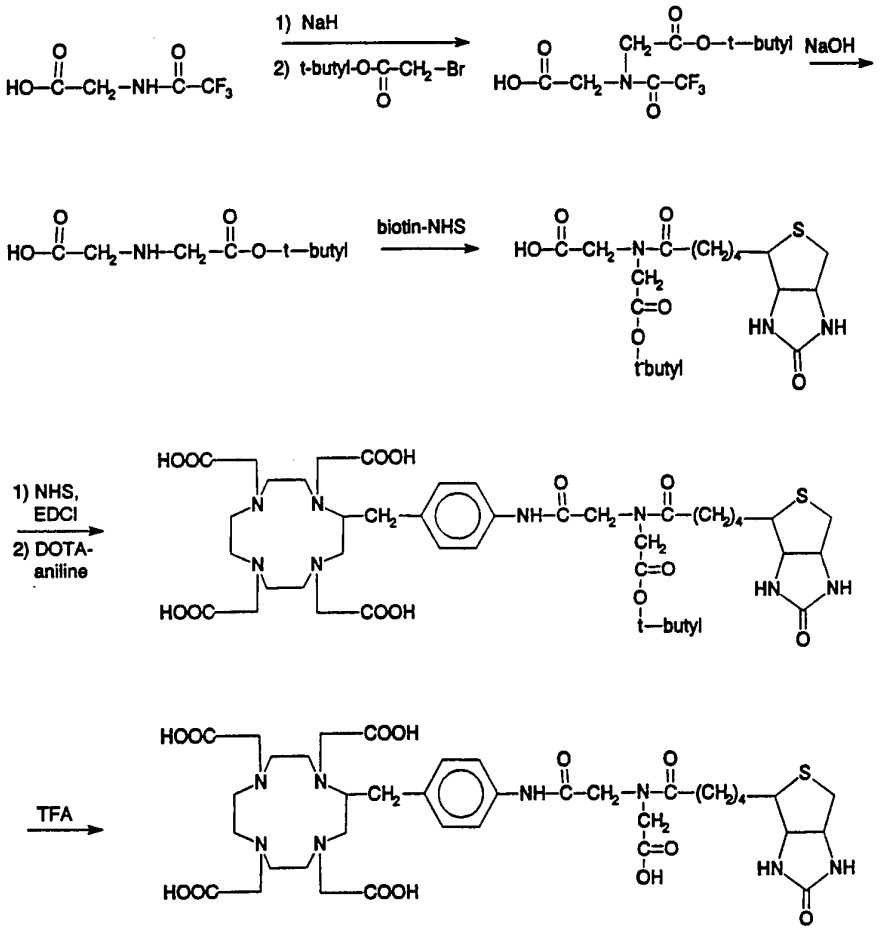
R^3 = NH_2 and R^4 = CH_3, when n = 0;

R^3 = H and R^4 = $-(CH_2)_5-CO-NH-$$-CH_2-DOTA$ when n = 4 (Bis-DOTA-LC-biotin), synthesizable from bromohexanoic acid as discussed in Example XV; and

R^3 = H and R^4 = $-(CH_2)-CO-NH-$$-CH_2-DOTA$ when n = 0 (bis-DOTA-SC-biotin), synthesizable from iminodiacetic acid.

The synthesis of a conjugate including a linker wherein R^3 is H and R^4 is $-CH_2CH_2OH$ and n is 0 is also described in Example XV. Schematically, the synthesis

of a conjugate of this embodiment of the present invention wherein n is 0, R^3 is H and R^4 is $-CH_2-COOH$ is shown below.



Bis-DOTA-LC-biotin, for example, offers the following advantages:

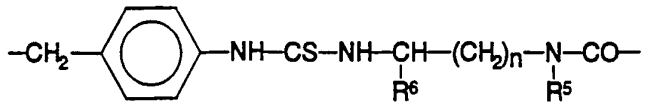
1) incorporation of two DOTA molecules on one biotin moiety increases the overall hydrophilicity of the biotin conjugate and thereby directs *in vivo* distribution to urinary excretion; and

2) substitution of the amide nitrogen adjacent to the biotin carboxyl group blocks peptide and/or biotinidase cleavage at that site.

Bis-DOTA-LC-biotin, the glycine-based linker and the N-methylated linker where R^3 = H, R^4 = CH_3, n = 4

are particularly preferred linkers of this embodiment of the present invention.

C. Another linker embodiment incorporates a thiourea moiety therein. Exemplary thiourea adducts of the present invention exhibit the following general formula:



where R^5 is selected from hydrogen or lower alkyl;

R^6 is selected from H and a hydrophilic moiety; and

n ranges from 0-4.

Preferred linkers of this embodiment of the present invention are as follows:

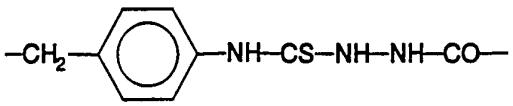
R^5 = H and R^6 = H when n = 5;

R^5 = H and R^6 = COOH when n = 5; and

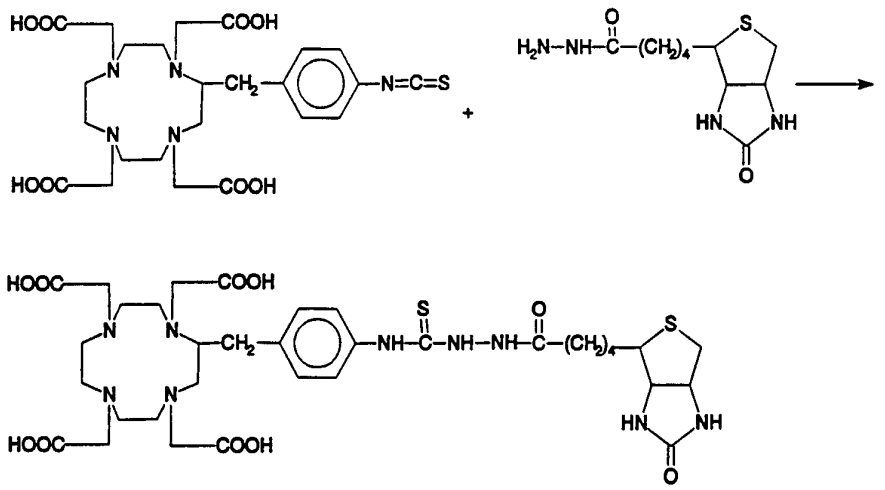
R^5 = CH_3 and R^6 = COOH when n = 5.

The second preferred linker recited above can be prepared using either L-lysine or D-lysine. Similarly, the third preferred linker can be prepared using either N-methyl-D-lysine or N-methyl-L-lysine.

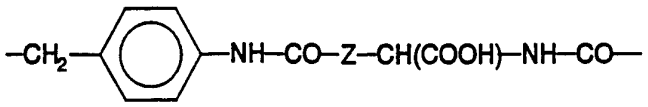
Another thiourea adduct of minimized lipophilicity is



which may be formed via the addition of biotinhydrazide (commercially available from Sigma Chemical Co., St. Louis, Missouri) and DOTA-benzyl-isothiocyanate (a known compound synthesized in one step from DOTA-aniline), with the thiourea-containing compound formed as shown below.



D. Amino acid-derived linkers of the present invention with substitution of the carbon adjacent to the cleavage susceptible amide have the general formula set forth below:



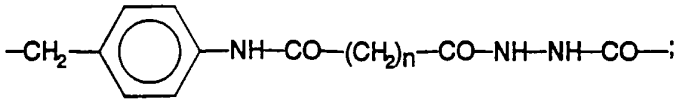
wherein Z is $-(CH_2)_2-$, conveniently synthesized form glutamic acid; or

Z = $-CH_2-S-CH_2-$, synthesizable from cysteine and iodo-acetic acid; or

Z = $-CH_2-$, conveniently synthesized form aspartic acid; or

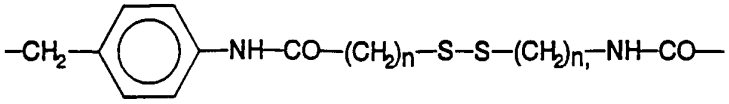
Z = $-(CH_2)_n-CO-O-CH_2-$, where n ranges from 1-4 and which is synthesizable from serine.

E. Another exemplary linker embodiment of the present invention has the general formula set forth below:



and n ranges from 1-5.

F. Another embodiment involves disulfide-containing linkers, which provide a metabolically cleavable moiety (-S-S-) to reduce non-target retention of the biotin-DOTA conjugate. Exemplary linkers of this type exhibit the following formula:

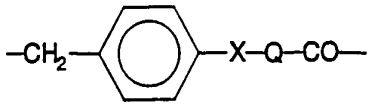


where n and n' preferably range between 0 and 5.

The advantage of using conditionally cleavable linkers is an improvement in target/non-target localization of the active agent. Conditionally cleavable linkers include enzymatically cleavable linkers, linkers that are cleaved under acidic conditions, linkers that are cleaved under basic conditions and the like. More specifically, use of linkers that are cleaved by enzymes, which are present in non-target tissues but reduced in amount or absent in target tissue, can increase target cell retention of active agent relative to non-target cell retention. Such conditionally cleavable linkers are useful, for example, in delivering therapeutic radionuclides to target cells, because such active agents do not require internalization for efficacy, provided that the linker is stable at the target cell surface or protected from target cell degradation.

Cleavable linkers are also useful to effect target site selective release of active agent at target sites. Active agents that are preferred for cleavable linker embodiments of the present invention are those that are substantially non-cytotoxic when conjugated to ligand or anti-ligand. Such active agents therefore require release from the ligand- or anti-ligand-containing conjugate to gain full potency. For example, such active agents, while conjugated, may be unable to bind to a cell surface receptor; unable to internalize either actively or passively; or unable to serve as a binding substrate for a soluble (intra- or inter-cellular) binding protein or enzyme. Exemplary of an active agent-containing conjugate of this type is chemotherapeutic drug-cis-aconityl-biotin. The cis-aconityl linker is acid sensitive. Other acid sensitive linkers useful in cleavable linker embodiments of the present invention include esters, thioesters and the like. Use of conjugates wherein an active agent and a ligand or an anti-ligand are joined by a cleavable linker will result in the selective release of the active agent at tumor cell target sites, for example, because the inter-cellular milieu of tumor tissue is generally of a lower pH (more highly acidic) than the inter-cellular milieu of normal tissue.

G. Ether, thioether, ester and thioester linkers are also useful in the practice of the present invention. Ether and thioether linkers are stable to acid and basic conditions and are therefore useful to deliver active agents that are potent in conjugated form, such as radionuclides and the like. Ester and thioesters are hydrolytically cleaved under acidic or basic conditions or are cleavable by enzymes including esterases, and therefore facilitate improved target:non-target retention. Exemplary linkers of this type have the following general formula:



where X is O or S; and

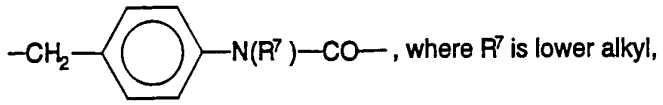
Q is a bond, a methylene group, a -CO- group or $-CO-(CH_2)_n-NH-$; and

n ranges from 1-5.

Other such linkers have the general formula:

$-CH_2-X-Q$, where Q and X are defined as set forth above.

H. Another amino-containing linker of the present invention is structured as follows:

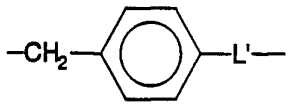


preferably methyl.

In this case, resistance to enzymatic cleavage is conferred by the alkyl substitution on the amine.

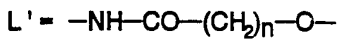
I. Polymeric linkers are also contemplated by the present invention. Dextran and cyclodextran are preferred polymers useful in this embodiment of the present invention as a result of the hydrophilicity of the polymer, which leads to favorable excretion of conjugates containing the same. Other advantages of using dextran polymers are that such polymers are substantially non-toxic and non-immunogenic, that they are commercially available in a variety of sizes and that they are easy to conjugate to other relevant molecules. Also, dextran-linked conjugates exhibit advantages when non-target sites are accessible to dextranase, an enzyme capable of cleaving dextran polymers into smaller units while non-target sites are not so accessible.

Other linkers of the present invention are produced prior to conjugation to DOTA and following the reduction of the biotin carboxy moiety. These linkers of the present invention have the following general formula:

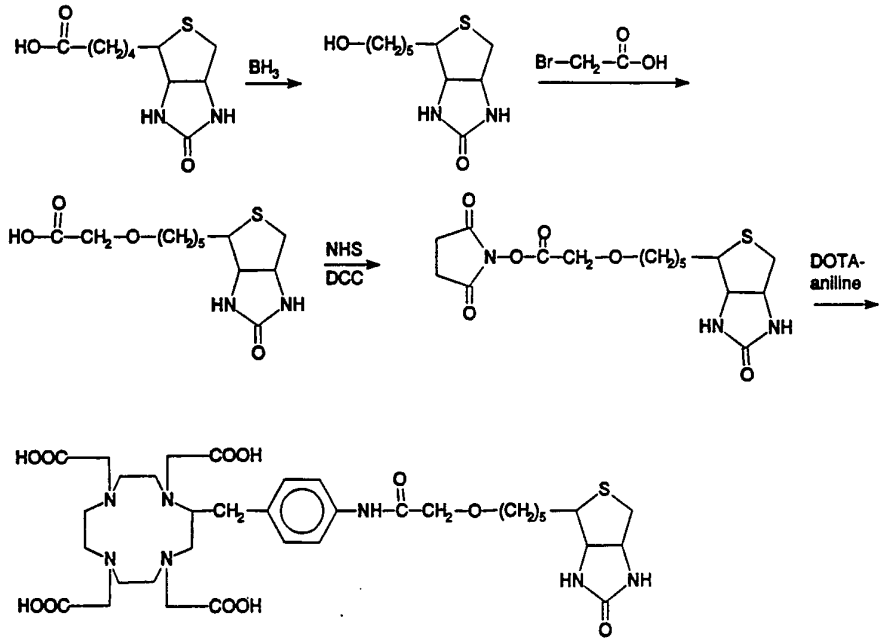


Embodiments of linkers of this aspect of the present invention include the following:

J. An ether linkage as shown below may be formed in a DOTA-biotin conjugate in accordance with the procedure indicated below.



where n ranges from 1 to 5, with 1 preferred.



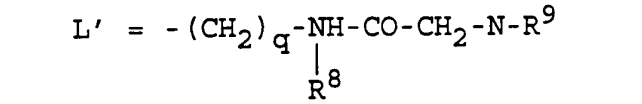
This linker has only one amide moiety which is bound directly to the DOTA aniline (as in the structure of DOTA-SC-biotin). In addition, the ether linkage imparts hydrophilicity, an important factor in facilitating renal excretion.

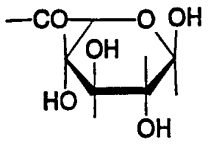
K. An amine linker formed from reduced biotin (hydroxybiotin or aminobiotin) is shown below, with conjugates containing such a linker formed, for example, in accordance with the procedure described in Example XV.

L' = -NH-

This linker contains no amide moieties and the unalkylated amine may impart favorable biodistribution properties since unalkylated DOTA-aniline displays excellent renal clearance.

L. Substituted amine linkers, which can form conjugates via amino-biotin intermediates, are shown below.



where R^8 is H; $-(CH_2)_2-OH$ or a sulfate or phosphonate derivative thereof; or  ; or the like;

and R^9 is a bond or $-(CH_2)_n-CO-NH-$, where n ranges from 0-5 and is preferably 1 and where q is 0 or 1. These moieties exhibit the advantages of an amide only directly attached to DOTA-aniline and either a non-amide amine imparting a positive charge to the linker *in vivo* or a N-alkylated glucuronide hydrophilic group, each alternative favoring renal excretion.

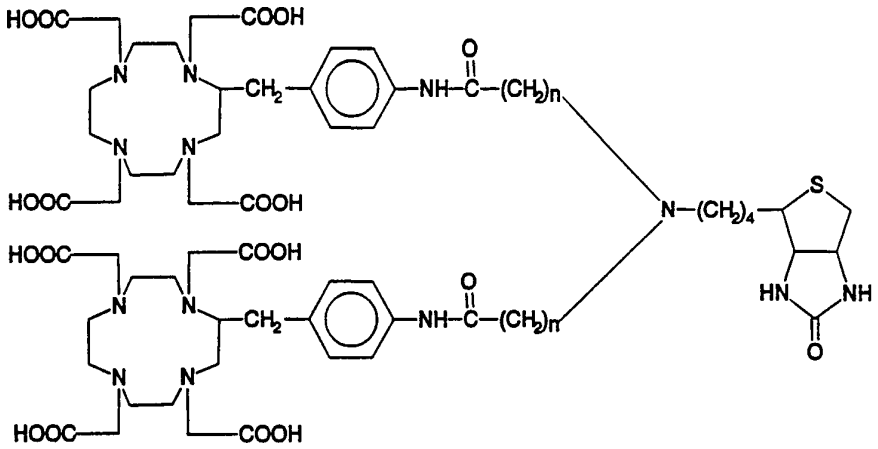
M. Amino biotin may also be used as an intermediate in the production of conjugates linked by

linkers having favorable properties, such as a thiourea-containing linker of the formula:

L' = -NH-CS-NH-

Conjugates containing this thiourea linker have the following advantages: no cleavable amide and a short, fairly polar linker which favors renal excretion.

A bis-DOTA derivative of the following formula can also be formed from amino-biotin.



where n ranges from 1 to 5, with 1 and 5 preferred. This molecule offers the advantages of the previously discussed bis-DOTA derivatives with the added advantage of no cleavable amides.

Additional linkers of the present invention which are employed in the production of conjugates characterized by a reduced biotin carboxy moiety are the following:

L = $-(CH_2)_4$-NH-, wherein the amine group is attached to the methylene group corresponding to the reduced biotin carboxy moiety and the methylene chain is attached to a core carbon in the DOTA ring. Such a linker is conveniently synthesizable from lysine.

L = $-(CH_2)_q$-CO-NH-, wherein q is 1 or 2, and wherein the amine group is attached to the methylene group corresponding to the reduced biotin carboxy moiety and the methylene group(s) are attached to a core carbon in the DOTA ring. This moiety is synthesizable from amino-biotin.

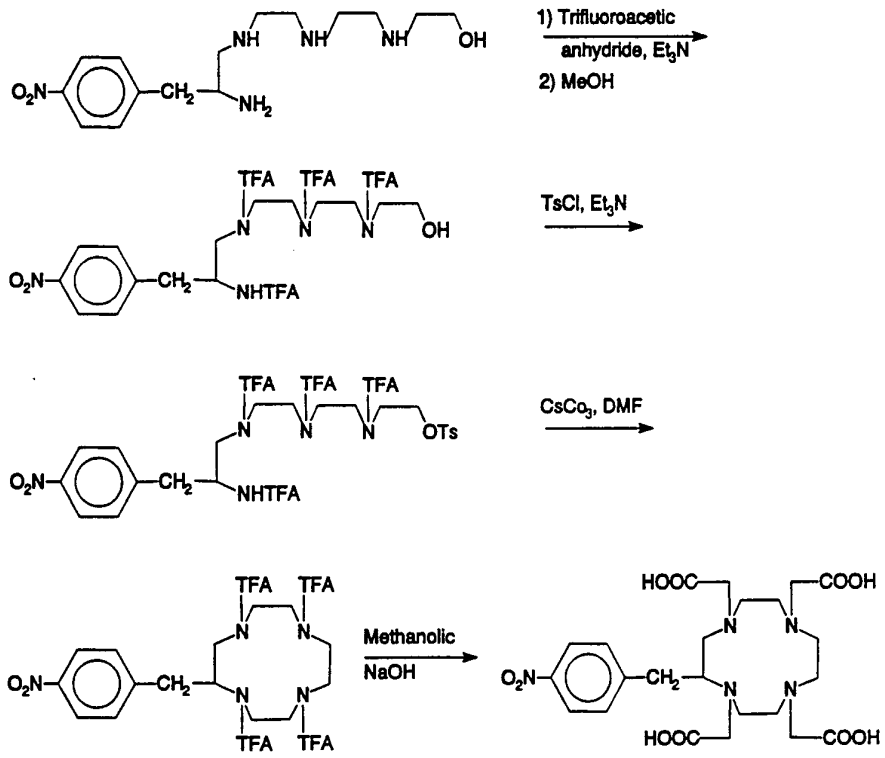
The linkers set forth above are useful to produce conjugates having one or more of the following advantages:

- bind avidin or streptavidin with the same or substantially similar affinity as free biotin;
- bind metal M^{+3} ions efficiently and with high kinetic stability;
- are excreted primarily through the kidneys into urine;
- are stable to endogenous enzymatic or chemical degradation (e.g., bodily fluid amidases, peptidases or the like);
- penetrate tissue rapidly and bind to pretargeted avidin or streptavidin; and
- are excreted rapidly with a whole body residence half-life of less than about 5 hours.

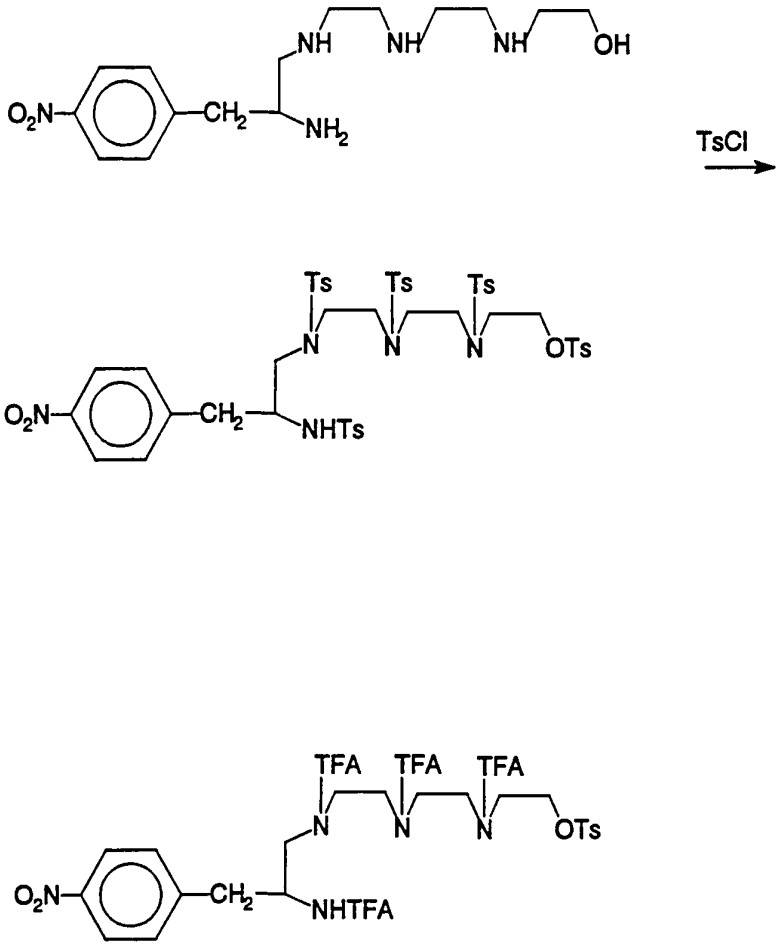
Synthetic routes to an intermediate of the DOTA-biotin conjugates depicted above, nitrobenzyl-DOTA, have been proposed. These proposed synthetic routes produce the intermediate compound in suboptimal yield, however. For example, Renn and Meares, "Large Scale Synthesis of Bifunctional Chelating Agent Q-(p-nitrobenzyl)-1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetra acetic acid, and the Determination of its Enantiomeric Purity by Chiral Chromatography," Bioconj. Chem., 3: 563-9, 1992, describe a nine-step synthesis of nitrobenzyl-DOTA, including reaction steps that either proceed in low yield or involve cumbersome transformations or purifications. More specifically, the sixth step proceeds in only 26% yield, and the product must be purified by preparative

HPLC. Additionally, step eight proceeds in good yield, but the process involves copious volumes of the coreactants.

These difficulties in steps 6-8 of the prior art synthesis are overcome in the practice of the present invention through the use of the following synthetic alternative therefor.



The poor yield in step six of the prior art synthesis procedure, in which a tetra amine alcohol is converted to a tetra-toluenesulfonamide toluenesulfonate as shown below, is the likely result of premature formation of the O-toluenesulfonate functionality (before all of the amine groups have been converted to their corresponding sulfonamides.

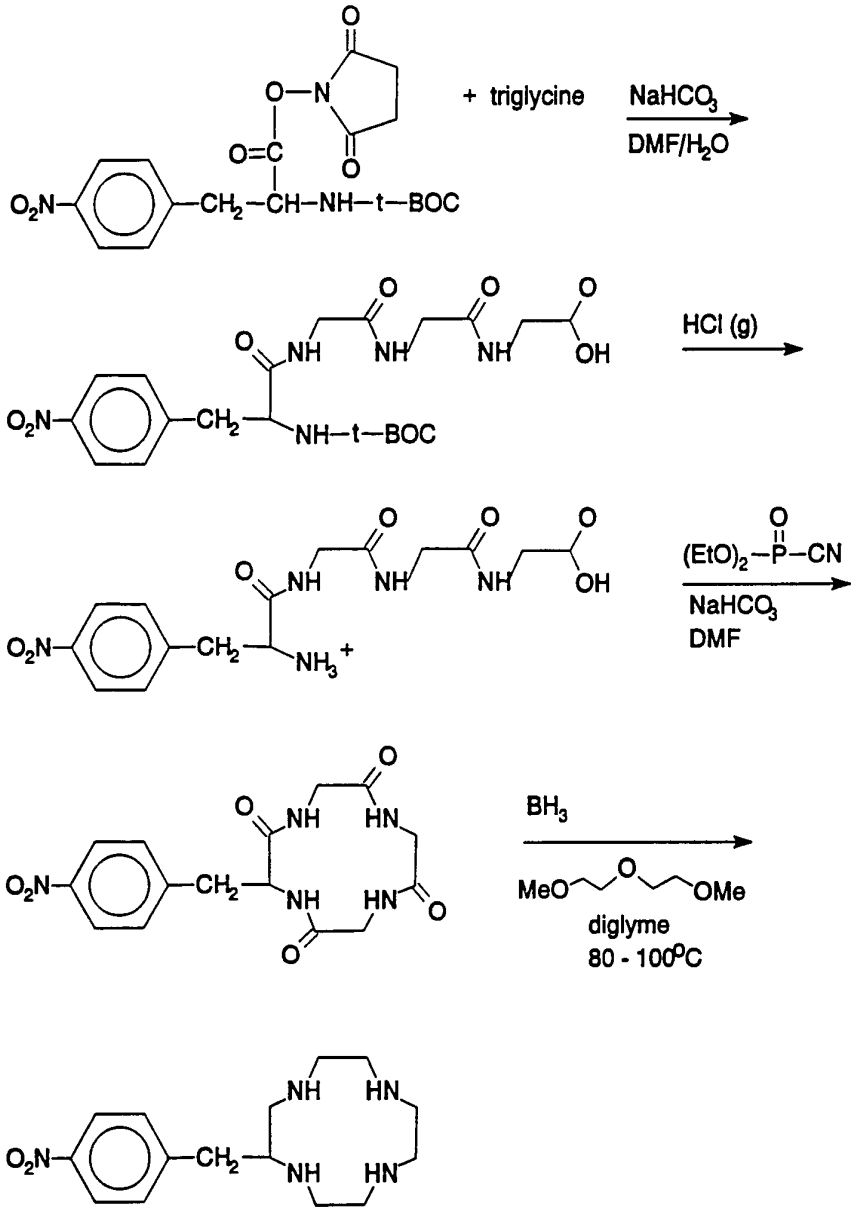


Such a sequence of events would potentially result in unwanted intra- or inter-molecular displacement of the reactive O-toluenesulfonate by unprotected amine groups, thereby generating numerous undesirable side-products.

This problem is overcome in the aforementioned alternative synthesis scheme of the present invention by reacting the tetra-amine alcohol with trifluoroacetic anhydride. Trifluoroacetates, being much poorer leaving groups than toluenesulfonates, are not vulnerable to analogous side reactions. In fact, the easy hydrolysis of trifluoroacetate groups, as reported in Greene and Wuts, "Protecting Groups in Organic Synthesis," John Wiley and Sons, Inc., New

York, p. 94, 1991, suggests that addition of methanol to the reaction mixture following consumption of all amines should afford the tetra-fluoroacetamide alcohol as a substantially exclusive product. Conversion of the tetra-fluoroacetamide alcohol to the corresponding toluenesulfonate provides a material which is expected to cyclize analogously to the tetra-toluenesulfonamide toluenesulfonate of the prior art. The cyclic tetra-amide product of the cyclization of the toluenesulfonate of tetra-fluoroacetamide alcohol, in methanolic sodium hydroxide at 15-25°C for 1 hour, should afford nitro-benzyl-DOTA as a substantially exclusive product. As a result, the use of trifluoracetamide protecting groups circumvents the difficulties associated with cleavage of the very stable toluenesulfonamide protecting group, which involves heating with a large excess of sulfuric acid followed by neutralization with copious volumes of barium hydroxide.

Another alternative route to nitro-benzyl-DOTA is shown on the next page.



This alternative procedure involves the cyclization of p-nitrophenylalanyltriglycine using a coupling agent, such as diethylcyanophosphate, to give the cyclic tetraamide. Subsequent borane reduction provides 2-(p-nitrobenzyl)-1,4,7,10-tetraazacyclododecane, a common precursor used in published routes to DOTA including the Renn and Meares article referenced above. This alternative procedure of the present

invention offers a synthetic pathway that is considerably shorter than the prior art Renn and Meares route, requiring two rather than four steps between p-nitrophenylalanyltriglycine to the tetraamine. The procedure of the present invention also avoids the use of tosyl amino protecting groups, which were prepared in low yield and required stringent conditions for removal. Also, the procedure of the present invention poses advantages over the route published by Gansow et al., U.S. Patent No. 4,923,985, because the crucial cyclization step is intramolecular rather than intermolecular. Intramolecular reactions typically proceed in higher yield and do not require high dilution techniques necessary for successful intermolecular reactions.

The present invention also provides an article of manufacture which includes packaging material and a clearing agent, such as a galactose-HSA-biotin, contained within the packaging material, wherein the clearing agent, upon administration to a mammalian recipient (which recipient has previously been administered a conjugate or moiety to be cleared), is capable of decreasing circulating conjugate or moiety concentration, and wherein the packaging material includes a label that identifies the clearing agent and the component parts thereof, if any, and indicates an appropriate use of the clearing agent in human recipients.

The packaging material indicates whether the clearing agent is limited to investigational use or identifies an indication for which the clearing agent has been approved by the U.S. Food and Drug Administration or other similar regulatory body for use in humans. The packaging material may also include additional information including the amount of clearing agent, the medium or environment in which the clearing agent is dispersed, if any, lot number or

other identifier, storage instructions, usage instructions, a warning with respect to any restriction upon use of the clearing agent, the name and address of the company preparing and/or packaging the clearing agent, and other information concerning the clearing agent.

The clearing agent is preferably contained within a vial which allows the clearing agent to be transported prior to use. Such clearing agent is preferably vialed in a sterile, pyrogen-free environment. Alternatively, the clearing agent may be lyophilized prior to packaging. In this circumstance, instructions for preparing the lyophilized clearing agent for administration to a recipient may be included on the label.

One component to be administered in a preferred two-step pretargeting protocol is a targeting moiety-anti-ligand or a targeting moiety-ligand conjugate. In three-step pretargeting, a preferred component for administration is a targeting moiety-ligand conjugate. A preferred targeting moiety useful in these embodiments of the present invention is a monoclonal antibody. Protein-protein conjugations are generally problematic due to the formation of undesirable byproducts, including high molecular weight and cross-linked species, however. A non-covalent synthesis technique involving reaction of biotinylated antibody with streptavidin has been reported to result in substantial byproduct formation. Also, at least one of the four biotin binding sites on the streptavidin is used to link the antibody and streptavidin, while another such binding site may be sterically unavailable for biotin binding due to the configuration of the streptavidin-antibody conjugate.

Thus, covalent streptavidin-antibody conjugation is preferred, but high molecular weight byproducts are often obtained. The degree of crosslinking and

aggregate formation is dependent upon several factors, including the level of protein derivitization using heterobifunctional crosslinking reagents. Sheldon et al., Appl. Radiat. Isot. 43: 1399-1402, 1992, discuss preparation of covalent thioether conjugates by reacting succinimidyl 4-(N-maleimidomethyl)cyclohexane -1-carboxylate (SMCC)-derivitized antibody and iminothiolane-derivitized streptavidin.

Streptavidin-proteinaceous targeting moiety conjugates are preferably prepared as described in Example XI below, with the preparation involving the steps of: preparation of SMCC-derivitized streptavidin; preparation of DTT-reduced proteinaceous targeting moiety; conjugation of the two prepared moieties; and purification of the monosubstituted or disubstituted (with respect to streptavidin) conjugate from crosslinked (antibody-streptavidin-antibody) and aggregate species and unreacted starting materials. The purified fraction is preferably further characterized by one or more of the following techniques: HPLC size exclusion, SDS-PAGE, immunoreactivity, biotin binding capacity and *in vivo* studies.

Alternatively, thioether conjugates useful in the practice of the present invention may be formed using other thiolating agents, such as SPDP, iminothiolane, SATA or the like, or other thio-reactive heterobifunctional cross linkers, such as m-maleimidobenzoyl-N-hydroxysuccinimide ester, N-succinimidyl(4-iodoacetyl)aminobenzoate or the like.

Streptavidin-proteinaceous targeting moiety conjugates of the present invention can also be formed by conjugation of a lysine epsilon amino group of one protein with a maleimide-derivitized form of the other protein. For example, at pH 8-10, lysine epsilon amino moieties react with protein maleimides, prepared, for instance, by treatment of the protein

with SMCC, to generate stable amine covalent conjugates. In addition, conjugates can be prepared by reaction of lysine epsilon amino moieties of one protein with aldehyde functionalities of the other protein. The resultant imine bond is reducible to generate the corresponding stable amine bond. Aldehyde functionalities may be generated, for example, by oxidation of protein sugar residues or by reaction with aldehyde-containing heterobifunctional cross linkers.

Another method of forming streptavidin-targeting moiety conjugates involves immobilized iminobiotin that binds SMCC-derivitized streptavidin. In this conjugation/purification method, the reversible binding character of iminobiotin (immobilized) to streptavidin is exploited to readily separate conjugate from the unreacted targeting moiety. Iminobiotin binding can be reversed under conditions of lower pH and elevated ionic strength, e.g., NH_2OAc, pH 4 (50 mM) with 0.5 M NaCl.

For streptavidin, for example, the conjugation/ purification proceeds as follows:

- SMCC-derivitized streptavidin is bound to immobilized iminobiotin (Pierce Chemical Co., St. Louis, Missouri), preferably in column format;
- a molar excess (with respect to streptavidin) of DTT-reduced antibody (preferably free of reductant) is added to the nitrogen-purged, phosphate-buffered iminobiotin column wherein the SMCC-streptavidin is bound (DTT-reduced antibody will saturate the bound SMCC-streptavidin, and unbound reduced antibody passing through the column can be reused);
- the column is washed free of excess antibody; and
- a buffer that lowers the pH and increases ionic strength is added to the column to elute streptavidin-antibody conjugate in pure form.

As indicated above, targeting moiety-mediated ligand-anti-ligand pretargeting involves the localization of either targeting moiety-ligand or targeting moiety-anti-ligand at target tissue. Often, peak uptake to such target tissue is achieved before the circulating level of targeting moiety-containing conjugate in the blood is sufficiently low to permit the attainment of an optimal target-to-non-target conjugate ratio. To obviate this problem, two approaches are useful. The first approach allows the targeting moiety-containing conjugate to clear from the blood by "natural" or endogenous clearance mechanisms. This method is complicated by variations in systemic clearance of proteins and by endogenous ligand or anti-ligand. For example, endogenous biotin may interfere with the preservation of biotin binding sites on a streptavidin-targeting moiety conjugate.

The second approach for improving targeting moiety-ligand or targeting moiety-anti-ligand conjugate target-to-blood ratio "chases" the conjugate from the circulation through *in vivo* complexation of conjugate with a molecule constituting or containing the complementary anti-ligand or ligand. When biotinylated antibodies are used as a ligand-targeting moiety conjugate, for example, avidin forms relatively large aggregated species upon complexation with the circulating biotinylated antibody, which aggregated species are rapidly cleared from the blood by the RES uptake. See, for example, U.S. Patent No. 4,863,713. One problem with this method, however, is the potential for cross-linking and internalizing tumor-bound biotinylated antibody by avidin.

When avidin-targeting moiety conjugates are employed, poly-biotinylated transferrin has been used to form relatively large aggregated species that are cleared by RES uptake. See, for example, Goodwin, J. Nucl. Med. 33(10):1816-18, 1992). Poly-biotinylated

transferrin also has the potential for cross-linking and internalizing tumor-bound avidinylated-targeting moiety, however. In addition, both "chase" methodologies involve the prolonged presence of aggregated moieties of intermediate, rather than large, size (which are not cleared as quickly as large size particles by RES uptake), thereby resulting in serum retention of subsequently administered ligand-active agent or anti-ligand active agent. Such serum retention unfavorably impacts the target cell-to-blood targeting ratio.

The present invention provides clearing agents of protein and non-protein composition having physical properties facilitating use for *in vivo* complexation and blood clearance of anti-ligand/ligand (e.g., avidin/biotin)-targeting moiety (e.g., antibody) conjugates. These clearing agents are useful in improving the target:blood ratio of targeting moiety conjugate. Other applications of these clearing agents include lesional imaging or therapy involving blood clots and the like, employing antibody-active agent delivery modalities. For example, efficacious anti-clotting agent provides rapid target localization and high target:non-target targeting ratio. Active agents administered in pretargeting protocols of the present invention using efficient clearing agents are targeted in the desirable manner and are, therefore, useful in the imaging/therapy of conditions such as pulmonary embolism and deep vein thrombosis.

Clearing agents useful in the practice of the present invention preferably exhibit one or more of the following characteristics:

- rapid, efficient complexation with targeting moiety-ligand (or anti-ligand) conjugate *in vivo*;

- rapid clearance from the blood of targeting moiety conjugate capable of binding a subsequently

administered complementary anti-ligand or ligand containing molecule;

- high capacity for clearing (or inactivating) large amounts of targeting moiety conjugate; and

- low immunogenicity.

Preferred clearing agents include hexose-based and non-hexose based moieties. Hexose-based clearing agents are molecules that have been derivatized to incorporate one or more hexoses (six carbon sugar moieties) recognized by Ashwell receptors or other receptors such as the mannose/N-acetylglucosamine receptor which are associated with endothelial cells and/or Kupffer cells of the liver or the mannose 6-phosphate receptor. Exemplary of such hexoses are galactose, mannose, mannose 6-phosphate, N-acetylglucosamine, pentamannosylphosphate, and the like. Other moieties recognized by Ashwell receptors, including glucose, N-galactosamine, N-acetylgalactosamine, pentamannosyl phosphate, thioglycosides of galactose and, generally, D-galactosides and glucosides or the like may also be used in the practice of the present invention. Galactose is the prototypical clearing agent hexose derivative for the purposes of this description. Galactose thioglycoside conjugation to a protein is preferably accomplished in accordance with the teachings of Lee et al., "2-Imino-2-methoxyethyl 1-Thioglycosides: New Reagents for Attaching Sugars to Proteins," Biochemistry, 15(18): 3956, 1976. Another useful galactose thioglycoside conjugation method is set forth in Drantz et al, "Attachment of Thioglycosides to Proteins: Enhancement of Liver Membrane Binding," Biochemistry, 15(18): 3963, 1976. Thus, galactose-based and non-galactose based molecules are discussed below.

Protein-type galactose-based clearing agents include proteins having endogenous exposed galactose

residues or which have been derivatized to expose or incorporate such galactose residues. Exposed galactose residues direct the clearing agent to rapid clearance by endocytosis into the liver through specific receptors therefor (Ashwell receptors). These receptors bind the clearing agent, and induce endocytosis into the hepatocyte, leading to fusion with a lysosome and recycle of the receptor back to the cell surface. This clearance mechanism is characterized by high efficiency, high capacity and rapid kinetics.

An exemplary clearing agent of the protein-based/galactose-bearing variety is the asialoorosomucoid derivative of human alpha-1 acid glycoprotein (orosomucoid, molecular weight = 41,000 Dal, isoelectric point = 1.8-2.7). The rapid clearance from the blood of asialoorosomucoid has been documented by Galli, et al., J. of Nucl. Med. Allied Sci. 32(2): 110-16, 1988.

Treatment of orosomucoid with neuraminidase removes sialic acid residues, thereby exposing galactose residues. Other such derivatized clearing agents include, for example, galactosylated albumin, galactosylated-IgM, galactosylated-IgG, asialohaptoglobin, asialofetuin, asialoceruloplasmin and the like. The present invention therefore provides clearing agents that do not incorporate ligand or anti-ligand molecules or derivatives thereof. For example, the present invention provides IgM molecules that are amenable to receptor-based clearance such as hexose residue-bearing IgM molecules. Preferred hexose residue-bearing clearing agents also incorporate a moiety that is recognized by a hepatocyte receptor, such as galactose, mannose, mannose 6-phosphate, N-acetylglucosamine, glucose, N-galactosamine, N-acetylgalactosamine, thioglycosides of galactose and, generally, D-galactosides and

glucosides or the like. The methods of derivatization of IgM with galactose or the like is analogous to those for derivatizing HSA therewith. In addition, desialyation, analogous to the procedure discussed herein with respect to orosomucoid, may be employed in appropriate circumstances.

The present invention further provides methods of increasing active agent localization at a target cell site of a mammalian recipient, which methods include:

administering to the recipient a first conjugate comprising a targeting moiety and a member of a ligand-anti-ligand binding pair;

thereafter administering to the recipient a clearing agent capable of directing the clearance of circulating first conjugate via hepatocyte receptors of the recipient, wherein the clearing agent does not incorporate a member of the ligand-anti-ligand binding pair or a lower binding affinity derivative thereof; and

subsequently administering to the recipient a second conjugate comprising an active agent and a ligand/anti-ligand binding pair member, wherein the second conjugate binding pair member is complementary to that of the first conjugate.

Human serum albumin (HSA), for example, may be employed in a ligand-bearing clearing agent of the present invention as follows:

$(Hexose)_m$--Human Serum Albumin (HSA)--$(Ligand)_n$, wherein n is an integer from 1 to about 10 and m is an integer from 1 to about 25 and wherein the hexose is recognized by Ashwell receptors. Other mammalian forms of human serum albumin, which differ from human serum albumin only by a few amino acid residues, may also be used in the practice of the present invention. Examples of such mammalian forms of serum albumin are bovine serum albumin, porcine serum albumin, and the like.

In a preferred embodiment of the present invention the ligand is biotin and the hexose is galactose. More preferably, HSA is derivatized with from 10-20 galactose residues and 1-5 biotin residues. Still more preferably, HSA clearing agents of the present invention are derivatized with from about 12 to about 15 galactoses and 3 biotins. Derivatization with both galactose and biotin are conducted in a manner sufficient to produce individual clearing agent molecules with a range of biotinylation levels that averages a recited whole number, such as 1, biotin. Derivatization with 3 biotins, for example, produces a product mixture made up of individual clearing agent molecules, substantially all of which having at least one biotin residue. Derivatization with 1 biotin produces a clearing agent product mixture, wherein a significant portion of the individual molecules are not biotin derivatized. The whole numbers used in this description refer to the average biotinylation of the clearing agents under discussion.

In addition, clearing agents based upon human proteins, especially human serum proteins, such as, for example, orosomucoid and human serum albumin, human IgG, human-anti-antibodies of IgG, IgA and IgM class, and the like, are less immunogenic upon administration into the serum of a human recipient. Another advantage of using asialoorosomucoid is that human orosomucoid is commercially available from, for example, Sigma Chemical Co, St. Louis, Missouri. Human HSA (Cutter Biological) and human IgG, IgA and IgM (Sigma Chemical Co.), for example, are also commercially available.

Another embodiment of the clearing agent of the present invention is a small molecule clearing agent. Such a small molecule clearing agent incorporates a hepatic clearance directing moiety; a liver retention moiety; and a member of a ligand/anti-ligand pair or a

lower affinity form thereof to facilitate binding to targeting moiety-ligand/anti-ligand conjugate. Preferably, small molecule clearing agents of the present invention range in molecular weight from between about 1,000 and about 20,000 daltons, more preferably from about 2,000 to 16,000 daltons.

Preferably, the clearance directing moiety component of the small molecule clearing agent of the present invention is a molecule that is recognized by a hepatocyte receptor. Exemplary molecules of this type have been discussed elsewhere herein.

The liver retention moiety of the small molecule clearing agent of the present invention promotes retention by the liver of the clearing agent which is directed to liver clearance by the clearance directing moiety component thereof. Exemplary liver retention moieties useful in the practice of the present invention include cyanuric chloride, cellobiose, polylysine, polyarginine and the like.

Exemplary ligand/anti-ligand pair members and lower affinity derivatives thereof have been discussed elsewhere herein.

One way to prevent clearing agent compromise of target-bound conjugate through direct complexation is through use of a clearing agent of a size sufficient to render the clearing agent less capable of diffusion into the extravascular space and binding to target-associated conjugate. This strategy is useful alone or in combination with the aforementioned recognition that exposed galactose residues direct rapid liver uptake. This size-exclusion strategy enhances the effectiveness of non-galactose-based clearing agents of the present invention. The combination (exposed galactose and size) strategy improves the effectiveness of "protein-type" or "polymer-type" galactose-based clearing agents.

Galactose-based clearing agents include galactosylated, biotinylated proteins (to remove circulating streptavidin-targeting moiety conjugates, for example) of intermediate molecular weight (ranging from about 40,000 to about 200,000 Dal), such as biotinylated asialoorosomucoid, galactosyl-biotinyl-human serum albumin or other galactosylated and biotinylated derivatives of non-immunogenic soluble natural proteins, as well as biotin- and galactose-derivatized polyglutamate, polylysine, polyarginine, polyaspartate and the like. High molecular weight moieties (ranging from about 200,000 to about 1,000,000 Dal) characterized by poor target access, including galactosyl-biotinyl-IgM or -IgG (approximately 150,000 Dal) molecules, as well as galactose- and biotin-derivatized transferrin conjugates of human serum albumin, IgG and IgM molecules and the like, can also be used as clearing agents of the claimed invention. Chemically modified polymers of intermediate or high molecular weight (ranging from about 40,000 to about 1,000,000 Dal), such as galactose- and biotin-derivatized dextran, hydroxypropylmethacrylamide polymers, polyvinylpyrrolidone-polystyrene copolymers, divinyl ether-maleic acid copolymers, pyran copolymers, or PEG, also have utility as clearing agents in the practice of the present invention. In addition, rapidly clearing biotinylated liposomes (high molecular weight moieties with poor target access) can be derivatized with galactose and biotin to produce clearing agents for use in the practice of the present invention.

Another embodiment of the present invention is the production of conjugates which do not provide for biotin release during usage.

A potential disadvantage associated with biotinylated galactosylated human serum albumin clearing agents is that metabolism thereof may result in the release of biotin. This is undesirable because it may result in poisoning of the targeted conjugate by biotin. Such biotin release may occur after uptake by the Ashwell receptor and catabolism of the protein. One means of alleviating this potential problem is to produce conjugates which are metabolically stable and therefore do not release any catabolized biotin. This may be effected, e.g., by the insertion of a non-cleavable linker comprised, e.g., of amino acid sequences, D-amino acids, teritary amines, sugars or highly charged or polar groups between the biotin linker and the HSA protein. Incorporation of such linkers should prevent biotin release, and the escape of biotin molecules from the hepatocytes and being released into the circulation.

The selection of suitable linkers which eliminate cleavage can be determined by one skilled in the art. Factors to be considered include, e.g., the relative ease of synthesis of the particular linker and its effects on biotin release. Most preferably, the linker sequence will eliminate the release of free biotin altogether, thereby eliminating the possibility of free biotin being released into the circulation and potentially adversely affecting the binding of active agent to tumor bound conjugates.

A further class of clearing agents useful in the present invention involve small molecules (ranging from about 500 to about 10,000 Dal) derivatized with galactose and biotin that are sufficiently polar to be confined to the vascular space as an *in vivo* volume of distribution. More specifically, these agents exhibit

a highly charged structure and, as a result, are not readily distributed into the extravascular volume, because they do not readily diffuse across the lipid membranes lining the vasculature. Exemplary of such clearing agents are mono- or poly-biotin-derivatized 6,6'-[(3,3'-dimethyl[1,1'-biphenyl]-4,4'-diyl)bis(azo) bis[4-amino-5-hydroxy-1,3-naphthalene disulfonic acid] tetrasodium salt, mono- or poly-biotinyl-galactose-derivatized polysulfated dextran-biotin, mono- or poly-biotinyl-galactose-derivatized dextran-biotin and the like.

The galactose-exposed or -derivatized clearing agents are preferably capable of (1) rapidly and efficiently complexing with the relevant ligand- or anti-ligand-containing conjugates via ligand-anti-ligand affinity; and (2) clearing such complexes from the blood via the galactose receptor, a liver specific degradation system, as opposed to aggregating into complexes that are taken up by the generalized RES system, including the lung and spleen. Additionally, the rapid kinetics of galactose-mediated liver uptake, coupled with the affinity of the ligand-anti-ligand interaction, allow the use of intermediate or even low molecular weight carriers.

Non-galactose residue-bearing moieties of low or intermediate molecular weight (ranging from about 40,000 to about 200,000 Dal) localized in the blood may equilibrate with the extravascular space and, therefore, bind directly to target-associated conjugate, compromising target localization. In addition, aggregation-mediated clearance mechanisms operating through the RES system are accomplished using a large stoichiometric excess of clearing agent. In contrast, the rapid blood clearance of galactose-based clearing agents used in the present invention prevents equilibration, and the high affinity ligand-anti-ligand binding allows the use of low

stoichiometric amounts of such galactose-based clearing agents. This feature further diminishes the potential for galactose-based clearing agents to compromise target-associated conjugate, because the absolute amount of such clearing agent administered is decreased.

A preferred embodiment of the present invention is the preparation of novel small molecule clearing agents and the use thereof in pretargeting protocols. More specifically, the present invention provides novel bispecific small molecule clearing agents which have utility for the clearance of streptavidin-targeting moiety or avidin-targeting moiety conjugates from non-targeted sites, e.g., the circulation, extravascular space, etc.

This aspect of the invention was developed while attempting to produce further improved galactosylated HSA-biotin clearing agents. While such clearing agents are effective as described *supra*, they have one potential adverse side effect. Specifically, the administration of such clearing agents may potentially result in biotin poisoning of the targeted conjugate as a result of endocytosis and degradation of the clearing agent. This may occur by endocytosis and degradation, the end result of which is the production and diffusion of the small molecule metabolite biotin into the circulation.

This potential problem may be obviated by use of a lower affinity analog of biotin, instead of biotin proper, in the construction of an HSA type clearing agent in that, even after hepatic processing, exocytosis and poisoning, the analog may be effectively competed off the targeted conjugate through the higher affinity of the biotin ligand. Incorporation of this concept on modification to small molecule type clearing agents should result in analogous elimination of the problem. Prior to

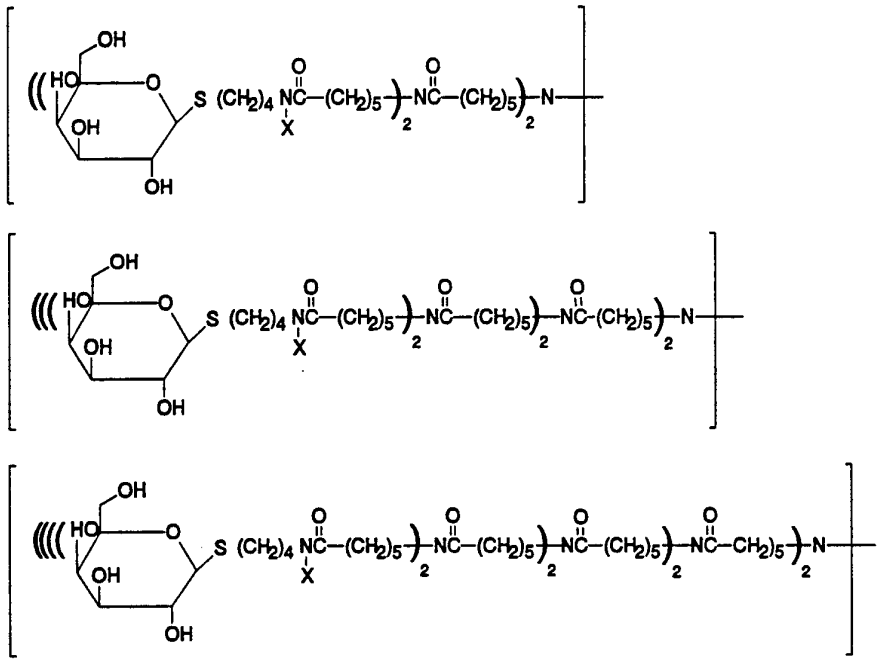
discussion of the particulars of such clearing agent embodiments, it is useful to further describe how they came to be produced.

The efficacy of biotin-HSA-galactose agents requires that such clearing agents effectively clear conjugate from the circulation. This clearance activity has been demonstrated previously. Conceptually, one reason why the inventors added galactose and biotin molecules to HSA, in particular, to produce a clearing agent was that, in theory, access of such a large "vehicle" to the extravascular space would be significantly restricted. Thus, because of its large size, there would be minimal danger of directly blocking extravascularly targeted, tumor-cell associated conjugate by the clearing agent.

On the other hand, operating on the assumption that lower affinity biotin-HSA-galactose agents can effectively clear conjugate from the circulation and that binding of the lower affinity biotin analog (produced by metabolism of the clearing agent by hepatocytes) to tumor associated conjugate is metastable, dissociating to regenerate the binding site for the active ligand (e.g., a DOTA-biotin ligand), it was hypothesized that there was no need to restrict access of the clearing agent to tumor associated conjugate (by use of large protein carriers which do not extravasate well) if a lower affinity biotin analog is used in clearing agent construction. Thus, it was theorized that lower molecular weight clearing agents could be designed to contain a sufficient number of hexose residues (e.g., galactoses) to facilitate directed clearance and which only transiently bind to conjugate (i.e., for an interval sufficient to clear non-targeted conjugate). Residual clearing agent initially bound to target associated conjugate could be expected to dissociate

over time, thus allowing access for the active agent biotinylated ligand.

In part, the inventor's beliefs have been proven valid, as evidenced by the demonstrated effective clearance by the small molecule clearing agents described *infra*. That is, lower molecular weight bispecific molecules, designed to contain appropriately spaced hexoses and to also contain biotin proper, when formulated, quickly cleared the streptavidin containing conjugate from the circulation.



where X is H, methyl, lower alkyl or lower alkyl with heteroatoms. The above structures bear 4, 8, and 16 galactose respectively. Further iteration in the branching allows expansion to include 32, 64, etc., galactose residues.

Thus, this embodiment of the invention involves the preparation and use of bispecific small molecule agents for use in clearance of streptavidin-targeting agent (antibody) or avidin-targeting agent (antibody)

from non-targeted sites, i.e., the circulation, and possibly extravascular space, etc. These bispecific small molecule clearing agents will preferably consist of a "low affinity" biotin analog arm, which can bind to avidin or streptavidin in a metastable fashion, to which has been attached one or more hexose residues which provide for targeted clearance, e.g., through hepatocyte receptors. Exemplary low affinity biotin molecules useful in this embodiment of the invention are identified elsewhere in this application.

As discussed previously, hepatocyte receptors which provide for effective clearance include in particular Ashwell receptors, mannose/N-acetylgalactosamine receptors associated with endothelial cells and/or Kupffer cells of the liver, the mannose 6-phosphate receptor, and the like. Hexoses which may be attached to such low affinity biotin analogs have been identified above and include by way of example galactose, mannose, mannose 6-phosphate, N-acetylgalactosamine, pentamannosylphosphate, and the like. Hexoses recognized by Ashwell receptors include glucose, galactose, N-galactosamine, N-acetylgalactosamine, pentamannosyl phosphate, thioglycosides of galactose, D-galactosides, galactosamine, N-acetylgalactosamine, mannosyl-6-phosphate and glucosides. A sufficient number of hexose residues will be attached to the selected biotin analog to provide for effective clearance, e.g., via the Ashwell receptors comprised on the surface of hepatocytes. Preferably, the clearance agents should be of a low enough molecular weight to provide for efficient diffusion into the extravascular space, thus providing for binding to both circulating and non-circulating conjugate. This molecular weight will preferably range from about 1,000 to about 20,000 daltons, more preferably about 2,000 to 16,000 daltons. This will enable the

conjugate in the circulation to be rapidly removed through the Ashwell receptors, internalized and metabolized. The conjugate at non-target sites will be removed as it is diffused back into the circulation as a complex with the small molecule clearance agent. The conjugate bound at the site of action, which is not susceptible to "rapid" diffusion should also complex with the small molecule clearing agent. However, by incorporation of an appropriate "lower affinity" biotin analog, this small molecule will readily dissociate from the conjugate as its circulating concentration diminishes due to hepatic clearance. The result therefore is an uncompromised, biotin binding conjugate at the target site.

Most preferably, the low affinity biotin analog will be bound to at least 3 hexose residues, e.g., galactose residues or N-acetylgalactosamine residues. However, the invention is not limited thereby and embraces the attachment of any number of hexose residues or mixture thereof which results in an efficacious bispecific small molecular weight clearance agent.

Selection of the ideal binding constant that the biotin analog should possess depends upon factors including:

(i) rate of clearance of conjugate small molecule complex by the liver; and

(ii) time before the cytotoxic ligand is administered.

With respect to i), the faster the rate of clearance, the lower (weaker) the binding constant needs to be. With respect to ii), the greater the amount of time between administration of the clearing agent and administration of the ligand, the greater (stronger) the binding constant can be as more time is available to permit dissociation of the conjugate of

the targeted site. In general, this interval should be minimized.

The design of the hexose portion of the small molecule, e.g., galactose or N-actylgalactosamine also depends upon a number of factors including:

(i) The number of hexose residues, e.g., galactose residues:

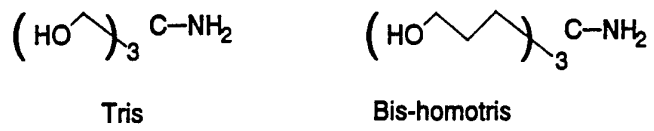
The literature suggests that galactose receptors are grouped on the surface of human hepatocytes as heterorimers and possibly bis-heterotrimers. Thus, for optimal affinity, the small molecule clearing agent should possess at least three galactose residues and preferably more, to provide for "galactose clusters." In general, the small molecule clearing agent will contain from about 3 to about 50 galactose residues, preferably from about 3 to 32, and most preferably 16 galactose residues.

(ii) Distance between galactose residues:

Each galactose receptor is separated by a distance of 15, 22 and 25 Å. Thus, the galactose residues within each small molecule should preferably be separated by a flexible linker which provides for a separation distance of at least 25 Å, to enable the sugars to be separated by at least said distance. It is expected that this minimum spacing will be more significant as the number of sugar residues, e.g., galactoses, are decreased. This is because larger numbers of galactoses will likely contain an appropriate spacing between sugars that are not immediately adjacent to one another, thus providing for the desired receptor interaction.

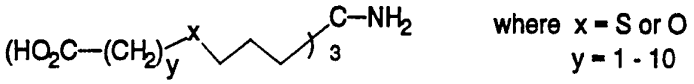
Assuming an average bond length of about 1.5 Å, this would mean that the sugar residues should ideally be separated by a spacer of not less than about 10 bond lengths, with at least 25 bond lengths being more preferred.

For example, the galactoses may be attached in a branched arrangement as follows, which is based on bis-homotris:

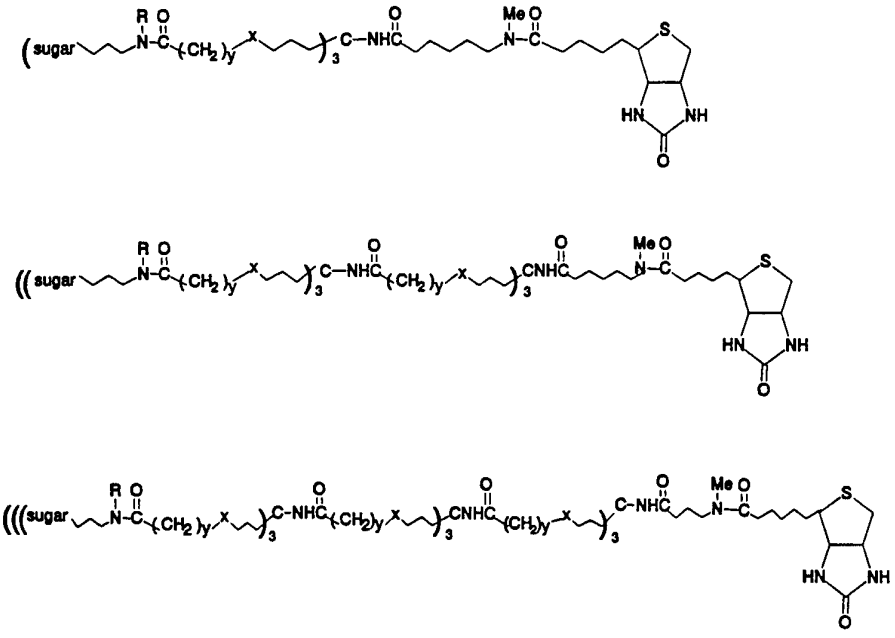


Tris Bis-homotris

Preferably, each arm is extended, and terminates in a carboxylic acid terminus as follows:



Exemplary clearing agents having such an arrangement are set forth below:



where R = H or Me; y = 1-10; x = O or S

Such an arrangement, with 0, 1 or 2 branched iterations, allows for the incorporation of 3, 9 or 27 sugars.

(iii) Distance between galactose cluster and biotin portion of small molecule:

If many galactose residues are linked to the biotin species, then the linker should be long enough to alleviate adverse steric effects which may result in diminished binding of the small molecule to the conjugate and/or diminished binding of the complex to the galactose receptor.

While the following parameters appear to be optimal for galactose it should be noted that these factors may vary with other hexoses or mixtures thereof, which may or may not bind to the same receptors, or may bind differently. Given the teachings in this application one skilled in the art can, using available synthesis techniques, attach biotin to other hexose residues, or a mixture of different hexose residues and ascertain those conjugates which provide for optimal rates of clearance.

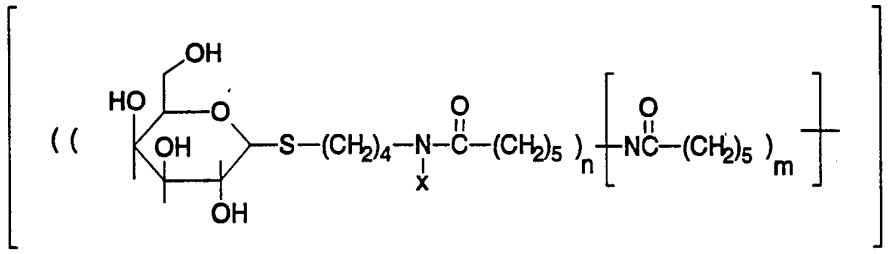
Also, one skilled in the art can additionally substitute other complementary ligands for biotin, ideally those having small molecular weight. Such ligands may also be modified to include suitable functional groups to allow for the attachment of other molecules of interest, e.g., peptides, proteins, nucleotides, and other small molecules.

For example, the clearing agent may be attached to a desired functional group via the end which is opposite to the sugar residues. Examples of suitable functional groups include, e.g., maleimides, activated esters, isocyanates, alkyl halides (e.g., iodoacetate), hydrazides, thiols, imidates and aldehydes.

In addition to the described therapeutic advantages of the described small molecule clearance agents, they are also superior from a cost, regulatory and safety perspective to proteinaceous clearing agents because biotin analogs are well defined, easily synthesized, and readily available. This is in contrast to protein based clearance agents which tend to be more expensive and less highly characterized.

The subject small molecule clearing agents may also be conjugated to active small molecules, e.g., radionuclides, peptides, small proteins and nucleotides, to provide for an active agent which is delivered to an active site which has been pretargeted with a first agent containing a targeting moiety attached to a ligand or anti-ligand which binds the ligand or anti-ligand contained in the small molecule clearing agent. Typically, the ligand in the small molecule clearing agent will be biotin or an analog and the anti-ligand contained in the pre-targeted conjugate will be streptavidin or avidin. Thus, this will provide for active agents which are delivered to active sites, and are rapidly eliminated from the circulation by virtue of the clearing directing moieties, e.g., galactose residues. This embodiment is particularly useful if the active agent is cytotoxic, e.g., a radionuclide.

Preferred galactose clusters contained in the subject small molecule clearing agents will be of the formula:



where x is H, methyl, lower alkyl or lower alkyl withi hetero atoms. The above stuctures bear 4, 8 and 16 galactose, respectively. Further iteration in the branching allows expansion to include 32, 64, etc. galactose residues.

Alternatively, branching structures may also be employed in the design of galactose clusters in accordance with the present invention. For example, a construct where each branching iteration results in galactose clusters bearing 3, 9, 27, 81, etc., galactose residues. The extender from the galactose to branching linker may be variable in length.

The ligand (e.g., biotin) and galactose cluster (and optionally an active agent) may be attached by use of suitable bifunctional or trifunctional linkers. Selection of suitable trifunctional and bifunctional linkers amenable to binding with functional groups on the ligand, galactose cluster, and optionally the active moiety, e.g., a chelate, is well within the level of skill in the art. Suitable bifunctional linkers include bis-N,N-(6-(1-hydroxycarbonylhexyl) amine.

Suitable trifunctional linkers include lysine.

Also, extender moieties may be utilized in the construction of the subject small molecule clearing agents. Suitable extenders include difunctional moieties capable of binding either the ligand component and the linker or the galactose cluster component and the linker. Suitable extender moieties include an aminocaproate moiety, 4 aminobutane thiol and the like. One of skill in the art can readily select appropriate extender molecules which promote bioavailability of the galactose cluster. Alternatively, the extender function may be served by an appropriately constructed linker.

Clearing agent evaluation experimentation involving galactose- and biotin-derivatized clearing

agents of the present invention is detailed in Examples XIII and XVI. Specific clearing agents of the present invention that were examined during the Example XVI experimentation are (1) asialoorosomucoid-biotin, (2) human serum albumin derivatized with galactose and biotin, and (3) a 70,000 dalton molecular weight dextran derivatized with both biotin and galactose. The experimentation showed that proteins and polymers are derivatizable to contain both galactose and biotin and that the resultant derivatized molecule is effective in removing circulating streptavidin-protein conjugate from the serum of the recipient. Biotin loading was varied to determine the effects on both clearing the blood pool of circulating avidin-containing conjugate and the ability to deliver a subsequently administered biotinylated isotope to a target site recognized by the streptavidin-containing conjugate. The effect of relative doses of the administered components with respect to clearing agent efficacy was also examined. Additionally, Examples XIX and XX relate to small molecule clearing agents comprising biotin and galactose residues.

Protein-type and polymer-type non-galactose-based clearing agents include the agents described above, absent galactose exposure or derivitization and the like. These clearing agents act through an aggregation-mediated RES mechanism. In these embodiments of the present invention, the clearing agent used will be selected on the basis of the target organ to which access of the clearing agent is to be excluded. For example, high molecular weight (ranging from about 200,000 to about 1,000,000 Dal) clearing agents will be used when tumor targets or clot targets are involved.

The present invention provides clearing agents that incorporate ligand derivatives or anti-ligand

derivatives, wherein such derivatives exhibit a lower affinity for the complementary ligand/anti-ligand pair member than the native form of the compound (i.e., lower affinity ligands or anti-ligands). In embodiments of the present invention employing a biotin-avidin or biotin-streptavidin ligand/anti-ligand pair, preferred clearing agents incorporate either lower affinity biotin (which exhibits a lower affinity for avidin or streptavidin than native biotin) or lower affinity avidin or a streptavidin (which exhibits a lower affinity for biotin than native avidin or streptavidin).

Clearing agents that employ a ligand or anti-ligand moiety that is complementary to the ligand/anti-ligand pair member (previously administered in conjunction with the targeting moiety) are useful in the practice of the present invention. When such clearing agents localize to hepatocytes, they are generally rapidly degraded. This degradation liberates a quantity of free ligand or free anti-ligand into the circulation. This bolus release of ligand or anti-ligand may compete for binding sites of targeting moiety-ligand or targeting moiety-anti-ligand with subsequently administered active agent-ligand or active agent-anti-ligand conjugate.

This competition can be addressed by using a clearing agent incorporating a lower affinity ligand or anti-ligand. In other words, the ligand or anti-ligand employed in the structure of the clearing agent more weakly binds to the complementary ligand/anti-ligand pair member than native ligand or anti-ligand. Consequently, lower affinity ligand or anti-ligand derivatives that bind to target-localized targeting moiety-anti-ligand or targeting moiety-ligand conjugate may be displaced by the subsequently administered, active agent-native (or higher binding

affinity ligand) or active agent-native (or higher binding affinity) anti-ligand conjugate.

In two-step pretargeting protocols employing the biotin-avidin or biotin-streptavidin ligand-anti-ligand pair, lower affinity biotin, lower affinity avidin or lower affinity streptavidin may be employed. Exemplary lower affinity biotin molecules, for example, exhibit the following properties: bind to avidin or streptavidin with an affinity less than that of native biotin (10^{-15}); retain specificity for binding to avidin or streptavidin; are non-toxic to mammalian recipients; and the like. Exemplary lower affinity avidin or streptavidin molecules, for example, exhibit the following properties: bind to biotin with an affinity less than native avidin or streptavidin; retain specificity for binding to biotin; are non-toxic to mammalian recipients; and the like.

Exemplary lower affinity biotin molecules include 2'-thiobiotin; 2'-iminobiotin; 1'-N-methoxycarbonyl-biotin; 3'-N-methoxycarbonylbiotin; 1-oxy-biotin; 1-oxy-2'-thiobiotin; 1-oxy-2'-iminobiotin; 1-sulfoxide-biotin; 1-sulfoxide-2'-thiobiotin; 1-sulfoxide-2'-iminobiotin; 1-sulfone-biotin; 1-sulfone-2'-thio-biotin; 1-sulfone-2'-iminobiotin; imidazolidone derivatives such as desthiobiotin (d and dl optical isomers), dl-desthiobiotin methyl ester, dl-desthiobiotinol, D-4-n-hexyl-imidazolidone, L-4-n-hexylimidazolidone, dl-4-n-butyl-imidazolidone, dl-4-n-propylimidazolidone, dl-4-ethyl-imidazolidone, dl-4-methylimidazolidone, imidazolidone, dl-4,5-dimethylimidazolidone, meso-4,5-dimethylimidazolidone, dl-norleucine hydantoin, D-4-n-hexyl-2-thiono-imidazolidine, d-4-n-hexyl-2-imino-imidazolidine and the like; oxazolidone derivatives such as D-4-n-hexyl-oxazolidone, D-5-n-hexyloxazolidone and the like; [5-(3,4-diamino-thiophan-2-yl] pentanoic acid; lipoic

acid; 4-hydroxy-azobenzene-2'-carboxylic acid; and the like. Preferred lower affinity biotin molecules for use in the practice of the present invention are 2'-thiobiotin, desthiobiotin, 1-oxy-biotin, 1-oxy-2'-thiobiotin, 1-sulfoxide-biotin, 1-sulfoxide-2'-thiobiotin, 1-sulfone-biotin, 1-sulfone-2'-thiobiotin, lipoic acid and the like. These exemplary lower affinity biotin molecules may be produced substantially in accordance with known procedures therefor. Conjugation of the exemplary lower affinity biotin molecules to HSA or other amino acid-based or polymeric moieties proceeds substantially in accordance with known procedures therefor and with procedures described herein with regard to biotin conjugation.

The present invention further provides methods of increasing active agent localization at a target cell site of a mammalian recipient, which methods include:

administering to the recipient a first conjugate comprising a targeting moiety and a member of a ligand-anti-ligand binding pair;

thereafter administering to the recipient a clearing agent capable of directing the clearance of circulating first conjugate via hepatocyte receptors of the recipient, wherein the clearing agent incorporates lower affinity complementary member of the ligand-anti-ligand binding pair; and

subsequently administering to the recipient a second conjugate comprising an active agent and a ligand/anti-ligand binding pair member, wherein the second conjugate binding pair member is complementary to that of the first conjugate and, preferably, constitutes a native or high affinity form of the member.

Certain active agents, e.g., certain cytokines, exert therapeutic activity in association with a receptor therefor on the target cell surface or on the

surface of other cells in the vicinity of target cells. The "sandwich" at the target cell surface including, for example, targeting moiety-anti-ligand-ligand-active agent may not provide optimal delivery of the active agent to the relevant receptor. In this circumstance, the sandwich is preferably structured to be conditionally cleavable.

One way to provide for conditional cleavage of the active agent is to employ lower affinity ligand or anti-ligand in the sandwich. After the sandwich is formed (e.g., from about 2 to about 8 hours following administration of ligand-active agent conjugate), a bolus dose of native or higher affinity ligand or anti-ligand is given. This native or higher affinity (from 3-6 orders of magnitude) ligand or anti-ligand will serve to displace its lower affinity counterpart in the sandwich, thereby releasing the active agent from the sandwich.

The present invention therefore provides methods of increasing active agent localization at a target cell site of a mammalian recipient, which methods include:

administering to the recipient a first conjugate comprising a targeting moiety and a member of a ligand-anti-ligand binding pair;

thereafter administering to the recipient a clearing agent capable of directing the clearance of circulating first conjugate via hepatocyte receptors of the recipient;

administering to the recipient a second conjugate comprising an active agent and a lower affinity ligand/anti-ligand binding pair member, wherein the second conjugate lower affinity binding pair member is complementary to that of the first conjugate; and

administering to the recipient native or higher affinity ligand or anti-ligand corresponding to the

lower affinity binding pair member of the second conjugate.

In addition, the present invention provides methods of increasing active agent localization at a target cell site of a mammalian recipient, which methods include:

administering to the recipient a receptor blocking agent in an amount sufficient to substantially block a subpopulation of hepatocyte receptors;

administering to the recipient a first conjugate comprising a targeting moiety, a hepatocyte receptor recognizing agent, and a member of a ligand-anti-ligand binding pair; and

subsequently administering to the recipient a second conjugate comprising an active agent and a ligand/anti-ligand binding pair member, wherein the second conjugate binding pair member is complementary to that of the first conjugate.

Exemplary hepatocyte receptors with respect to which this block/deblock protocol may be employed include Ashwell receptors; other receptors such as the mannose/N-acetylglucosamine receptor which are associated with endothelial cells and/or Kupffer cells of the liver; the mannose 6-phosphate receptor; or the like.

Exemplary receptor blocking agents of the present invention exhibit one or more of the following structural or functional characteristics: low immunogenicity; low toxicity; are recognized by a hepatocyte receptor and are processed thereby; and the like. For this embodiment of the present invention, preferred receptor blocking agents include IgG-galactose; human IgG-galactose; asialoorosomucoid, galactose-HSA, with human or other mammalian HSA.

Preferably, the receptor blocking agent is administered via intravenous, intraarterial or like routes of administration, with intravenous

administration preferred. Such administration is preferably conducted in continuous or via multiple administrations for a time sufficient to substantially block the relevant hepatocyte receptors and to permit localization of the targeting moiety to target sites, e.g., generally ranging from about 18 to about 72 hours. In this manner, the blocking agent is occupying the relevant hepatocyte receptor population to permit localization of the first conjugate.

After the final administration or the cessation of administration of the blocking agent, the hepatocyte receptor population processes the remaining blocking agent and the hepatocyte receptor recognizing agent-bearing first conjugate. Therefore, the second conjugate is preferably administered after a time sufficient to permit receptor-based clearance of receptor blocking agent to deblock the receptors and receptor-based clearance of circulating first conjugate, e.g., generally ranging from about 2 to about 8 hours post-cessation of administration or post-final administration of receptor blocking agent and from about 24 to about 72 hours post-administration of first conjugate.

Another class of clearing agents includes agents that do not remove circulating ligand or anti-ligand/targeting moiety conjugates, but instead "inactivate" the circulating conjugates by blocking the relevant anti-ligand or ligand binding sites thereon. These "cap-type" clearing agents are preferably small (500 to 10,000 Dal) highly charged molecules, which exhibit physical characteristics that dictate a volume of distribution equal to that of the plasma compartment (i.e., do not extravasate into the extravascular fluid volume). Exemplary cap-type clearing agents are poly-biotin-derivatized 6,6'-[(3,3'-dimethyl[1,1'-biphenyl]-4,4'-diyl)bis(azo) bis[4-amino-5-hydroxy-1,3-naphthalene disulfonic acid]

tetrasodium salt, poly-biotinyl-derivatized polysulfated dextran-biotin, mono- or poly-biotinyl-derivatized dextran-biotin and the like.

Cap-type clearing agents are derivatized with the relevant anti-ligand or ligand, and then administered to a recipient of previously administered ligand/ or anti-ligand/targeting moiety conjugate. Clearing agent-conjugate binding therefore diminishes the ability of circulating conjugate to bind any subsequently administered active agent-ligand or active agent-anti-ligand conjugate. The ablation of active agent binding capacity of the circulating conjugate increases the efficiency of active agent delivery to the target, and increases the ratio of target-bound active agent to circulating active agent by preventing the coupling of long-circulating serum protein kinetics with the active agent. Also, confinement of the clearing agent to the plasma compartment prevents compromise of target-associated ligand or anti-ligand.

Clearing agents of the present invention may be administered in single or multiple doses. A single dose of biotinylated clearing agent, for example, produces a rapid decrease in the level of circulating targeting moiety-streptavidin, followed by a small increase in that level, presumably caused, at least in part, by re-equilibration of targeting moiety-streptavidin within the recipient's physiological compartments. A second or additional clearing agent doses may then be employed to provide supplemental clearance of targeting moiety-streptavidin. Alternatively, clearing agent may be infused intravenously for a time period sufficient to clear targeting moiety-streptavidin in a continuous manner.

Other types of clearing agents and clearance systems are also useful in the practice of the present invention to remove circulating targeting moiety-

ligand or -anti-ligand conjugate from the recipient's circulation. Particulate-based clearing agents, for example, are discussed in Example IX. In addition, extracorporeal clearance systems are discussed in Example IX. *In vivo* clearance protocols employing arterially inserted proteinaceous or polymeric multiloop devices are also described in Example IX.

One embodiment of the present invention in which rapid acting clearing agents are useful is in the delivery of Auger emitters, such as I-125, I-123, Er-165, Sb-119, Hg-197, Ru-97, Tl-201 and I-125 and Br-77, or nucleus-binding drugs to target cell nuclei. In these embodiments of the present invention, targeting moieties that localize to internalizing receptors on target cell surfaces are employed to deliver a targeting moiety-containing conjugate (i.e., a targeting moiety-anti-ligand conjugate in the preferred two-step protocol) to the target cell population. Such internalizing receptors include EGF receptors, transferrin receptors, HER2 receptors, IL-2 receptors, other interleukins and cluster differentiation receptors, somatostatin receptors, other peptide binding receptors and the like.

After the passage of a time period sufficient to achieve localization of the conjugate to target cells, but insufficient to induce internalization of such targeted conjugates by those cells through a receptor-mediated event, a rapidly acting clearing agent is administered. In a preferred two-step protocol, an active agent-containing ligand or anti-ligand conjugate, such as a biotin-Auger emitter or a biotin-nucleus acting drug, is administered as soon as the clearing agent has been given an opportunity to complex with circulating targeting moiety-containing conjugate, with the time lag between clearing agent and active agent administration being less than about 24 hours. In this manner, active agent is readily

internalized through target cell receptor-mediated internalization. While circulating Auger emitters are thought to be non-toxic, the rapid, specific targeting afforded by the pretargeting protocols of the present invention increases the potential of shorter half-life Auger emitters, such as I-123, which is available and capable of stable binding.

In order to more effectively deliver a therapeutic or diagnostic dose of radiation to a target site, the radionuclide is preferably retained at the tumor cell surface. Loss of targeted radiation occurs as a consequence of metabolic degradation mediated by metabolically active target cell types, such as tumor or liver cells.

Preferable agents and protocols within the present invention are therefore characterized by prolonged residence of radionuclide at the target cell site to which the radionuclide has localized and improved radiation absorbed dose deposition at that target cell site, with decreased targeted radioactivity loss resulting from metabolism. Radionuclides that are particularly amenable to the practice of this aspect of the present invention are rhenium, iodine and like "non +3 charged" radiometals which exist in chemical forms that easily cross cell membranes and are not, therefore, inherently retained by cells. In contrast, radionuclides having a +3 charge, such as In-111, Y-90, Lu-177 and Ga-67, exhibit natural target cell retention as a result of their containment in high charge density chelates.

Evidence exists that streptavidin is resistant to metabolic degradation. Consequently, radionuclides bound directly or indirectly to streptavidin, rather than, for example, directly to the targeting moiety, are retained at target cell sites for extended periods of time. Streptavidin-associated radionuclides can be administered in pretargeting protocols intravenously,

intraarterially or the like or injected directly into lesions.

The efficacy of pretargeting protocols employing streptavidin/ or avidin/biotin anti-ligand/ligand systems may be diminished by the presence of endogenous biotin. Endogenous biotin exhibits D stereochemistry. Natural avidin and streptavidin are formed of L-amino acids to bind to D-biotin. The affinity of natural avidin and streptavidin for L-biotin is so low that L-biotin is non-competitive with D-biotin for such binding. See, for example, Green, Advances in Protein Chemistry, 29: 85-131, 1975. Other biotin binding peptides (BBPs) are also specific for D-biotin. BBPs include peptides containing the motif represented by CXWXPPF (K or R) XXC; peptides containing the previously identified motif without one or both terminal cysteine residues; biotin operon repressor; biotin holoenzyme synthetase; and biotin carboxylase. Biotin binding peptides are well known in the art.

Enzymes are chiral molecules having strict selectivity for substrates with the correct stereochemical configuration. Natural enzymes are made up of L amino acids and may recognize substrates of either L or D configuration. *See*, for example, White et al., "Principles of Biochemistry," 5th ed., McGraw Hill, 1973.

Natural enzymes of L configuration prepared synthetically using D-amino acids exhibit specificity for substrates with the opposite stereochemistry compared to that of the natural substrate. Also, these "mirror image" enzymes convert the opposite stereochemical substrate with substantially the same efficiency or turnover rate as the naturally occurring enzyme acts on the natural substrate. *See*, for example, Milton et al., Science, 256: 1445-1447, 1992,

for a discussion of preparation of D-enzymes having reciprocal chiral substrate specificity.

Natural streptavidin and natural avidin recognizes D-biotin. Consequently, streptavidin or avidin formed with D-amino acids in the manner described by Milton et al. therefore interact with L-biotin rather than D-biotin (endogenous biotin). Consequently, any inefficiencies in *in vivo* operation of pretargeting protocols caused by endogenous biotin can be obviated or substantially reduced by forming streptavidin or avidin of D-amino acids. The D-amino acid forms of streptavidin or avidin will show binding specificity for L-biotin rather than naturally occurring, endogenous D-biotin. Moreover, the high affinity avidin- and streptavidin-binding will be preserved in the mirror image format. Biotin binding peptides may also be converted to mirror image configuration in this manner to bind L-biotin rather than D-biotin. Preparation of mirror image biotin binding peptides may be conducted by solid phase peptide synthesis in accordance with known techniques therefor.

Binding of mirror image streptavidin, avidin or BBPs to targeting moieties can be accomplished via the same techniques described herein and known in the art for natural protein-targeting moiety binding. Also, mirror image BBPs can be incorporated into fusion proteins substantially as described herein for L stereochemistry BBPs. In addition, the use of targeting moiety-avidin, -streptavidin, -BBP conjugates or fusion proteins in pretargeting protocols is accomplished as described herein for natural protein-containing forms of such conjugates and fusion proteins.

Monovalent antibody fragment-streptavidin conjugate may be used to pretarget streptavidin, preferably in additional embodiments of the two-step aspect of the present invention. Exemplary monovalent

antibody fragments useful in these embodiments are Fv, Fab, Fab' and the like. Monovalent antibody fragments, typically exhibiting a molecular weight ranging from about 25 kD (Fv) to about 50 kD (Fab, Fab'), are smaller than whole antibody and, therefore, are generally capable of greater target site penetration. Moreover, monovalent binding can result in less binding carrier restriction at the target surface (occurring during use of bivalent antibodies, which bind strongly and adhere to target cell sites thereby creating a barrier to further egress into sublayers of target tissue), thereby improving the homogeneity of targeting.

In addition, smaller molecules are more rapidly cleared from a recipient, thereby decreasing the immunogenicity of the administered small molecule conjugate. A lower percentage of the administered dose of a monovalent fragment conjugate localizes to target in comparison to a whole antibody conjugate. The decreased immunogenicity may permit a greater initial dose of the monovalent fragment conjugate to be administered, however.

A multivalent, with respect to ligand, moiety is preferably then administered. This moiety also has one or more radionuclides associated therewith. As a result, the multivalent moiety serves as both a clearing agent for circulating anti-ligand-containing conjugate (through cross-linking or aggregation of conjugate) and as a therapeutic agent when associated with target bound conjugate. In contrast to the internalization caused by cross-linking described above, cross-linking at the tumor cell surface stabilizes the monovalent fragment-anti-ligand molecule and, therefore, enhances target retention, under appropriate conditions of antigen density at the target cell. In addition, monovalent antibody fragments generally do not internalize as do bivalent

or whole antibodies. The difficulty in internalizing monovalent antibodies permits cross-linking by a monovalent moiety serves to stabilize the bound monovalent antibody through multipoint binding. This two-step protocol of the present invention has greater flexibility with respect to dosing, because the decreased fragment immunogenicity allows more streptavidin-containing conjugate, for example, to be administered, and the simultaneous clearance and therapeutic delivery removes the necessity of a separate controlled clearing step.

Another embodiment of the pretargeting methodologies of the present invention involves the route of administration of the ligand- or anti-ligand-active agents. In these embodiments of the present invention, the active agent-ligand (e.g., radiolabeled biotin) or -anti-ligand is administered intraarterially using an artery supplying tissue that contains the target. In the radiolabeled biotin example, the high extraction efficiency provided by avidin-biotin interaction facilitates delivery of very high radioactivity levels to the target cells, provided the radioactivity specific activity levels are high. The limit to the amount of radioactivity delivered therefore becomes the biotin binding capacity at the target (i.e., the amount of antibody at the target and the avidin equivalent attached thereto).

For these embodiments of the pretargeting methods of the present invention, particle emitting therapeutic radionuclides resulting from transmutation processes (without non-radioactive carrier forms present) are preferred. Exemplary radionuclides include Y-90, Re-188, At-211, Bi-212 and the like. Other reactor-produced radionuclides are useful in the practice of these embodiments of the present invention, if they are able to bind in amounts

delivering a therapeutically effective amount of radiation to the target. A therapeutically effective amount of radiation ranges from about 1500 to about 10,000 cGy depending upon several factors known to nuclear medicine practitioners.

Intraarterial administration pretargeting can be applied to targets present in organs or tissues for which supply arteries are accessible. Exemplary applications for intraarterial delivery aspects of the pretargeting methods of the present invention include treatment of liver tumors through hepatic artery administration, brain primary tumors and metastases through carotid artery administration, lung carcinomas through bronchial artery administration and kidney carcinomas through renal artery administration. Intraarterial administration pretargeting can be conducted using chemotherapeutic drug, toxin and anti-tumor active agents as discussed below. High potency drugs, lymphokines, such as IL-2 and tumor necrosis factor, drug/lymphokine-carrier-biotin molecules, biotinylated drugs/lymphokines, and drug/lymphokine/toxin-loaded, biotin-derivitized liposomes are exemplary of active agents and/or dosage forms useful for the delivery thereof in the practice of this embodiment of the present invention.

In embodiments of the present invention employing radionuclide therapeutic agents, the rapid clearance of nontargeted therapeutic agent decreases the exposure of non-target organs, such as bone marrow, to the therapeutic agent. Consequently, higher doses of radiation can be administered absent dose limiting bone marrow toxicity. In addition, pretargeting methods of the present invention optionally include administration of short duration bone marrow protecting agents, such as WR 2721. As a result, even higher doses of radiation can be given, absent dose limiting bone marrow toxicity.

It is another object of the present invention to produce novel conjugates for use in pretargeting methods which contain at least one member of a complementary binding pair, selected from the group consisting of S-peptide/S-protein, head activator peptide (which binds to itself), cystatin C/cathepsin B, and antibody/hapen pairs and to use said conjugates in pretargeting methods.

Conjugates containing said peptides have utility in all aspects of pretargeting methods, i.e., they may be administered in the initial pretargeting step, they may be used as novel clearing agents, and they may be administered in order to direct an active agent, e.g., a therapeutic or diagnostic agent, to a targeted site, e.g., a tumor.

The S-peptide/S-protein complementary binding pair members have particular applicability in pretargeting methods given the fact that both of these moieties are well characterized, e.g., the complete amino acid sequences of both S-peptide and S-protein have been reported in the literature. Moreover, both the S-peptide and the S-protein are commercially available from Sigma Chemical (St. Louis, Missouri).

S-peptide and S-protein are enzymatically inactive products obtained by limited digestion of ribonuclease A with subtilisin. These moieties bind to one another with an affinity of about 10^{-9}M to produce a ribonuclease S complex which catalyzes the hydrolytic cleavage of RNA similar to ribonuclease A. (*See*, e.g., Kim et al. Protein Science, 2, 348-356, (1993); Kim et al., Anal. Biochem., 219, 165-166, (1994) which describe the S-peptide/S-protein system and the incorporation of S-peptide in fusion proteins).

The present invention embraces the use of conjugates containing S-peptide and/or S-protein in pretargeting methods, as well as derivatives and analogs thereof. The only prerequisite is that such

S-peptide or S-protein derivatives and analogs retain their ability to bind either S-peptide or S-protein with sufficient affinity to be useful in pretargeting methods. An especially preferred S-peptide is a truncated form known in the art as S15 which consists of the following peptide sequence:

Lys-Gly-Thr-Ala-Ala-Ala-Lys-Phe-Glu-Arg-Gln-His-met-Asp-Ser.

Other suitable S-peptides and S-protein conjugates and derivatives thereof are also known in the literature. For example, Thomson et al., Biochem, 33(28), 8587-8593, (1994) describes methylene derivatized S-peptides and truncated forms which effectively complex with S-protein; Kim et al., Protein Science, 2(3), 348-356, (1993) describes functional S-peptide derivatives where the aspartic acid at position 14 is changed to an asparagine, Varadarjan et al., Biochem., 31(49) 12315-12327, (1992) describes variants of S-peptide modified at position 13, and Pease et al., Proc. Natl. Acad. Sci., USA, 87(15), 5643-5647 (1990) describe hybrid peptides derived from S-peptide containing the bee venom peptide apamin. Additionally, S-peptide analogs are described in each of the following references; Teno et al., Chem. Pharm. Bull., 35(2), 468-478, (1987); Mitchinson et al., Protein & Struct. Funct. Genet., 1(1), 23-33, (1986); Voskuyl-Holtkamp et al., Int. J. Pept. Protein Res., 13(2), 185-194, (1979); Scoffone et al., Med. Chem., Spec. Contrib. Int. Symp., 3rd, Editor Pratesi, 83-104, (1973); Rocchi et al., Biochem., Vol. 11(1), 50-57, (1972); and Marchioni et al., J. Am. Chem. Soc., Vol. 90 (21), 5889-5894, (1968).

In the preferred embodiments, the conjugates will contain S-peptide or S15 and/or S-protein because all of these moieties have been extensively characterized and are commercially available. Moreover, since the

amino acid sequence of each of these moieties is known, and all of these moieties are relatively small, i.e., S-peptide is 20 amino acid residues, S15 is 15 amino acid residues, and S-protein is only 104 amino acid residues, all of these moieties can readily be made synthetically, e.g., by solid-state synthesis or by recombinant methods. Alternatively, the S-peptide and S-protein may be obtained by limited digestion of ribonuclease A with subtilisin to generate a peptide fragment containing the first 20 amino acid residues of ribonuclease A (S-peptide) and a protein fragment containing residues 21 to 124 (S-protein).

The S-peptide, S15 peptide and/or S-protein or derivatives and analogs thereof may be used in lieu of other ligand/anti-ligand in conjugates which are used in pretargeting strategies or in combination therewith. For example, S-peptide/S-protein may be used in lieu of biotin/avidin or biotin/streptavidin or in combination therewith.

The use of S-peptide and S-protein and derivatives and analogs as the ligand/anti-ligand pair in pretargeting methods should afford numerous advantages given their ready availability, relatively, low cost; high degree of characterization; the fact that they bind to one another with relatively high affinity (about 10^{-9}M); their relatively small size; and the fact that they are derived by cleavage of a mammalian protein, i.e., bovine pancreatic ribonuclease A. For example, given their small size and mammalian origin, it is expected that immunogenicity should not be as significantly reduced compared to bacterial proteins such as streptavidin. Also, bovine ribonuclease is about 70% homologous to human (Beintena et al., Anal. Biochem., 136, 48-64, (1984)). Moreover, unlike the biotin/avidin or biotin/streptavidin system, there also should not be the problem of endogenously

circulating ligand or anti-ligand (S-peptide or S-protein).

S-peptide and S-protein have particular applicability in the preparation of novel clearing agents. For example, either S-peptide or S-protein may be conjugated to or derivatized with clearance directing moieties to produce compounds which provide for enhanced clearance of a previously, concurrently or subsequently administered conjugate.

S-peptide or S-protein may be attached to any of the afore-described clearance directing agents. As described previously, a clearing agent is any agent capable of binding, complexing or otherwise associating with an administered moiety, e.g., targeting moiety-ligand, targeting moiety-anti-ligand or anti-ligand alone, present in the recipient's circulation, thereby facilitating circulating moiety clearance from the recipient's body, removal from blood circulation, or inactivation thereof in the circulation. However, preferably the clearing agent will comprise hepatocyte receptor binding moiety or moieties.

For example, S-peptide, S-15 peptide or S-protein may be conjugated or derivatized with hexose-based or non-hexose based moieties such as are described *supra*. Hexose-based clearing agents are molecules that have been derivatized to contain one or more hexoses (six carbon moieties), which are preferably recognized by receptor, i.e., Ashwell receptors or other receptors such as the mannose/N-acetylgalactosamine receptor which are associated with endothelial cells and/or the mannose 6-phosphate receptor. S-peptide or S-protein may be directly or indirectly attached to one or more hexoses selected from galactose, mannose, mannose 6-phosphate, N-acetylgalactosamine, pentamannosyl phosphate, thioglycosides of galactose, and more generally, D-galactosides and glucosides or the like,

as well as combinations thereof. As described previously, galactose is the prototypical hexose clearing agent.

One or more such hexoses or several different hexoses may be directly or indirectly attached to an S-peptide or S-protein to provide for an effective clearance agent. In the case of galactose, it appears that at least three galactose residues are necessary, with about 3 to 32 being preferred. Methods of attachment of hexose residues to proteins and peptides are well known in the art. For example, if galactose residues are to be attached, this may be accomplished, e.g., by galactose thioglycoside conjugation such as is described *supra*.

The efficacy of the resultant clearing agent of course depends upon the ability of the resultant agent, e.g., galactose derivatized S-peptide or S-protein to effectively bind its binding partner, i.e., S-protein or S-peptide. With respect to the S-protein, it is expected that galactose or other hexose-derivatization should not adversely affect the ability of the resultant galactose-derivatized S-protein to bind S-peptide and conjugates containing S-peptide. However, in the event that this is not the case, or in the case of S-peptide, the S-peptide or S-protein may instead be indirectly attached to galactose or other hexoses by attachment to a moiety or moieties which contain one or more exposed hexoses, e.g., galactose residues. Preferably, the galactose will be arranged in clusters as described elsewhere in this application.

Examples of such moieties include proteinaceous hexose-based clearing agents which endogenously contain or have been derivatized to contain one or more exposed hexose residues. Exposed hexose residues, e.g., galactose residues, direct rapid

clearance by endocytosis into the liver through specific receptors (Ashwell receptors).

By way of example, S-peptide or S-protein may be attached to the asialoorsomucoid derivative of human alpha-1 acid glycoprotein (orosomucoid), galactosylated albumins such as galactosylated HSA, galactosylated-IgM, galactosylated-IgG, asialohaptoglobin, asialofetuin, asialoceruloplasmin and the like. Preferably, S-peptide or S-protein will be attached to a hexose residue bearing proteinaceous clearing agent which effectively binds to hepatocyte receptors such as galactose, mannose 6-phosphate, N-acetylglucosamine, glucose, N-galactosamine, N-acetylgalactosamine, thioglycosides of galactose, and more generally D-galactosides and glucosides or the like.

It is known in the art that S-peptide may be C-or N-terminally fused to proteinaceous moieties without loss of S-protein binding function. Thus, it is expected that attachment of hexose containing proteinaceous moieties should result in conjugates which effectively bind to S-protein or S-peptide and to conjugates which contain S-peptide or S-protein. Attachment of either S-peptide or S-protein to hexose derivatized proteins, e.g., galactosylated human serum albumin may be effected using conventional heterobifunctional cross-linking agents.

After synthesis, such conjugates will be screened to assess their ability to effectively bind conjugates containing the complementary binding partner with a sufficient binding affinity to provide for effective clearance. Such conjugates will further preferably be designed so as to contain a number of hexose residues, e.g., galactose, which provides for optimal clearance, e.g., by the Ashwell receptor mechanism. This can be determined by variation of the number of attached hexose residues on the S-peptide or S-protein

derivative and comparing clearance rates as a function of the number of attached hexose residues, e.g., galactose, after *in vivo* administration.

When S-protein conjugate is utilized as the clearing agent, exemplary embodiments are described schematically below:

(i) a pretargeting step comprising the administration of a conjugate or fusion protein comprising an S-peptide, e.g., S15 peptide N-or C-terminally attached to a targeting moiety, e.g., an antibody or antibody fragment, which is optionally attached to another ligand or anti-ligand, e.g., streptavidin, avidin or biotin; and

(ii) a clearance step comprising the administration of a clearing agent comprising S-protein derivatized with one or more hexose-based derivatives, e.g., galactose residues, or a hexose containing or derivatized protein, e.g., galactosylated HSA.

A particular application for the S-peptide is for imaging of clots by modification of an annexin with S-peptide. More specifically, it should be useful in enhancing target and background ratio for improved clot/thrombus imaging with Tc-99m annexin.

Technetium-99m labeled annexin has been shown to effectively localize in clots that have been induced in the pig animal model. This is based on the ability of annexin to bind to the membranes of activated platelets. Because of the occurrence of clots in the vascular system and thus in the presence of blood radioactivity background, the ability to visualize smaller clots in areas of the heart, lung or brain would be enhanced if the clot to blood background could be further increased over that resulting from the natural organ clearance of Tc-99m annexin from the blood.

The enzyme subtilisin cleaves RNase A into S-protein and S-peptide. These are of 103 and 20 residues respectively. The S-protein and S-peptide bind with high affinity. This has been exploited *in vitro* for detection of small amounts of recombinant proteins by attaching the S-peptide to the end of the recombinant protein and allowing the S-protein to bind to the peptide thus providing an affinity tag for detection or purification of the recombinant protein. While the detection need has been usefully met, the affinity is high enough such that separation of S-protein from purified material is problematic. (*See* Kim and Raines, Anal. Biochem, 219, 165-166, (1994)).

Based on the foregoing, it is proposed that annexin be modified with S-peptide. Recombinant annexin is available and addition of the 20-mer S-peptide to annexin should be facile. This can be done to either the carboxyl or amino terminus with the choice presumably resulting in minimal impact on annexin activated platelet binding. S-protein would be modified with a liver targeting moiety such as galactose. This would allow a procedure as follows: (i) Tc-99m annexin-S-peptide is administered, allowed to target clots and determination of sufficient information for diagnosis made by scintigraphic imaging. (ii) If higher clot to blood background ratio is needed for diagnosis, S-protein-liver targeting moiety is injected. Tc-99m annexin in circulation would be bound by the S-protein portion while the liver targeting moiety would cause liver uptake of the bound complex.

Initial studies of galactose modified annexin resulted in loss of binding affinity of the annexin for activated platelets. This approach would allow controlled modification in such a way that interference with binding is unlikely as S-peptide can be modified at either end and retain affinity for S-

protein. An advantage of this system would be that maximum targeting of the Tc-99m annexin would be retained with improved blood clearance as a subsequent, perhaps optional step. In the case of directly modified annexin, the increased hepatic clearance resulting from the modification may result in reduced bioavailability and decrease the fraction of dose localizing at the clot.

However, a potential problem is that once S-peptide and S-protein are rebound (reconnected), active RNase results. Thus, targeting the complex to the liver via galactose modification as an example, may result in toxicity to the hepatocytes. As a result, it may by necessary to use S-peptide and/or S-protein analogs which complex, but which upon complexation do not result in an active RNase.

As discussed, the S-peptide and S-protein and derivatives thereof may be used alone or in combination with or as a substitute for streptavidin, avidin or biotin in pretargeting methods. Also, the S-peptide or S-protein may be mutagenized, e.g., by site-specific mutagenesis to produce analogs which bind either S-peptide or S-protein with higher affinity than the unmodified S-peptide or S-protein or complex to produce non-enzymatically active derivaties. Also, the S-peptide may be derivatized to effectively "lock in" the peptide by modification of an appropriate amino acid side chain. This may be accomplished, e.g., by reaction with an active halide such as chloromethylketone. This will enable rapid binding of the mutagenized S-peptide (on rate), without significant loss of the bound S-peptide ligand (off-rate). Thus, the use of such S-peptide derivatives should result in better retention of the active agent at the target site, attributable to non-reversible binding of the modified S-peptide and S-protein containing conjugate at the target site.

Because the S-peptide is so small in size, it should also be possible to attach several of these moieties to the targeting moiety, e.g., antibody or antibody fragment, thereby increasing receptor target at the target site, e.g., a tumor. For example, the S-peptide may be modified by linking two or more together to provide for multivalent binding. This will increase affinity at the *in vivo* target site. Still further, the linking peptide may contain an additional linkage to the effector moiety. Such conjugates should enhance binding affinity by providing for the crosslinking of the S-protein or S-peptide which is attached to the antibody or other targeting moiety, or by increasing the probability that the conjugate rebinds its complementary binding partner.

If S-peptide is used in clearing agents it may particularly exhibit some liver toxicity. If toxic, ribonuclease inhibitors can be used to obviate cytotoxicity. (*See*, e.g., White et al., Principles Biochem, McGraw Hill, 5th ed., p. 258 in this regard).

The S-protein may also be oligomerized to produce conjugates having increased binding stoichiometry and affinity. Incorporation of such oligomers in conjugates for use in pretargeting should provide for both better delivery and retention of the active agent at the targeted site. However, one disadvantage is that this may result in enhanced immunogenicity to the S-protein.

Yet another advantage of the S-peptide/S-protein system is that these moieties complex to produce an enzymatically active ribonuclease S complex. This enzyme activity may be exploited to provide for targeted cytotoxic activity. In this regard, a non-toxic form of a RNase toxin that is cytotoxic when internalized into cells via proteins such as transferrin (following binding to the transferrin

receptor) has been described by Youle and Rybak (*See*, Proc. Natl. Acad. Sci., 89, 3165-3169 (1992); and J. Biol. Chem., 266, 21202-21207, (1991)). Therefore, the use of S-peptide and S-protein containing conjugates in pretargeting methods may be exploited to provide for RNase activity at a target site, e.g., tumor cells. Moreover, if an internalizing antibody, e.g., slowly internalizing antibody, is selected as the targeting moiety, this will enable the enzymatically active complex to internalize onto the cell together with the antibody. Such internalization provides for selective cytotoxicity at the targeted site attributable to the RNase activity of the S-peptide/S-protein complex. If S-peptide is used as a clearing agent it may potentially by cytotoxized. However, this may potentially be obviated by administration of ribonuclease inhibitors, inhibit A??? or RNase. Also, iodoacetate reaction with ribonuclease inactivates the enzyme by alkylation of histidine 119. (White et al., Principles Biochem., McGraw Hill, Sin ed., p. 258).

Another ligand/anti-ligand system useful in the present invention is the head activator (HA) peptide. The HA peptide is derived from the freshwater coelenterate, *Hydra*. This peptide acts as a morphogen which controls the coelenterate's head-specific growth and differentiation process.

The entire HA peptide consists of the following amino acid sequence:

pGlu-Pro-Pro-Gly-Gly-Ser-Lys-Val-Ile-Leu-Phe.

It is known in the literature (*See*, Bodenmuller et al., EMBO J., Vol. 5(8), 1825-1829, (1986)), that this peptide self-aggregates to produce dimers which, unlike the HA peptide monomer, are inactive. It is additionally known that the affinity between two HA molecules is extremely high (*Id.*). Under physiological conditions, the HA dimer does not

dissociate into monomers, even at concentrations as low as 10^{-13} M (Id.). Moreover, it is also known that carboxyl fragments of the HA peptide, in particular a fragment containing the last six carboxyl amino acids of the HA peptide, dimerize equal to or even more efficiently than the intact HA peptide (Id.). Given the foregoing, the HA peptide, as well as fragments and derivatives thereof, most particularly a hexameric peptide consisting of the following amino acid sequence: Ser-Lys-Val-Ile-Leu-Phe are particularly well suited as both the ligand and anti-ligand binding partners in pretargeting methods.

The HA peptide affords numerous advantages to other known ligands and anti-ligands in pretargeting methods. For example, given its small size, the HA peptide and fragments thereof should not be very immunogenic. Thus, the HA peptide is especially well suited for therapeutic pretargeting methods wherein immunogenicity may be a potential concern. Also, the HA peptide binds to itself with very high affinity. Therefore, it should enhance binding and retention of active agents at targeted sites.

The HA peptide or fragments thereof may be fused to a targeting moiety, e.g., an antibody or antibody fragment and then administered in the initial pretargeting step. Another advantage of the HA peptide and fragments thereof is that its small size should enable it to be inserted into targeting moiety sequences, e.g., antibody sequences and fragments thereof. Such insertion may be effected by recombinant methods or by solid state synthesis. However, recombinant methods are preferred.

Recombinant methods of expressing antibodies and binding fragments thereof are well known in the art. For example, methods are known in the art for the recombinant expression of antibodies, fragments and derivatives, e.g., Fab fragments, Fv's, humanized

antibodies, chimeric antibodies, single chain antibodies and bispecific antibodies (*See*, e.g., U.S. Patent No. 4,816,567 to Cabibly et al.; U.S. Patent No. 5,132,405 to Huston et al; U.S. Patent No. 4,704,692 to Ladner, U.S. Patent No. 4,946,778 to Ladner et al.; U.S. Patent No. 5,091,513 to Huston et al.; U.S. Patent No. 4,816,397 to Boss et al.; U.S. Patent No. 5,169,939 to Gefter et al.; U.S. Patent No. 5,196,320 to Gillies et al.; U.S. Patent No. 5,225,539; U.S. Patent No. 4,642,334 to Moore et al.; U.S. Patent No. 5,202,238 to Fell, Jr.; U.S. Patent No. 5,204,244 to Fell et al., all of which are incorporated by reference herein. Accordingly, an oligonucleotide encoding the subject HA peptide, or the above-described hexameric peptide, can be inserted into a DNA sequence encoding a desired antibody sequence or antibody fragment and expressed to produce a recombinant antibody or antibody fragment capable of dimerizing with another conjugate containing the HA peptide. Alternatively, such a sequence may be created by site specific mutagenesis of a recombinant antibody or antibody fragment DNA sequence. Such sequences will preferably be inserted or created in portions of the antibody molecule which are non-essential for antigen binding.

Functional antigen-binding sequences can be selected by inserting the HA peptide encoding sequences into different regions of a particular antibody DNA sequence and then screening the resultant expression products in binding assays to identify those particular recombinant sequences which bind antigen with sufficient affinity.

Yet another application of the HA peptide and truncated and derivative forms thereof is for "cementing" the light and variable domains of a recombinant Fv molecule. This may be accomplished by expression of fusion peptides respectively comprising

the heavy variable region fused to at least one HA peptide and a light variable region fused to at least one HA peptide. These fusion peptides may be separately or co-expressed, with co-expression being preferred since this may result in formation of Fv's in the host cell. The presence of the HA peptide on each of the light and variable region should facilitate the formation of a highly stable Fv molecules, given the high autoaffinity of HA peptides, e.g., the HA hexameric peptide sequence identified *supra*. Additionally, the resultant monovalent Fv sequence can additionally be dimerized by fusing several HA peptide sequences onto either or both of the variable heavy and light sequence fusion proteins. This will provide for the formation of divalent or higher valency Fv's.

Still another application of the HA peptide, and derivatives thereof is for increasing the avidity of single chain antibody molecules to antigen molecules. To date, single chain antibodies have not been widely used given their typically low antigen avidity relative to native antibodies and to Fab fragments. While their small size and single chain form affords some intrinsic advantages, e.g., the ability to be internalized by tumor cells, e.g., extravascular tumors and rapid renal clearance, their low avidity to antigen renders their therapeutic and diagnostic use disadvantageous.

Incorporation of one or more HA peptide sequences into a single chain antibody molecule will result in dimerization of the single chain antibody molecule, or even multimerization if more than one HA peptide sequence is incorporated into or fused to the single chain antibody molecule. This will result in single chain antibodies containing more than one antigen binding site. Therefore, this should result in single chain antibodies having higher avidity to antigen.

Yet another application of HA peptide sequences is for the preparation of bispecific antibodies. Bispecific antibodies comprise the antigenic binding sequences of antibodies having two different antigen specificities. Therefore, such antibodies have the ability to bind to two different antigens. The present invention provides a novel method for the formation of bispecific antibodies by the attachment of one or more HA peptide sequences to Fv sequences, single chain antibody sequences, or Fab sequences, wherein the fused antigen binding sequences possess different antigenic specificity. These fusion proteins may be made by recombinant methods or by synthetic means with recombinant methods being preferred. Said HA containing sequences may be separately or co-expressed in recombinant cells. Co-expression is preferred since this may enable the fusion proteins to dimerize in the recombinant host cell to produce bispecific antibody molecules comprised of Fv's, single chain antibodies or Fab's of two different specificities.

Alternatively, if these sequences are separately expressed, or if dimerization does not occur *in vivo*, the resistant HA containing antigen binding sequences may alternatively be dimerized by mixing in solution, or alternatively by contacting a solid phase to which one of the antigen binding fusion proteins has been immobilized with the other HA peptide containing antigen binding sequence having different antigenic specificity.

It is yet another object of the invention to produce fusion proteins which contain the HA peptide or fragments thereof which provide for the formation of multimeric proteins having dual or even higher functionality. For example, HA may be fused to another member of a complementary binding pair, e.g., biotin. This HA-biotin fusion protein may be used to

produce a highly stable linkage with an HA-antibody fusion protein, e.g., which has been pretargeted to a target site, e.g., a tumor cell. Moreover, the presence of the biotin in the fusion protein will in addition provide for the stable attachment of avidin or streptavidin. The use of two ligands in combination will also enable several different moieties to be directed to a targeted site, e.g., tumor cells.

The HA peptide may be incorporated in conjugates which are used in all steps of pre-targeting methods. For example, the HA peptide or a fragment thereof may be attached to or inserted in a targeting moiety, e.g., an antibody, antibody fragment or receptor binding moiety as described previously, and used in the initial pretargeting step. Also, an active agent, e.g., the diagnostic or therapeutic agent, can also be attached to an HA peptide. The HA peptide-active agent will bind the pretargeted HA-targeting moiety because of the affinity of the HA peptide to the HA peptide contained in the pretargeted conjugate. Also, because of its small size, several HA peptides may be attached to an active agent, or the HA peptide may be attached to different active agents. This should enable more or several different active agents to be delivered to a targeted site. This is advantageous because some therapies may require delivery of several active agents, (because of synergistic cytotoxic effects) or high dosages of the particular cytotoxin agent to be effective.

Moreover, the HA peptide may also be utilized for the preparation of novel clearing agents. In this embodiment, the HA peptide will be directly or indirectly attached to one of the clearance directing moieties described *supra*, e.g., a galactosylated protein such as galactosylated human serum albumin.

With respect to all the above-described usages of the HA peptide, it should be noted that the HA peptide dimerizes in an antiparallel fashion. In most cases, this should not adversely affect the binding function of the particular conjugates which are being dimerized. In fact, in many instances such an anti-parallel binding arrangement is expected to enhance the binding activity of the resulting complexes, e.g., an Fv dimer. However, assuming that this is problematic in some instances, e.g., if dimerization creates steric constraints which adversely affect antigen binding, this may be alleviated or obviated by the additional attachment of additional amino acid residues which eliminate steric constraints. For example, proline residues may be engineered onto the particular HA peptide fusion protein given their known efficacy in enhancing the flexibility of proteins and in particular antibody fusion proteins.

An additional aspect of the present invention is directed to the use of targeting moieties that are monoclonal antibodies or fragments thereof that localize to an antigen that is recognized by the antibody NR-LU-10. Such monoclonal antibodies or fragments may be murine or of other non-human mammalian origin, chimeric, humanized or human.

NR-LU-10 is a 150 kilodalton molecular weight IgG2b monoclonal antibody that recognizes an approximately 40 kilodalton glycoprotein antigen expressed on most carcinomas. *In vivo* studies in mice using an antibody specific for the NR-LU-10 antigen revealed that such antibody was not rapidly internalized, which would have prevented localization of the subsequently administered active-agent-containing conjugate to the target site.

NR-LU-10 is a well characterized pancarcinoma antibody that has been safely administered to over 565 patients in human clinical trials. The hybridoma

secreting NR-LU-10 was developed by fusing mouse splenocytes immunized with intact cells of a human small cell lung carcinoma with P3 x 63/Ag8UI murine myeloma cells. After establishing a seed lot, the hybridoma was grown via *in vitro* cell culture methods, purified and verified for purity and sterility.

Radioimmunoassays, immunoprecipitation and Fluorescence-Activated Cell Sorter (FACS) analysis were used to obtain reactivity profiles of NR-LU-10. The NR-LU-10 target antigen was present on either fixed cultured cells or in detergent extracts of various types of cancer cells. For example, the NR-LU-10 antigen is found in small cell lung, non-small cell lung, colon, breast, renal, ovarian, pancreatic, and other carcinoma tissues. Tumor reactivity of the NR-LU-10 antibody is set forth in Table A, while NR-LU-10 reactivity with normal tissues is set forth in Table B. The values in Table B are obtained as described below. Positive NR-LU-10 tissue reactivity indicates NR-LU-10 antigen expression by such tissues. The NR-LU-10 antigen has been further described by Varki et al., "Antigens Associated with a Human Lung Adenocarcinoma Defined by Monoclonal Antibodies," Cancer Research, 44: 681-687, 1984, and Okabe et al., "Monoclonal Antibodies to Surface Antigens of Small Cell Carcinoma of the Lung," Cancer Research, 44: 5273-5278, 1984.

The tissue specimens were scored in accordance with three reactivity parameters: (1) the intensity of the reaction; (2) the uniformity of the reaction within the cell type; and (3) the percentage of cells reactive with the antibody. These three values are combined into a single weighted comparative value between 0 and 500, with 500 being the most intense reactivity. This comparative value facilitates comparison of different tissues. Table B includes a summary reactivity value, the number of tissue samples

examined and the number of samples that reacted positively with NR-LU-10.

Methods for preparing antibodies that bind to epitopes of the NR-LU-10 antigen are described in U.S. Patent No. 5,084,396. Briefly, such antibodies may be prepared by the following procedure:

- absorbing a first monoclonal antibody directed against a first epitope of a polyvalent antigen onto an inert, insoluble matrix capable of binding immunoglobulin, thereby forming an immunosorbent;
- combining the immunosorbent with an extract containing polyvalent NR-LU-10 antigen, forming an insolubilized immune complex wherein the first epitope is masked by the first monoclonal antibody;
- immunizing an animal with the insolubilized immune complex;
- fusing spleen cells from the immunized animal to myeloma cells to form a hybridoma capable of producing a second monoclonal antibody directed against a second epitope of the polyvalent antigen;
- culturing the hybridoma to produce the second monoclonal antibody; and
- collecting the second monoclonal antibody as a product of the hybridoma.

Consequently, monoclonal antibodies NR-LU-01, NR-LU-02 and NR-LU-03, prepared in accordance with the procedures described in the aforementioned patent, are exemplary targeting moieties useful in this aspect of the present invention.

Additional antibodies reactive with the NR-LU-10 antigen may also be prepared by standard hybridoma production and screening techniques. Any hybridoma clones so produced and identified may be further

screened as described above to verify antigen and tissue reactivity.

Table A

Organ/Cell Type Tumor	#Pos/ Exam	Intensity[a] Avg.	Range	Percent[b] Avg.	Range	Uniformity[c] Avg.	Range
Pancreas Carcinoma	6/6	3	3	100	100	2.3	2-3
Prostate Carcinoma	9/9	2.8	2-3	95	80-100	2	1-3
Lung Adenocarcinoma	8/8	3	3	100	100	2.2	1-3
Lung Small Cell Carcinoma	2/2	3	3	100	100	2	2
Lung Squamous Cell Carcinoma	8/8	2.3	2-3	73	5-100	1.8	1-3
Renal Carcinoma	8/9	2.2	2-3	83	75-100	1	1
Breast Adenocarcinoma	23/23	2.9	2-3	97	75-100	2.8	1-3
Colon Carcinoma	12/12	2.9	2-3	98	95-100	2.9	2-3
Malignant Melanoma Ocular	0/2	0	0	0	0	0	0
Malignant Melanoma	0/11	0	0	0	0	0	0
Ovarian Carcinoma	35/35	2.9	2-3	200	100	2.2	1-3
Undifferentiated Carcinoma	1/1	2	2	90	90	2	2
Osteosarcoma	1/1	2	2	20	20	1	1
Synovial Sarcoma	0/1	0	0	0	0	0	0
Lymphoma	0/2	0	0	0	0	0	0
Liposarcoma	0/2	0	0	0	0	0	0
Uterine Leiomyosarcoma	0/1	0	0	0	0	0	0

[a] Rated from 0-3, with 3 representing highest intensity.
[b] Percentage of cells stained within the examined tissue section.
[c] Rates from 0-3, with 3 representing highest uniformity.

Table B

Organ/Cell Type	# Pos/Exam	Summary Reactivity
Adenoid	3/3	433
Epithelium	0/3	0
Lymphoid Follicle-Central	0/3	0
Lymphoid Follicle-Peripheral	2/2	400
Adipose Tissue		
Fat Cells	0/3	0
Adrenal		
Zona Fasciculata Cortex	0/3	0
Zona Glomerulosa Cortex	0/3	0
Zona Reticularis Cortex	0/3	0
Medulla	0/3	0
Aorta		
Endothelium	0/3	0
Elastic Interna	0/3	0
Tunica Adventitia	0/3	0
Tunica Media	0/3	0
Brain-Cerebellum		
Axons, Myelinated	0/3	0
Microglia	0/3	0
Neurons	0/3	0
Purkenje's Cells	0/3	0
Brain-Cerebrum		
Axons, Myelinated	0/3	0
Microglia	0/3	0
Neurons	0/3	0
Brain-Midbrain		
Axons, Myelinated	0/3	0
Microglia	0/3	0
Neurons	0/3	0
Colon		
Mucosal Epithelium	3/3	500

Organ/Cell Type	# Pos/Exam	Summary Reactivity
Muscularis Externa	0/3	0
Muscularis Mucosa	0/3	0
Nerve Ganglia	0/3	0
Serosa	0/1	0
Duodenum		
Mucosal Epithelium	3/3	500
Muscularis Mucosa	0/3	0
Epididymis		
Epithelium	3/3	419
Smooth Muscle	0/3	0
Spermatozoa	0/1	0
Esophagus		
Epithelium	3/3	86
Mucosal Gland	2/2	450
Smooth Muscle	0/3	0
Gall Bladder		
Mucosal Epithelium	0/3	467
Smooth Muscle	0/3	0
Heart		
Myocardium	0/3	0
Serosa	0/1	0
Ileum		
Lymph Node	0/2	0
Mucosal Epithelium	0/2	0
Muscularis Externa	0/1	0
Muscularis Mucosa	0/2	0
Nerve Ganglia	0/1	0
Serosa	0/1	0
Jejunum		
Lymph Node	0/1	0
Mucosal Epithelium	2/2	400
Muscularis Externa	0/2	0

Organ/Cell Type	# Pos/Exam	Summary Reactivity
Muscularis Mucosa	0/2	0
Nerve Ganglia	0/2	0
Serosa	0/1	0
Kidney		
Collecting Tubules	2/3	160
Distal Convoluted Tubules	3/3	500
Glomerular Eipthelium	0/3	0
Mesangial	0/3	0
Proximal Convoluted Tubules	3/3	500
Liver		
Bile Duct	3/3	500
Central Lobular Hepatocyte	1/3	4
Periportal Hepatocyte	1/3	40
Kupffer Cells	0/3	0
Lung		
Alveolar Macrophage	0/3	0
Bronchial Epithelium	0/2	0
Bronchial Smooth Muscle	0/2	0
Pneumocyte Type I	3/3	354
Pneumocyte Type II	3/3	387
Lymph Node		
Lymphoid Follicle-Central	0/3	0
Lymphoid Follicle-Peripheral	0/3	0
Mammary Gland		
Alveolar Epithelium	3/3	500
Duct Epithelium	3/3	500
Myoepithelium	0/3	0
Muscle Skeletal		
Muscle Fiber	0/3	0
Nerve		
Axon, Myelinated	0/2	0
Endoneurium	0/2	0

Organ/Cell Type	# Pos/Exam	Summary Reactivity
Neurolemma	0/2	0
Neuron	0/2	0
Perineurium	0/2	0
Ovary		
Corpus Luteum	0/3	0
Eipthelium	1/1	270
Granulosa	1/3	400
Serosa	0/3	0
Theca	0/3	0
Oviduct		
Epithelium	1/1	500
Smooth Muscle	0/3	0
Pancreas		
Acinar Cell	3/3	500
Duct Epthelium	3/3	500
Islet Cell	3/3	500
Peritoneum		
Mesothelium	0/1	0
Pituitary		
Adenohypophysis	2/2	500
Neurohypophysis	0/2	0
Placenta		
Trophoblasts	0/3	0
Prostate		
Concretions	0/3	0
Glandular Epithelium	3/3	400
Smooth Muscle	0/3	0
Rectum		
Lymph Node	0/2	0
Mucosal Epithelium	0/2	0
Muscularis Externa	0/1	0
Muscularis Mucosa	0/3	0

Organ/Cell Type	# Pos/Exam	Summary Reactivity
Nerve Ganglia	0/3	0
Salivary Gland		
Acinar Epithelium	3/3	500
Duct Epithelium	3/3	500
Skin		
Apocrine Glands	3/3	280
Basal Layer	3/3	33
Epithelium	1/3	10
Follicle	1/1	190
Stratum Corneum	0/3	0
Spinal Cord		
Axons, Myelinated	0/2	0
Microglial	0/2	0
Neurons	0/2	0
Spleen		
Lymphoid Follicle-Central	0/3	0
Lymphoid Follicle-Peripheral	0/3	0
Trabecular Smooth Muscle	0/3	0
Stomach		
Chief Cells	3/3	290
Mucosal Epithelium	3/3	367
Muscularis Mucosa/Externa	0/3	0
Parietal Cells	3/3	290
Smooth Muscle	0/3	0
Stromal Tissue		
Adipose	0/63	0
Arteriolar Smooth Muscle	0/120	0
Endothelium	0/120	0
Fibrous Connective Tissue	0/120	0
Macrophages	0/117	0
Mast Cells/Eosinophils	0/86	0
Testis		

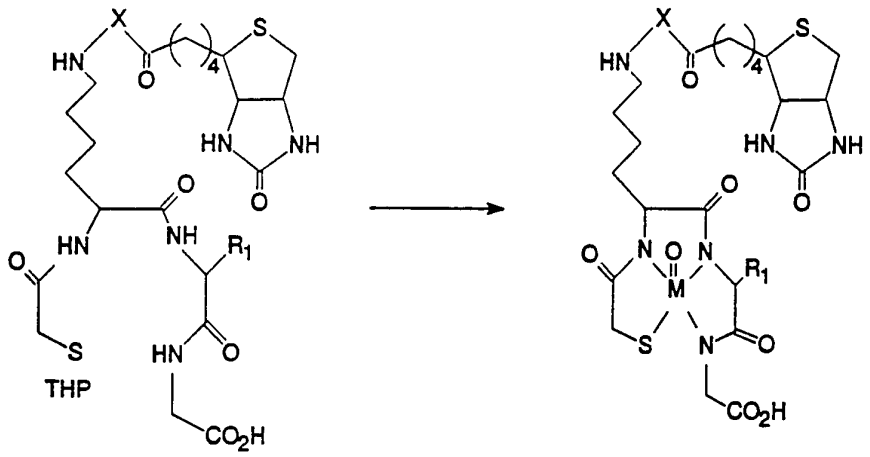
Organ/Cell Type	# Pos/Exam	Summary Reactivity
Interstitial Cells	0/3	0
Sertoli Cells	3/3	93
Thymus		
Hassal's Epithelium	3/3	147
Hassal's Keratin	3/3	333
Lymphoid Cortex	0/3	0
Lymphoid Medulla	3/3	167
Thyroid		
C-Cells	0/3	0
Colloid	0/3	0
Follicular Epithelium	3/3	500
Tonsil		
Epithelium	1/3	500
Lymphoid Follicle-Central	0/3	0
Lymphoid Follicle-Peripheral	0/3	0
Mucus Gland	1/1	300
Striated Muscle	0/3	0
Umbilical Cord		
Epithelium	0/3	0
Urinary Bladder		
Mucosal Epithelium	3/3	433
Serosa	0/1	0
Smooth Muscle	0/3	0
Uterus		
Endometrial Epithelium	3/3	500
Endometrial Glands	3/3	500
Smooth Muscle	0/3	0
Vagina/Cervix		
Epithelial Glands	1/1	500
Smooth Muscle	0/2	0
Squamous Epithelium	1/1	200

The invention is further described through presentation of the following examples. These examples are offered by way of illustration, and not by way of limitation.

Example I

Synthesis of a Chelate-Biotin Conjugate

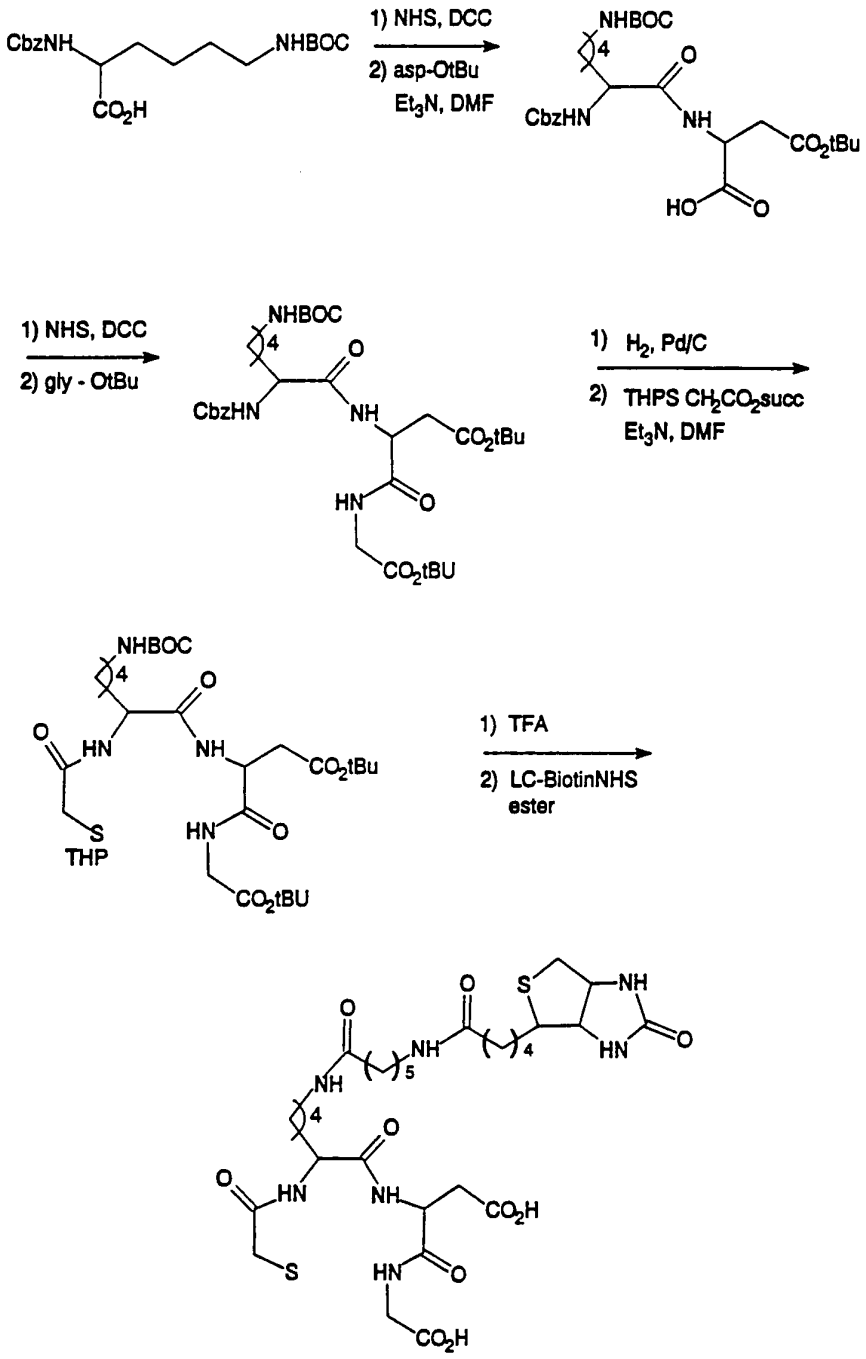
A chelating compound that contains an N_3S chelating core was attached via an amide linkage to biotin. Radiometal labeling of an exemplary chelate-biotin conjugate is illustrated below.



The spacer group "X" permits the biotin portion of the conjugate to be sterically available for avidin binding. When "R^1" is a carboxylic acid substituent (for instance, CH_2COOH), the conjugate exhibits improved water solubility, and further directs *in vivo* excretion of the radiolabeled biotin conjugate toward renal rather than hepatobiliary clearance.

Briefly, N-α-Cbz-N-Σ-t-BOC protected lysine was converted to the succinimidyl ester with NHS and DCC, and then condensed with aspartic acid β--t-butyl

ester. The resultant dipeptide was activated with NHS and DCC, and then condensed with glycine t-butyl ester. The Cbz group was removed by hydrogenolysis, and the amine was acylated using tetrahydropyranyl mercaptoacetic acid succinimidyl ester, yielding S-(tetrahydropyranyl)-mercaptoacetyl-lysine. Trifluoroacetic acid cleavage of the N-t-BOC group and t-butyl esters, followed by condensation with LC-biotin-NHS ester provided (Σ-caproylamide biotin)-aspartyl glycine. This synthetic method is illustrated on the following page.



1H NMR: (CD_3OD; 200 MHz Varian): 1.25-1.95 (m, 24H), 2.15-2.25 (broad t, 4H), 2.65-3.05 (m, 4H), 3.30-3.45 (dd, 2H), 3.50-3.65 (ddd, 2H), 3.95 (broad s, 2H), 4.00-4.15 (m, 1H), 4.25-4.35 (m, 1H), 4.45-4.55 (m, 1H), 4.7-5.05 (m overlapping with HOD).

Elemental Analysis: C, H, N for $C_{35}H_{57}N_7O_{11}S_2 \cdot H_2O$
calculated: 50.41, 7.13, 11.76
found: 50.13, 7.14, 11.40

Example II

Preparation of a Technetium or Rhenium Radiolabeled Chelate-Biotin Conjugate

The chelate-biotin conjugate of Example I was radiolabeled with either ^{99m}Tc pertechnetate or ^{186}Re perrhenate. Briefly, ^{99m}Tc pertechnetate was reduced with stannous chloride in the presence of sodium gluconate to form an intermediate Tc-gluconate complex. The chelate-biotin conjugate of Example I was added and heated to 100°C for 10 min at a pH of about 1.8 to about 3.3. The solution was neutralized to a pH of about 6 to about 8, and yielded an N_3S-coordinated ^{99m}Tc-chelate-biotin conjugate. C-18 HPLC gradient elution using 5-60% acetonitrile in 1% acetic acid demonstrated two anomers at 97% or greater radiochemical yield using δ (gamma ray) detection.

Alternatively, ^{186}Re perrhenate was spiked with cold ammonium perrhenate, reduced with stannous chloride, and complexed with citrate. The chelate-biotin conjugate of Example I was added and heated to 90°C for 30 min at a pH of about 2 to 3. The solution was neutralized to a pH of about 6 to about 8, and yielded an N_3S-coordinated ^{186}Re-chelate-biotin conjugate. C-18 HPLC gradient elution using 5-60% acetonitrile in 1% acetic acid resulted in radiochemical yields of 85-90%. Subsequent purification over a C-18 reverse phase hydrophobic column yielded material of 99% purity.

Example III

***In vitro* Analysis of Radiolabeled Chelate-Biotin Conjugates**

Both the ^{99m}Tc- and ^{186}Re-chelate-biotin conjugates were evaluated *in vitro*. When combined with excess

avidin (about 100-fold molar excess), 100% of both radiolabeled biotin conjugates complexed with avidin.

A ^{99m}Tc-biotin conjugate was subjected to various chemical challenge conditions. Briefly, ^{99m}Tc-chelate-biotin conjugates were combined with avidin and passed over a 5 cm size exclusion gel filtration column. The radiolabeled biotin-avidin complexes were subjected to various chemical challenges (see Table 1), and the incubation mixtures were centrifuged through a size exclusion filter. The percent of radioactivity retained (indicating avidin-biotin-associated radiolabel) is presented in Table 1. Thus, upon chemical challenge, the radiometal remained associated with the macromolecular complex.

TABLE 1

Chemical Challenge of ^{99m}Tc-Chelate-Biotin-Avidin Complexes

Challenge Medium	pH	% Radioactivity Retained 1 h, 37°C	18 h, RT
PBS	7.2	99	99
Phosphate	8.0	97	97
10 mM cysteine	8.0	92	95
10 mM DTPA	8.0	99	98
0.2 M carbonate	10.0	97	94

In addition, each radiolabeled biotin conjugate was incubated at about 50 μg/ml with serum; upon completion of the incubation, the samples were subjected to instant thin layer chromatography (ITLC) in 80% methanol. Only 2-4% of the radioactivity remained at the origin (i.e., associated with protein); this percentage was unaffected by the addition of exogenous biotin. When the samples were analyzed using size exclusion H-12 FPLC with 0.2 M phosphate as mobile phase, no association of radioactivity with serum macromolecules was observed.

Each radiolabeled biotin conjugate was further examined using a competitive biotin binding assay. Briefly, solutions containing varying ratios of D-biotin to radiolabeled biotin conjugate were combined with limiting avidin at a constant total biotin:avidin ratio. Avidin binding of each radiolabeled biotin conjugate was determined by ITLC, and was compared to the theoretical maximum stoichiometric binding (as determined by the HABA spectrophotometric assay of Green, Biochem. J. 94:23c-24c, 1965). No significant difference in avidin binding was observed between each radiolabeled biotin conjugate and D-biotin.

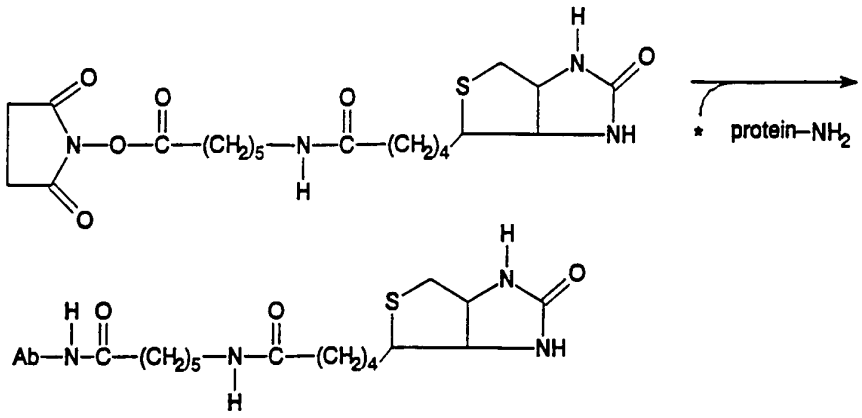
Example IV

in vivo Analysis of Radiolabeled Chelate-Biotin Conjugates Administered After Antibody Pretargeting

The ^{186}Re-chelate-biotin conjugate of Example I was studied in an animal model of a three-step antibody pretargeting protocol. Generally, this protocol involved: (i) prelocalization of biotinylated monoclonal antibody; (ii) administration of avidin for formation of a "sandwich" at the target site and for clearance of residual circulating biotinylated antibody; and (iii) administration of the 186Re-biotin conjugate for target site localization and rapid blood clearance.

A. Preparation and Characterization of Biotinylated Antibody

Biotinylated NR-LU-10 was prepared according to either of the following procedures. The first procedure involved derivitization of antibody via lysine ϵ-amino groups. NR-LU-10 was radioiodinated at tyrosines using chloramine T and either ^{125}I or ^{131}I sodium iodide. The radioiodinated antibody (5-10 mg/ml) was then biotinylated using biotinamido caproate NHS ester in carbonate buffer, pH 8.5, containing 5% DMSO, according to the scheme below.



The impact of lysine biotinylation on antibody immunoreactivity was examined. As the molar offering of biotin:antibody increased from 5:1 to 40:1, biotin incorporation increased as expected (measured using the HABA assay and pronase-digested product) (Table 2, below). Percent of biotinylated antibody immunoreactivity as compared to native antibody was assessed in a limiting antigen ELISA assay. The immunoreactivity percentage dropped below 70% at a measured derivitization of 11.1:1; however, at this level of derivitization, no decrease was observed in antigen-positive cell binding (performed with LS-180 tumor cells at antigen excess). Subsequent experiments used antibody derivitized at a biotin:antibody ratio of 10:1.

TABLE 2

Effect of Lysine Biotinylation on Immunoreactivity

Molar Offering (Biotins/Ab)	Measured Derivitization (Biotins/Ab)	Immunoassessment (%) ELISA	Cell Binding
5:1	3.4	86	
10:1	8.5	73	100
13:1	11.1	69	102
20:1	13.4	36	106
40:1	23.1	27	

Alternatively, NR-LU-10 was biotinylated using thiol groups generated by reduction of cystines. Derivitization of thiol groups was hypothesized to be less compromising to antibody immunoreactivity. NR-LU-10 was radioiodinated using p-aryltin phenylate NHS ester (PIP-NHS) and either ^{125}I or ^{131}I sodium iodide. Radioiodinated NR-LU-10 was incubated with 25 mM dithiothreitol and purified using size exclusion chromatography. The reduced antibody (containing free thiol groups) was then reacted with a 10- to 100-fold molar excess of N-iodoacetyl-n'-biotinyl hexylene diamine in phosphate-buffered saline (PBS), pH 7.5, containing 5% DMSO (v/v).

TABLE 3

Effect of Thiol Biotinylation on Immunoreactivity

Molar Offering (Biotins/Ab)	Measured Derivitization (Biotins/Ab)	Immunoassessment (%) ELISA	Cell Binding
10:1	4.7	114	
50:1	6.5	102	100
100:1	6.1	95	100

As shown in Table 3, at a 50:1 or greater biotin:antibody molar offering, only 6 biotins per antibody were incorporated. No significant impact on immunoreactivity was observed.

The lysine- and thiol-derivitized biotinylated antibodies ("antibody (lysine)" and "antibody (thiol)", respectively) were compared. Molecular sizing on size exclusion FPLC demonstrated that both biotinylation protocols yielded monomolecular (monomeric) IgGs. Biotinylated antibody (lysine) had an apparent molecular weight of 160 kD, while biotinylated antibody (thiol) had an apparent molecular weight of 180 kD. Reduction of endogenous sulfhydryls (i.e., disulfides) to thiol groups,

followed by conjugation with biotin, may produce a somewhat unfolded macromolecule. If so, the antibody (thiol) may display a larger hydrodynamic radius and exhibit an apparent increase in molecular weight by chromatographic analysis. Both biotinylated antibody species exhibited 98% specific binding to immobilized avidin-agarose.

Further comparison of the biotinylated antibody species was performed using non-reducing SDS-PAGE, using a 4% stacking gel and a 5% resolving gel. Biotinylated samples were either radiolabeled or unlabeled and were combined with either radiolabeled or unlabeled avidin or streptavidin. Samples were not boiled prior to SDS-PAGE analysis. The native antibody and biotinylated antibody (lysine) showed similar migrations; the biotinylated antibody (thiol) produced two species in the 50-75 kD range. These species may represent two thiol-capped species. Under these SDS-PAGE conditions, radiolabeled streptavidin migrates as a 60 kD tetramer. When 400 μg/ml radiolabeled streptavidin was combined with 50 μg/ml biotinylated antibody (analogous to "sandwiching" conditions *in vivo*), both antibody species formed large molecular weight complexes. However, only the biotinylated antibody (thiol)-streptavidin complex moved from the stacking gel into the resolving gel, indicating a decreased molecular weight as compared to the biotinylated antibody (lysine)-streptavidin complex.

B. Blood Clearance of Biotinylated Antibody Species

Radioiodinated biotinylated NR-LU-10 (lysine or thiol) was intravenously administered to non-tumored nude mice at a dose of 100 μg. At 24 h post-administration of radioiodinated biotinylated NR-LU-10, mice were intravenously injected with either saline or 400 μg of avidin. With saline

administration, blood clearances for both biotinylated antibody species were biphasic and similar to the clearance of native NR-LU-10 antibody.

In the animals that received avidin intravenously at 24 h, the biotinylated antibody (lysine) was cleared (to a level of 5% of injected dose) within 15 min of avidin administration (avidin:biotin = 10:1). With the biotinylated antibody (thiol), avidin administration (10:1 or 25:1) reduced the circulating antibody level to about 35% of injected dose after two hours. Residual radiolabeled antibody activity in the circulation after avidin administration was examined *in vitro* using immobilized biotin. This analysis revealed that 85% of the biotinylated antibody was complexed with avidin. These data suggest that the biotinylated antibody (thiol)-avidin complexes that were formed were insufficiently crosslinked to be cleared by the RES.

Blood clearance and biodistribution studies of biotinylated antibody (lysine) 2 h post-avidin or post-saline administration were performed. Avidin administration significantly reduced the level of biotinylated antibody in the blood (see Figure 1), and increased the level of biotinylated antibody in the liver and spleen. Kidney levels of biotinylated antibody were similar.

Example V

In vivo Characterization of ^{186}Re-Chelate-Biotin Conjugates In a Three-Step Pretargeting Protocol

A ^{186}Re-chelate-biotin conjugate of Example I (MW ≈ 1000; specific activity = 1-2 mCi/mg) was examined in a three-step pretargeting protocol in an animal model. More specifically, 18-22 g female nude mice were implanted subcutaneously with LS-180 human colon tumor xenografts, yielding 100-200 mg tumors within 10 days of implantation.

NR-LU-10 antibody (MW ~ 150 kD) was radiolabeled with ^{125}I/Chloramine T and biotinylated via lysine residues (as described in Example IV.A, above). Avidin (MW ~ 66 kD) was radiolabeled with ^{131}I/PIP-NHS (as described for radioiodination of NR-LU-10 in Example IV.A., above). The experimental protocol was as follows:

Group 1:	**Time 0**, inject 100 μg ^{125}I-labeled, biotinylated NR-LU-10 **Time 24 h**, inject 400 μg ^{131}I-labeled avidin **Time 26 h**, inject 60 μg ^{186}Re-chelate-biotin conjugate
Group 2: (control)	**Time 0**, inject 400 μg ^{131}I-labeled avidin **Time 2 h**, inject 60 μg ^{186}Re-chelate-biotin conjugate
Group 3: (control)	**Time 0**, inject 60 μg ^{186}Re-chelate-biotin conjugate

The three radiolabels employed in this protocol are capable of detection in the presence of each other. It is also noteworthy that the sizes of the three elements involved are logarithmically different -- antibody ≅ 150,000; avidin ≅ 66,000; and biotin ≅ 1,000. Biodistribution analyses were performed at 2, 6, 24, 72 and 120 h after administration of the ^{186}Re-chelate-biotin conjugate.

Certain preliminary studies were performed in the animal model prior to analyzing the ^{186}Re-chelate-biotin conjugate in a three-step pretargeting protocol. First, the effect of biotinylated antibody on blood clearance of avidin was examined. These experiments showed that the rate and extent of avidin clearance was similar in the presence or absence of biotinylated antibody. Second, the effect of biotinylated antibody and avidin on blood clearance of the ^{186}Re-chelate-biotin conjugate was examined; blood clearance was similar in the presence or absence of

biotinylated antibody and avidin. Further, antibody immunoreactivity was found to be uncompromised by biotinylation at the level tested.

Third, tumor uptake of biotinylated antibody administered at time 0 or of avidin administered at time 24 h was examined. The results of this experimentation are shown in Fig. 1. At 25 h, about 350 pmol/g biotinylated antibody was present at the tumor; at 32 h the level was about 300 pmol/g; at 48 h, about 200 pmol/g; and at 120 h, about 100 pmol/g. Avidin uptake at the same time points was about 250, 150, 50 and 0 pmol/g, respectively. From the same experiment, tumor to blood ratios were determined for biotinylated antibody and for avidin. From 32 h to 120 h, the ratios of tumor to blood were very similar.

Rapid and efficient removal of biotinylated antibody from the blood by complexation with avidin was observed. Within two hours of avidin administration, a 10-fold reduction in blood pool antibody concentration was noted (Fig. 1), resulting in a sharp increase in tumor to blood ratios. Avidin is cleared rapidly, with greater than 90% of the injected dose cleared from the blood within 1 hour after administration. The Re-186-biotin chelate is also very rapidly cleared, with greater than 99% of the injected dose cleared from the blood by 1 hour after administration.

The three-step pretargeting protocol (described for Group 1, above) was then examined. More specifically, tumor uptake of the ^{186}Re-chelate-biotin conjugate in the presence or absence of biotinylated antibody and avidin was determined. In the absence of biotinylated antibody and avidin, the ^{186}Re-chelate-biotin conjugate displayed a slight peak 2 h post-injection, which was substantially cleared from the tumor by about 5 h. In contrast, at 2 h post-injection in the presence of biotinylated antibody and

avidin (specific), the ^{186}Re-chelate-biotin conjugate reached a peak in tumor approximately 7 times greater than that observed in the absence of biotinylated antibody and avidin. Further, the specifically bound ^{186}Re-chelate-biotin conjugate was retained at the tumor at significant levels for more than 50 h. Tumor to blood ratios determined in the same experiment increased significantly over time (i.e., T:B = ~ 8 at 30 h; ~ 15 at 100 h; ~ 35 at 140 h).

Tumor uptake of the ^{186}Re-chelate-biotin conjugate has further been shown to be dependent on the dose of biotinylated antibody administered. At 0 μg of biotinylated antibody, about 200 pmol/g of ^{186}Re-chelate-biotin conjugate was present at the tumor at 2 h after administration; at 50 μg antibody, about 500 pmol/g of ^{186}Re-chelate-biotin conjugate; and at 100 μg antibody, about 1,300 pmol/g of ^{186}Re-chelate-biotin conjugate.

Rhenium tumor uptake via the three-step pretargeting protocol was compared to tumor uptake of the same antibody radiolabeled through chelate covalently attached to the antibody (conventional procedure). The results of this comparison are depicted in Figure 2. Blood clearance and tumor uptake were compared for the chelate directly labeled rhenium antibody conjugate and for the three-step pretargeted sandwich. Areas under the curves (AUC) and the ratio of AUC_{tumor}/AUC_{blood} were determined. For the chelate directly labeled rhenium antibody conjugate, the ratio of AUC_{tumor}/AUC_{blood} = 24055/10235 or 2.35; for the three-step pretargeted sandwich, the ratio of AUC_{tumor}/AUC_{blood} = 46764/6555 or 7.13.

Tumor uptake results are best taken in context with radioactivity exposure to the blood compartment, which directly correlates with bone marrow exposure. Despite the fact that 100-fold more rhenium was administered to animals in the three-step protocol,

the very rapid clearance of the small molecule (Re-186-biotin) from the blood minimizes the exposure to Re-186 given in this manner. In the same matched antibody dose format, direct labeled (conventional procedure) NR-LU-10 whole antibody yielded greater exposure to rhenium than did the 100-fold higher dose given in the three-step protocol. A clear increase in the targeting ratio (tumor exposure to radioactivity:blood exposure to radioactivity--AUC_{tumor}:AUC_{blood}) was observed for three-step pretargeting (approximately 7:1) in comparison to the direct labeled antibody approach (approximately 2.4:1).

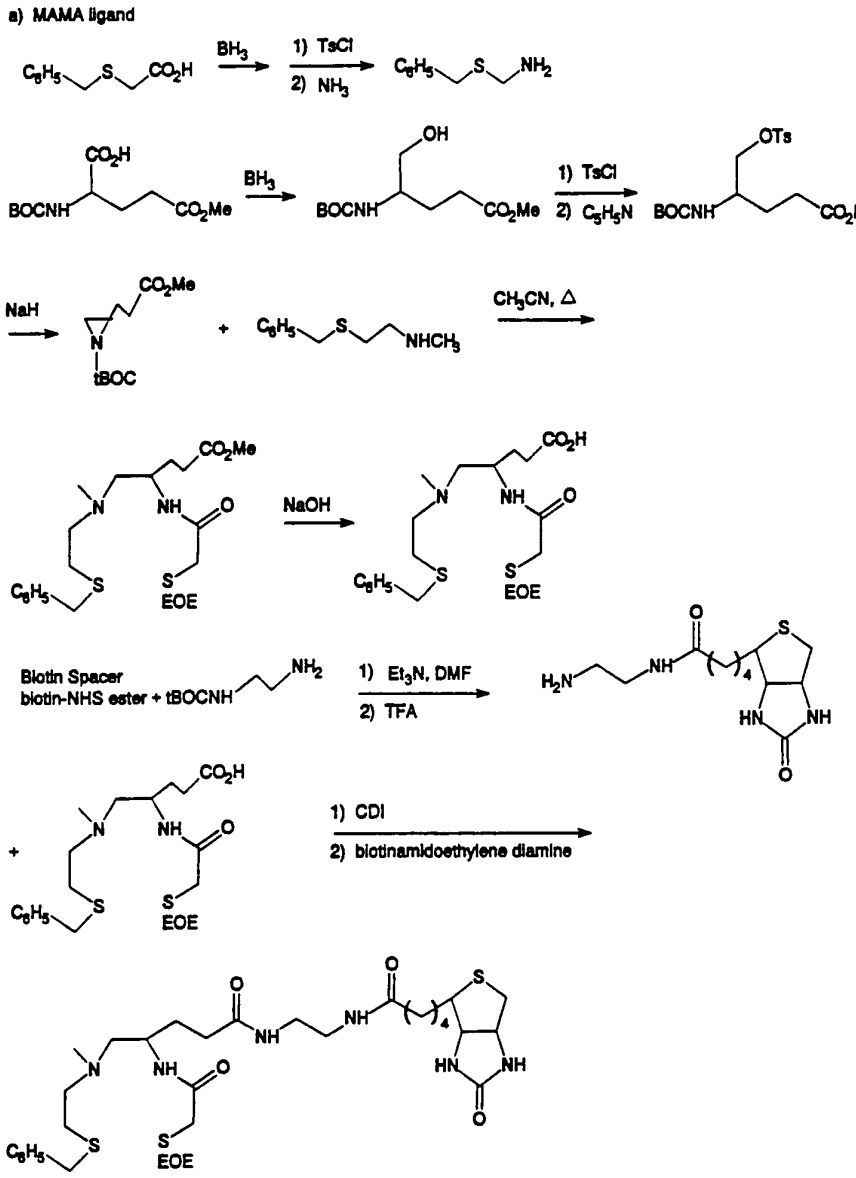
Example VI

Preparation of Chelate-Biotin Conjugates Having Improved Biodistribution Properties

The biodistribution of ^{111}In-labeled-biotin derivatives varies greatly with structural changes in the chelate and the conjugating group. Similar structural changes may affect the biodistribution of technetium- and rhenium-biotin conjugates. Accordingly, methods for preparing technetium- and rhenium-biotin conjugates having optimal clearance from normal tissue are advantageous.

A. Neutral MAMA Chelate/Conjugate

A neutral MAMA chelate-biotin conjugate is prepared according to the following scheme.

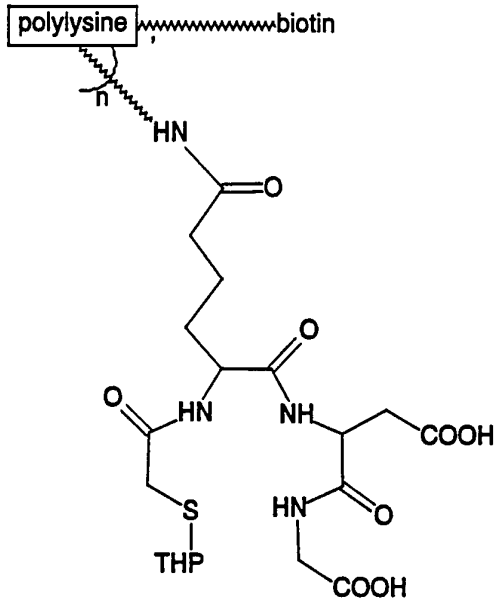


The resultant chelate-biotin conjugate shows superior kidney excretion. Although the net overall charge of the conjugate is neutral, the polycarboxylate nature of the molecule generates regions of hydrophilicity and hydrophobicity. By altering the number and nature of the carboxylate groups within the conjugate, excretion may be shifted from kidney to gastrointestinal routes. For instance, neutral compounds are generally cleared by the kidneys;

anionic compounds are generally cleared through the GI system.

B. Polylysine Derivitization

Conjugates containing polylysine may also exhibit beneficial biodistribution properties. With whole antibodies, derivitization with polylysine may skew the biodistribution of conjugate toward liver uptake. In contrast, derivitization of Fab fragments with polylysine results in lower levels of both liver and kidney uptake; blood clearance of these conjugates is similar to that of Fab covalently linked to chelate. An exemplary polylysine derivitized chelate-biotin conjugate is illustrated below.



Inclusion of polylysine in radiometal-chelate-biotin conjugates is therefore useful for minimizing or eliminating RES sequestration while maintaining good liver and kidney clearance of the conjugate. For improved renal excretion properties, polylysine derivatives are preferably succinylated following biotinylation. Polylysine derivatives offer the

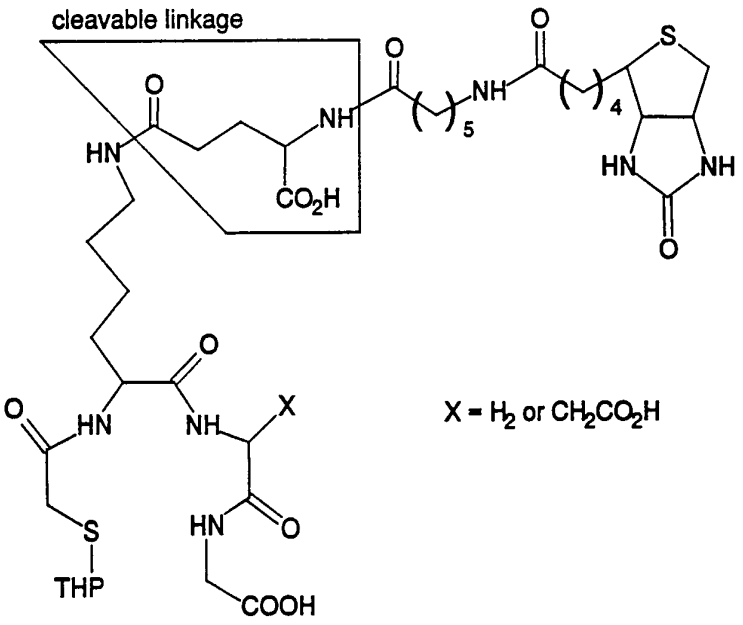
further advantages of: (1) increasing the specific activity of the radiometal-chelate-biotin conjugate; (2) permitting control of rate and route of blood clearance by varying the molecular weight of the polylysine polymer; and (3) increasing the circulation half-life of the conjugate for optimal tumor interaction.

Polylysine derivitization is accomplished by standard methodologies. Briefly, poly-L-lysine is acylated according to standard amino group acylation procedures (aqueous bicarbonate buffer, pH 8, added biotin-NHS ester, followed by chelate NHS ester). Alternative methodology involves anhydrous conditions using nitrophenyl esters in DMSO and triethyl amine. The resultant conjugates are characterized by UV and NMR spectra.

The number of biotins attached to polylysine is determined by the HABA assay. Spectrophotometric titration is used to assess the extent of amino group derivitization. The radiometal-chelate-biotin conjugate is characterized by size exclusion.

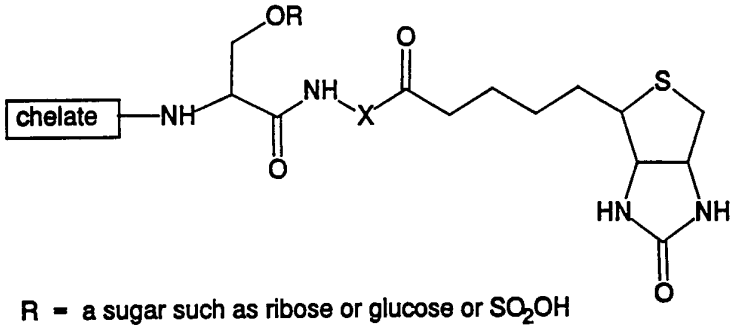
C. Cleavable Linkage

Through insertion of a cleavable linker between the chelate and biotin portion of a radiometal-chelate-biotin conjugate, retention of the conjugate at the tumor relative to normal tissue may be enhanced. More specifically, linkers that are cleaved by enzymes present in normal tissue but deficient or absent in tumor tissue can increase tumor retention. As an example, the kidney has high levels of γ-glutamyl transferase; other normal tissues exhibit *in vivo* cleavage of γ-glutamyl prodrugs. In contrast, tumors are generally deficient in enzyme peptidases. The glutamyl-linked biotin conjugate depicted below is cleaved in normal tissue and retained in the tumor.



D. Serine Linker With O-Polar Substituent

Sugar substitution of N_3S chelates renders such chelates water soluble. Sulfonates, which are fully ionized at physiological pH, improve water solubility of the chelate-biotin conjugate depicted below.



This compound is synthesized according to the standard reaction procedures. Briefly, biocytin is condensed with N-t-BOC-(O-sulfonate or O-glucose) serine NHS ester to give N-t-BOC-(O-sulfonate or O-glucose) serine biocytinamide. Subsequent cleavage of the N-t-

BOC group with TFA and condensation with ligand NHS ester in DMF with triethylamine provides ligand-amidoserine(O-sulfonate or O-glucose)biocytinamide.

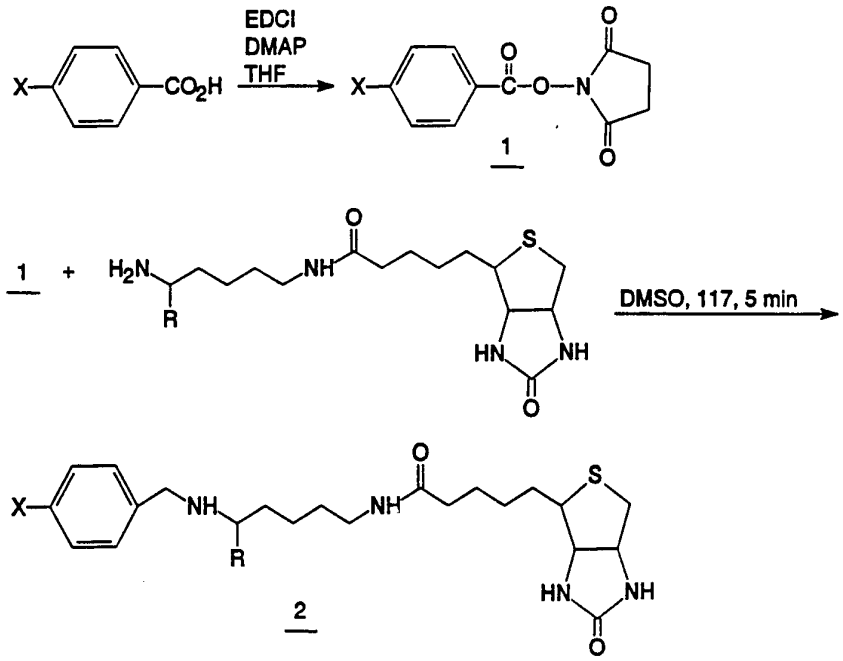
Example VII

Preparation and Characterization of PIP-Radioiodinated Biotin

Radioiodinated biotin derivatives prepared by exposure of poly-L-lysine to excess NHS-LC-biotin and then to Bolton-Hunter N-hydroxysuccinimide esters in DMSO has been reported. After purification, this product was radiolabeled by the iodogen method (see, for instance, Del Rosario et al., J. Nucl. Med., 32:5, 1991, 993 (abstr.)). Because of the high molecular weight of the resultant radioiodinated biotin derivative, only limited characterization of product (i.e., radio-HPLC and binding to immobilized streptavidin) was possible.

Preparation of radioiodinated biotin according to the present invention provides certain advantages. First, the radioiodobiotin derivative is a low molecular weight compound that is amenable to complete chemical characterization. Second, the disclosed methods for preparation involve a single step and eliminate the need for a purification step.

Briefly, iodobenzamide derivatives corresponding to biocytin (R = COOH) and biotinamidopentylamine (R = H) were prepared according to the following scheme. In this scheme, "X" may be any radiohalogen, including ^{125}I, ^{131}I, ^{123}I, ^{211}At and the like.



Preparation of 1 was generally according to Wilbur et al., J. Nucl. Med., 30:216-26, 1989, using a tributyltin intermediate. Water soluble carbodiimide was used in the above-depicted reaction, since the NHS ester 1 formed intractable mixtures with DCU. The NHS ester was not compatible with chromatography; it was insoluble in organic and aqueous solvents and did not react with biocytin in DMF or in buffered aqueous acetonitrile. The reaction between 1 and biocytin or 5-(biotinamido) pentylamine was sensitive to base. When the reaction of 1 and biocytin or the pentylamine was performed in the presence of triethylamine in hot DMSO, formation of more than one biotinylated product resulted. In contrast, the reaction was extremely clean and complete when a suspension of 1 and biocytin (4 mg/ml) or the pentylamine (4 mg/ml) was heated in DMSO at 117°C for about 5 to about 10 min. The resultant ^{125}I-biotin derivatives were obtained in 94% radiochemical yield. Optionally, the radioiodinated products may be purified using C-18 HPLC and a reverse

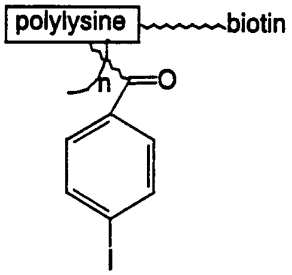
phase hydrophobic column. Hereinafter, the resultant radioiodinated products 2 are referred to as PIP-biocytin (R = COOH) and PIP-pentylamine (R = H).

Both iodobiotin derivatives 2 exhibited ≥95% binding to immobilized avidin. Incubation of the products 2 with mouse serum resulted in no loss of the ability of 2 to bind to immobilized avidin. Biodistribution studies of 2 in male BALB/c mice showed rapid clearance from the blood (similar to ^{186}Re-chelate-biotin conjugates described above). The radioiodobiotin 2 had decreased hepatobiliary excretion as compared to the ^{186}Re-chelate-biotin conjugate; urinary excretion was increased as compared to the ^{186}Re-chelate-biotin conjugate. Analysis of urinary metabolites of 2 indicated deiodination and cleavage of the biotin amide bond; the metabolites showed no binding to immobilized avidin. In contrast, metabolites of the ^{186}Re-chelate-biotin conjugate appear to be excreted in urine as intact biotin conjugates. Intestinal uptake of 2 is <50% that of the ^{186}Re-chelate-biotin conjugate. These biodistribution properties of 2 provided enhanced whole body clearance of radioisotope and indicate the advantageous use of 2 within pretargeting protocols.

^{131}I-PIP-biocytin was evaluated in a two-step pretargeting procedure in tumor-bearing mice. Briefly, female nude mice were injected subcutaneously with LS-180 tumor cells; after 7 d, the mice displayed 50-100 mg tumor xenografts. At t = 0, the mice were injected with 200 μg of NR-LU-10-avidin conjugate labeled with ^{125}I using PIP-NHS (see Example IV.A.). At t = 36 h, the mice received 42 μg of ^{131}I-PIP-biocytin. The data showed immediate, specific tumor localization, corresponding to ≈ 1.5 ^{131}I-PIP-biocytin molecules per avidin molecule.

The described radiohalogenated biotin compounds are amenable to the same types of modifications

described in Example VI above for ^{186}Re-chelate-biotin conjugates. In particular, the following PIP-polylysine-biotin molecule is made by trace labeling polylysine with ^{125}I-PIP, followed by extensive biotinylation of the polylysine.



Assessment of ^{125}I binding to immobilized avidin ensures that all radioiodinated species also contain at least an equivalent of biotin.

Example VIII

Preparation of Biotinylated Antibody (Thiol) Through Endogenous Antibody Sulfhydryl Groups Or Sulfhydryl-Generating Compounds

Certain antibodies have available for reaction endogenous sulfhydryl groups. If the antibody to be biotinylated contains endogenous sulfhydryl groups, such antibody is reacted with N-iodoacetyl-n'-biotinyl hexylene diamine (as described in Example IV.A., above). The availability of one or more endogenous sulfhydryl groups obviates the need to expose the antibody to a reducing agent, such as DTT, which can have other detrimental effects on the biotinylated antibody.

Alternatively, one or more sulfhydryl groups are attached to a targeting moiety through the use of chemical compounds or linkers that contain a terminal sulfhydryl group. An exemplary compound for this purpose is iminothiolane. As with endogenous sulfhydryl groups (discussed above), the detrimental

effects of reducing agents on antibody are thereby avoided.

Example IX

Two-Step Pretargeting Methodology That Does Not Induce Internalization

A NR-LU-13-avidin conjugate is prepared as follows. Initially, avidin is derivitized with N-succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC). SMCC-derived avidin is then incubated with NR-LU-13 in a 1:1 molar ratio at pH 8.5 for 16 h. Unreacted NR-LU-13 and SMCC-derived avidin are removed from the mixture using preparative size exclusion HPLC. Two conjugates are obtained as products -- the desired 1:1 NR-LU-13-avidin conjugate as the major product; and an incompletely characterized component as the minor product.

A ^{99m}Tc-chelate-biotin conjugate is prepared as in Example II, above. The NR-LU-13-avidin conjugate is administered to a recipient and allowed to clear from the circulation. One of ordinary skill in the art of radioimmunoscintigraphy is readily able to determine the optimal time for NR-LU-13-avidin conjugate tumor localization and clearance from the circulation. At such time, the ^{99m}Tc-chelate-biotin conjugate is administered to the recipient. Because the ^{99m}Tc-chelate-biotin conjugate has a molecular weight of ≈ 1,000, crosslinking of NR-LU-13-avidin molecules on the surface of the tumor cells is dramatically reduced or eliminated. As a result, the ^{99m}Tc diagnostic agent is retained at the tumor cell surface for an extended period of time. Accordingly, detection of the diagnostic agent by imaging techniques is optimized; further, a lower dose of radioisotope provides an image comparable to that resulting from the typical three-step pretargeting protocol.

Optionally, clearance of NR-LU-13-avidin from the circulation may be accelerated by plasmapheresis in

combination with a biotin affinity column. Through use of such column, circulating NR-LU-13-avidin will be retained extracorporeally, and the recipient's immune system exposure to a large, proteinaceous immunogen (i.e., avidin) is minimized.

Exemplary methodology for plasmapheresis/column purification useful in the practice of the present invention is discussed in the context of reducing radiolabeled antibody titer in imaging and in treating tumor target sites in U.S. Patent 5,078,673. Briefly, for the purposes of the present invention, an example of an extracorporeal clearance methodology may include the following steps:

administering a ligand- or anti-ligand-targeting moiety conjugate to a recipient;

after a time sufficient for localization of the administered conjugate to the target site, withdrawing blood from the recipient by, for example, plasmapheresis;

separating cellular element from said blood to produce a serum fraction and returning the cellular elements to the recipient; and

reducing the titer of the administered conjugate in the serum fraction to produce purified serum;

infusing the purified serum back into the recipient.

Clearance of NR-LU-13-avidin is also facilitated by administration of a particulate-type clearing agent (e.g., a polymeric particle having a plurality of biotin molecules bound thereto). Such a particulate clearing agent preferably constitutes a biodegradable polymeric carrier having a plurality of biotin molecules bound thereto. Particulate clearing agents of the present invention exhibit the capability of binding to circulating administered conjugate and removing that conjugate from the recipient. Particulate clearing agents of this aspect of the

present invention may be of any configuration suitable for this purpose. Preferred particulate clearing agents exhibit one or more of the following characteristics:

- microparticulate (e.g., from about 0.5 micrometers to about 100 micrometers in diameter, with from about 0.5 to about 2 micrometers more preferred), free flowing powder structure;

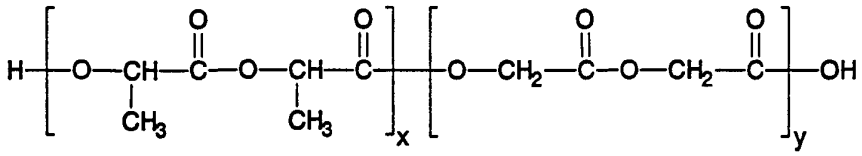
- biodegradable structure designed to biodegrade over a period of time between from about 3 to about 180 days, with from about 10 to about 21 days more preferred, or non-biodegradable structure;

- biocompatible with the recipients physiology over the course of distribution, metabolism and excretion of the clearing agent, more preferably including biocompatible biodegradation products;

- and capability to bind with one or more circulating conjugates to facilitate the elimination or removal thereof from the recipient through one or more binding moieties (preferably, the complementary member of the ligand/anti-ligand pair). The total molar binding capacity of the particulate clearing agents depends upon the particle size selected and the ligand or anti-ligand substitution ratio. The binding moieties are capable of coupling to the surface structure of the particulate dosage form through covalent or non-covalent modalities as set forth herein to provide accessible ligand or anti-ligand for binding to its previously administered circulating binding pair member.

Preferable particulate clearing agents of the present invention are biodegradable or non-biodegradable microparticulates. More preferably, the particulate clearing agents are formed of a polymer containing matrix that biodegrades by random, nonenzymatic, hydrolytic scissioning.

Polymers derived from the condensation of alpha hydroxycarboxylic acids and related lactones are more preferred for use in the present invention. A particularly preferred moiety is formed of a mixture of thermoplastic polyesters (e.g., polylactide or polyglycolide) or a copolymer of lactide and glycolide components, such as poly(lactide-co-glycolide). An exemplary structure, a random poly(DL-lactide-co-glycolide), is shown below, with the values of x and y being manipulable by a practitioner in the art to achieve desirable microparticulate properties.



Other agents suitable for forming particulate clearing agents of the present invention include polyorthoesters and polyacetals (Polymer Letters, 18:293, 1980) and polyorthocarbonates (U.S. Patent No. 4,093,709) and the like.

Preferred lactic acid/glycolic acid polymer containing matrix particulates of the present invention are prepared by emulsion-based processes, that constitute modified solvent extraction processes such as those described by Cowsar et al., "Poly(Lactide-Co-Glycolide) Microcapsules for Controlled Release of Steroids," Methods Enzymology, 112:101-116, 1985 (steroid entrapment in microparticulates); Eldridge et al., "Biodegradable and Biocompatible Poly(DL-Lactide-Co-Glycolide) Microspheres as an Adjuvant for Staphylococcal Enterotoxin B Toxoid Which Enhances the Level of Toxin-Neutralizing Antibodies," Infection and Immunity, 59:2978-2986, 1991 (toxoid entrapment);

Cohen et al., "Controlled Delivery Systems for Proteins Based on Poly(Lactic/Glycolic Acid) Microspheres," Pharmaceutical Research, 8(6):713-720, 1991 (enzyme entrapment); and Sanders et al., "Controlled Release of a Luteinizing Hormone-Releasing Hormone Analogue from Poly(D,L-Lactide-Co-Glycolide) Microspheres," J. Pharmaceutical Science, 73(9):1294-1297, 1984 (peptide entrapment).

In general, the procedure for forming particulate clearing agents of the present invention involves dissolving the polymer in a halogenated hydrocarbon solvent and adding an additional agent that acts as a solvent for the halogenated hydrocarbon solvent but not for the polymer. The polymer precipitates out from the polymer-halogenated hydrocarbon solution. Following particulate formation, they are washed and hardened with an organic solvent. Water washing and aqueous non-ionic surfactant washing steps follow, prior to drying at room temperature under vacuum.

For biocompatibility purposes, particulate clearing agents are sterilized prior to packaging, storage or administration. Sterilization may be conducted in any convenient manner therefor. For example, the particulates can be irradiated with gamma radiation, provided that exposure to such radiation does not adversely impact the structure or function of the binding moiety attached thereto. If the binding moiety is so adversely impacted, the particulate clearing agents can be produced under sterile conditions.

The preferred lactide/glycolide structure is biocompatible with the mammalian physiological environment. Also, these preferred sustained release dosage forms have the advantage that biodegradation thereof forms lactic acid and glycolic acid, both normal metabolic products of mammals.

Functional groups required for binding moiety - particulate bonding, are optionally included in the particulate structure, along with the non-degradable or biodegradable polymeric units. Functional groups that are exploitable for this purpose include those that are reactive with ligands or anti-ligands, such as carboxyl groups, amine groups, sulfhydryl groups and the like. Preferred binding enhancement moieties include the terminal carboxyl groups of the preferred (lactide-glycolide) polymer containing matrix or the like. A practitioner in the art is capable of selecting appropriate functional groups and monitoring conjugation reactions involving those functional groups.

Advantages garnered through the use of particulate clearing agents of the type described above are as follows:

- particles in the "micron" size range localize in the RES and liver, with galactose derivatization or charge modification enhancement methods for this capability available, and, preferably, are designed to remain in circulation for a time sufficient to perform the clearance function;

- the size of the particulates facilitates central vascular compartment retention thereof, substantially precluding equilibration into the peripheral or extravascular compartment;

- desired substituents for ligand or anti-ligand binding to the particulates can be introduced into the polymeric structure;

- ligand- or anti-ligand-particulate linkages having desired properties (e.g., serum biotinidase resistance thereby reducing the release of biotin metabolite from a particle-biotin clearing agent) and

- multiple ligands or anti-ligands can be bound to the particles to achieve optimal cross-linking of circulating targeting agent-ligand or -anti-ligand

conjugate and efficient clearance of cross-linked species. This advantage is best achieved when care is taken to prevent particulate aggregation both in storage and upon *in vivo* administration.

Clearance of NR-LU-13-avidin may also be accelerated by an arterially inserted proteinaceous or polymeric multiloop device. A catheter-like device, consisting of thin loops of synthetic polymer or protein fibers derivitized with biotin, is inserted into a major artery (e.g., femoral artery) to capture NR-LU-13-avidin. Since the total blood volume passes through a major artery every 70 seconds, the *in situ* clearing device is effective to reduce circulating NR-LU-13-avidin within a short period of time. This device offers the advantages that NR-LU-13-avidin is not processed through the RES; removal of NR-LU-13-avidin is controllable and measurable; and fresh devices with undiminished binding capacity are insertable as necessary. This methodology is also useful with intraarterial administration embodiments of the present invention.

An alternative procedure for clearing NR-LU-13-avidin from the circulation without induction of internalization involves administration of biotinylated, high molecular weight molecules, such as liposomes, IgM and other molecules that are size excluded from ready permeability to tumor sites. When such biotinylated, high molecular weight molecules aggregate with NR-LU-13-avidin, the aggregated complexes are readily cleared from the circulation via the RES.

Example X

Enhancement of Therapeutic Agent Internalization Through Avidin Crosslinking

The ability of multivalent avidin to crosslink two or more biotin molecules (or chelate-biotin conjugates) is advantageously used to improve delivery

of therapeutic agents. More specifically, avidin crosslinking induces internalization of crosslinked complexes at the target cell surface.

Biotinylated NR-CO-04 (lysine) is prepared according to the methods described in Example IV.A., above. Doxorubicin-avidin conjugates are prepared by standard conjugation chemistry. The biotinylated NR-CO-04 is administered to a recipient and allowed to clear from the circulation. One of ordinary skill in the art of radioimmunotherapy is readily able to determine the optimal time for biotinylated NR-CO-04 tumor localization and clearance from the circulation. At such time, the doxorubicin-avidin conjugate is administered to the recipient. The avidin portion of the doxorubicin-avidin conjugate crosslinks the biotinylated NR-CO-04 on the cell surface, inducing internalization of the complex. Thus, doxorubicin is more efficiently delivered to the target cell.

In a first alternative protocol, a standard three-step pretargeting methodology is used to enhance intracellular delivery of a drug to a tumor target cell. By analogy to the description above, biotinylated NR-LU-05 is administered, followed by avidin (for blood clearance and to form the middle layer of the sandwich at the target cell-bound biotinylated antibody). Shortly thereafter, and prior to internalization of the biotinylated NR-LU-05-avidin complex, a methotrexate-biotin conjugate is administered.

In a second alternative protocol, biotinylated NR-LU-05 is further covalently linked to methotrexate. Subsequent administration of avidin induces internalization of the complex and enhances intracellular delivery of drug to the tumor target cell.

In a third alternative protocol, NR-CO-04-avidin is administered to a recipient and allowed to clear

from the circulation and localize at the target site. Thereafter, a polybiotinylated species (such as biotinylated poly-L-lysine, as in Example IV.B., above) is administered. In this protocol, the drug to be delivered may be covalently attached to either the antibody-avidin component or to the polybiotinylated species. The polybiotinylated species induces internalization of the (drug)-antibody-avidin-polybiotin-(drug) complex.

Example XI

Targeting Moiety-Anti-Ligand Conjugate for Two-Step Pretargeting *in vivo*

A. Preparation of SMCC-derivitized streptavidin.

31 mg (0.48 μmol) streptavidin was dissolved in 9.0 ml PBS to prepare a final solution at 3.5 mg/ml. The pH of the solution was adjusted to 8.5 by addition of 0.9 ml of 0.5 M borate buffer, pH 8.5. A DMSO solution of SMCC (3.5 mg/ml) was prepared, and 477 μl (4.8 μmol) of this solution was added dropwise to the vortexing protein solution. After 30 minutes of stirring, the solution was purified by G-25 (PD-10, Pharmacia, Piscataway, New Jersey) column chromatography to remove unreacted or hydrolyzed SMCC. The purified SMCC-derivitized streptavidin was isolated (28 mg, 1.67 mg/ml).

B. Preparation of DTT-reduced NR-LU-10. To 77 mg NR-LU-10 (0.42 μmol) in 15.0 ml PBS was added 1.5 ml of 0.5 M borate buffer, pH 8.5. A DTT solution, at 400 mg/ml (165 μl) was added to the protein solution. After stirring at room temperature for 30 minutes, the reduced antibody was purified by G-25 size exclusion chromatography. Purified DTT-reduced NR-LU-10 was obtained (74 mg, 2.17 mg/ml).

C. Conjugation of SMCC-streptavidin to DTT-reduced NR-LU-10. DTT-reduced NR-LU-10 (63 mg, 29 ml, 0.42 μmol) was diluted with 44.5 ml PBS. The solution

of SMCC-streptavidin (28 mg, 17 ml, 0.42 μmol) was added rapidly to the stirring solution of NR-LU-10. Total protein concentration in the reaction mixture was 1.0 mg/ml. The progress of the reaction was monitored by HPLC (Zorbax® GF-250, available from MacMod). After approximately 45 minutes, the reaction was quenched by adding solid sodium tetrathionate to a final concentration of 5 mM.

D. Purification of conjugate. For small scale reactions, monosubstitutedor disubstituted (with regard to streptavidin) conjugate was obtained using HPLC Zorbax (preparative) size exclusion chromatography. The desired monosubstituted or disubstituted conjugate product eluted at 14.0-14.5 min (3.0 ml/min flow rate), while unreacted NR-LU-10 eluted at 14.5-15 min and unreacted derivitized streptavidin eluted at 19-20 min.

For larger scale conjugation reactions, monosubstituted or disubstituted adduct is isolatable using DEAE ion exchange chromatography. After concentration of the crude conjugate mixture, free streptavidin was removed therefrom by eluting the column with 2.5% xylitol in sodium borate buffer, pH 8.6. The bound unreacted antibody and desired conjugate were then sequentially eluted from the column using an increasing salt gradient in 20 mM diethanolamine adjusted to pH 8.6 with sodium hydroxide.

E. Characterization of Conjugate.

1. HPLC size exclusion was conducted as described above with respect to small scale purification.

2. SDS-PAGE analysis was performed using 5% polyacrylamide gels under non-denaturing conditions. Conjugates to be evaluated were not boiled in sample buffer containing SDS to avoid dissociation of streptavidin into its 15 kD subunits. Two product

bands were observed on the gel, which correspond to the mono- and di- substituted conjugates.

3. Immunoreactivity was assessed, for example, by competitive binding ELISA as compared to free antibody. Values obtained were within 10% of those for the free antibody.

4. Biotin binding capacity was assessed, for example, by titrating a known quantity of conjugate with p-[I-125]iodobenzoylbiocytin. Saturation of the biotin binding sites was observed upon addition of 4 equivalences of the labeled biocytin.

5. *In vivo* studies are useful to characterize the reaction product, which studies include, for example, serum clearance profiles, ability of the conjugate to target antigen-positive tumors, tumor retention of the conjugate over time and the ability of a biotinylated molecule to bind streptavidin conjugate at the tumor. These data facilitate determination that the synthesis resulted in the formation of a 1:1 streptavidin-NR-LU-10 whole antibody conjugate that exhibits blood clearance properties similar to native NR-LU-10 whole antibody, and tumor uptake and retention properties at least equal to native NR-LU-10.

For example, Figure 3 depicts the tumor uptake profile of the NR-LU-10-streptavidin conjugate (LU-10-StrAv) in comparison to a control profile of native NR-LU-10 whole antibody. LU-10-StrAv was radiolabeled on the streptavidin component only, giving a clear indication that LU-10-StrAv localizes to target cells as efficiently as NR-LU-10 whole antibody itself.

Example XII

Two-Step Pretargeting *in vivo*

A ^{186}Re-chelate-biotin conjugate (Re-BT) of Example I (MW ~ 1000; specific activity = 1-2 mCi/mg) and a biotin-iodine-131 small molecule, PIP-Biocytin (PIP-

BT, MW approximately equal to 602; specific activity = 0.5-1.0 mCi/mg), as discussed in Example VII above, were examined in a three-step pretargeting protocol in an animal model, as described in Example V above. Like Re-BT, PIP-BT has the ability to bind well to avidin and is rapidly cleared from the blood, with a serum half-life of about 5 minutes. Equivalent results were observed for both molecules in the two-step pretargeting experiments described herein.

NR-LU-10 antibody (MW ~ 150 kD) was conjugated to streptavidin (MW ~ 66 kD) (as described in Example XI above) and radiolabeled with ^{125}I/PIP-NHS (as described for radioiodination of NR-LU-10 in Example IV.A., above). The experimental protocol was as follows:

Time 0 inject (i.v.) 200 μg NR-LU-10-StrAv conjugate;

Time 24-48 h inject (i.v.) 60-70 fold molar excess of radiolabeled biotinyl molecule ;

and perform biodistributions at 2, 6, 24, 72, 120 hours after injection of radiolabeled biotinyl molecule

NR-LU-10-streptavidin has shown very consistent patterns of blood clearance and tumor uptake in the LS-180 animal model. A representative profile is shown in Figure 4. When either PIP-BT or Re-BT is administered after allowing the LU-10-StrAv conjugate to localize to target cell sites for at least 24 hours, the tumor uptake of therapeutic radionuclide is high in both absolute amount and rapidity. For PIP-BT administered at 37 hours following LU-10-StrAv (I-125) administration, tumor uptake was above 500 pMOL/G at the 40 hour time point and peaked at about 700 pMOL/G at 45 hours post-LU-10-StrAv administration.

This almost instantaneous uptake of a small molecule therapeutic into tumor in stoichiometric amounts comparable to the antibody targeting moiety facilitates utilization of the therapeutic radionuclide at its highest specific activity. Also, the rapid clearance of radionuclide that is not bound to LU-10-StrAv conjugate permits an increased targeting ratio (tumor:blood) by eliminating the slow tumor accretion phase observed with directly labeled antibody conjugates. The pattern of radionuclide tumor retention is that of whole antibody, which is very persistent.

Experimentation using the two-step pretargeting approach and progressively lower molar doses of radiolabeled biotinyl molecule was also conducted. Uptake values of about 20% ID/G were achieved at no-carrier added (high specific activity) doses of radiolabeled biotinyl molecules. At less than saturating doses, circulating LU-10-StrAv was observed to bind significant amounts of administered radiolabeled biotinyl molecule in the blood compartment.

Example XIII

Asialoorosomucoid Clearing Agent and Two-Step Pretargeting

In order to maximize the targeting ratio (tumor:blood), clearing agents were sought that are capable of clearing the blood pool of targeting moiety-anti-ligand conjugate (e.g., LU-10-StrAv), without compromising the ligand binding capacity thereof at the target sites. One such agent, biotinylated asialoorosomucoid, which employs the avidin-biotin interaction to conjugate to circulating LU-10-StrAv, was tested.

A. Derivitization of orosomucoid. 10 mg human orosomucoid (Sigma N-9885) was dissolved in 3.5 ml of

pH 5.5 0.1 M sodium acetate buffer containing 160 mM NaCl. 70 μl of a 2% (w/v) CaCl solution in deionized (D.I.) water was added and 11 μl of neuraminidase (Sigma N-7885), 4.6 U/ml, was added. The mixture was incubated at 37°C for 2 hours, and the entire sample was exchanged over a Centricon-10® ultrafiltration device (available from Amicon, Danvers, Massachusetts) with 2 volumes of PBS. The asialoorosomucoid and orosomucoid starting material were radiolabeled with I-125 using PIP technology, as described in Example IV above.

The two radiolabeled preparations were injected i.v. into female BALB/c mice (20-25 g), and blood clearance was assessed by serial retro-orbital eye bleeding of each group of three mice at 5, 10, 15 and 30 minutes, as well as at 1, 2 and 4 hours post-administration. The results of this experiment are shown in Figure 5, with asialoorosomucoid clearing more rapidly than its orosomucoid counterpart.

In addition, two animals receiving each compound were sacrificed at 5 minutes post-administration and limited biodistributions were performed. These results are shown in Figure 6. The most striking aspects of these data are the differences in blood levels (78% for orosomucoid and 0.4% for asialoorosomucoid) and the specificity of uptake of asialoorosomucoid in the liver (86%), as opposed to other tissues.

B. Biotinylation of asialoorosomucoid clearing agent and orosomucoid control. 100 μl of 0.2 M sodium carbonate buffer, pH 9.2, was added to 2 mg (in 1.00 ml PBS) of PIP-125-labeled orosomucoid and to 2 mg PIP-125-labeled asialoorosomucoid. 60 μl of a 1.85 mg/ml solution of NHS-amino caproate biotin in DMSO was then added to each compound. The reaction mixtures were vortexed and allowed to sit at room temperature for 45 minutes. The material was purified

by size exclusion column chromatography (PD-10, Pharmacia) and eluted with PBS. 1.2 ml fractions were taken, with fractions 4 and 5 containing the majority of the applied radioactivity (>95%). Streptavidin-agarose beads (Sigma S-1638) or -pellets were washed with PBS, and 20 μg of each biotinylated, radiolabeled protein was added to 400 μl of beads and 400 μl of PBS, vortexed for 20 seconds and centrifuged at 14,000 rpm for 5 minutes. The supernatant was removed and the pellets were washed with 400 μl PBS. This wash procedure was repeated twice more, and the combined supernatants were assayed by placing them in a dosimeter versus their respective pellets. The values are shown below in Table 4.

TABLE 4

Compound	Supernatant	Pellet
orosomucoid	90%	10%
biotin-oroso	7.7%	92.%
asialoorosomucoid	92%	8.0%
biotin-asialo	10%	90%

C. Protein-Streptavidin Binding in vivo. Biotin-asialoorosomucoid was evaluated for the ability to couple with circulating LU-10-StrAv conjugate *in vivo* and to remove it from the blood. Female BALB/c mice (20-25 g) were injected i.v. with 200 μg LU-10-StrAv conjugate. Clearing agent (200 μl PBS - group 1; 400 μg non-biotinylated asialoorosomucoid - group 2; 400 μg biotinylated asialoorosomucoid - group 3; and 200 μg biotinylated asialoorosomucoid- group 4) was administered at 25 hours following conjugate administration. A fifth group received PIP-I-131-LU-10-StrAv conjugate which had been saturated prior to injection with biotin - group 5. The 400 μg dose constituted a 10:1 molar excess of clearing agent over the initial dose of LU-10-StrAv conjugate, while the

200 μg dose constituted a 5:1 molar excess. The saturated PIP-I-131-LU-10-StrAv conjugate was produced by addition of a 10-fold molar excess of D-biotin to 2 mg of LU-10-StrAv followed by size exclusion purification on a G-25 PD-10 column.

Three mice from each group were serially bled, as described above, at 0.17, 1, 4 and 25 hours (pre-injection of clearing agent), as well as at 27, 28, 47, 70 and 90 hours. Two additional animals from each group were sacrificed at 2 hours post-clearing agent administration and limited biodistributions were performed.

The blood clearance data are shown in Figure 7. These data indicate that circulating LU-10-StrAv radioactivity in groups 3 and 4 was rapidly and significantly reduced, in comparison to those values obtained in the control groups 1, 2 and 5. Absolute reduction in circulating antibody-streptavidin conjugate was approximately 75% when compared to controls.

Biodistribution data are shown in tabular form in Figure 8. The biodistribution data show reduced levels of conjugate for groups 3 and 4 in all tissues except the liver, kidney and intestine, which is consistent with the processing and excretion of radiolabel associated with the conjugate after complexation with biotinylated asialoorosomucoid.

Furthermore, residual circulating conjugate was obtained from serum samples by cardiac puncture (with the assays conducted in serum + PBS) and analyzed for the ability to bind biotin (immobilized biotin on agarose beads), an indicator of functional streptavidin remaining in the serum. Group 1 animal serum showed conjugate radiolabel bound about 80% to immobilized biotin. Correcting the residual circulating radiolabel values by multiplying the remaining percent injected dose (at 2 hours after

clearing agent administration) by the remaining percent able to bind immobilize biotin (the amount of remaining functional conjugate) leads to the graph shown in Figure 9. Administration of 200 μg biotinylated asialoorosomucoid resulted in a 50-fold reduction in serum biotin-binding capacity and, in preliminary studies in tumored animals, has not exhibited cross-linking and removal of prelocalized LU-10-StrAv conjugate from the tumor. Removal of circulating targeting moiety-anti-ligand without diminishing biotin-binding capacity at target cell sites, coupled with an increased radiation dose to the tumor resulting from an increase in the amount of targeting moiety-anti-ligand administered, results in both increased absolute rad dose to tumor and diminished toxicity to non-tumor cells, compared to what is currently achievable using conventional radioimmunotherapy.

A subsequent experiment was executed to evaluate lower doses of asialoorosomucoid-biotin. In the same animal model, doses of 50, 20 and 10 μg asialoorosomucoid-biotin were injected at 24 hours following administration of the LU-10-StrAv conjugate. Data from animals serially bled are shown in Figure 10, and data from animals sacrificed two hours after clearing agent administration are shown in Figures 11A (blood clearance) and 11B (serum biotin-binding), respectively. Doses of 50 and 20 μg asialoorosomucoid-biotin effectively reduced circulating LU-10-StrAv conjugate levels by about 65% (Figure 11A) and, after correction for binding to immobilized biotin, left only 3% of the injected dose in circulation that possessed biotin-binding capacity, compared with about 25% of the injected dose in control animals (Figure 11B). Even at low doses (approaching 1:1 stoichiometry with circulating LU-10-StrAv conjugate), asialoorosomucoid-biotin was highly

effective at reducing blood levels of circulating streptavidin-containing conjugate by an *in vivo* complexation that was dependent upon biotin-avidin interaction.

Example XIV

Tumor Uptake of PIP-Biocytin

PIP-Biocytin, as prepared and described in Example VII above, was tested to determine the fate thereof *in vivo*. The following data are based on experimentation with tumored nude mice (100 mg LS-180 tumor xenografts implanted subcutaneously 7 days prior to study) that received, at time 0, 200 μg of I-125 labeled NR-LU-10-Streptavidin conjugate (950 pmol), as discussed in Example XI above. At 24 hours, the mice received an i.v. injection of PIP-I-131-biocytin (40 μCi) and an amount of cold carrier PIP-I-127 biocytin corresponding to doses of 42 μg (69,767 pmol), 21 μg (34,884 pmol), 5.7 μg (9468 pmol), 2.85 μg (4734 pmol) or 0.5 μg (830 pmol). Tumors were excised and counted for radioactivity 4 hours after PIP-biocytin injection.

The three highest doses produced PIP-biocytin tumor localizations of about 600 pmol/g. Histology conducted on tissues receiving the two highest doses indicated that saturation of tumor-bound streptavidin was achieved. Equivalent tumor localization observed at the 5.7 μg dose is indicative of streptavidin saturation as well. In contrast, the two lowest doses produced lower absolute tumor localization of PIP-biocytin, despite equivalent localization of NR-LU-10-Streptavidin conjugate (tumors in all groups averaged about 40% ID/G for the conjugate).

The lowest dose group (0.5 μg) exhibited high efficiency tumor delivery of PIP-I-131-biocytin, which efficiency increased over time. A peak uptake of 85.0 % ID/G was observed at the 120 hour time point (96

hours after administration of PIP-biocytin). Also, the absolute amount of PIP-biocytin, in terms of % ID, showed a continual increase in the tumor over all of the sampled time points. The decrease in uptake on a % ID/G basis at the 168 hour time point resulted from significant growth of the tumors between the 120 and 168 hour time points.

In addition, the co-localization of NR-LU-10-Streptavidin conjugate (LU-10-StrAv) and the subsequently administered PIP-Biocytin at the same tumors over time was examined. The localization of radioactivity at tumors by PIP-biocytin exhibited a pattern of uptake and retention that differed from that of the antibody-streptavidin conjugate (LU-10-StrAv). LU-10-StrAv exhibited a characteristic tumor uptake pattern that is equivalent to historical studies of native NR-LU-10 antibody, reaching a peak value of 40% ID/G between 24 and 48 hours after administration. In contrast, the PIP-Biocytin exhibited an initial rapid accretion in the tumor, reaching levels greater than those of LU-10-StrAv by 24 hours after PIP-Biocytin administration. Moreover, the localization of PIP-Biocytin continued to increase out to 96 hours, when the concentration of radioactivity associated with the conjugate has begun to decrease. The slightly greater amounts of circulating PIP-Biocytin compared to LU-10-StrAv at these time points appeared insufficient to account for this phenomenon.

The ratio of PIP-Biocytin to LU-10-StrAv in the tumor increased continually during the experiment, while the ratio in the blood decreased continually. This observation is consistent with a process involving continual binding of targeting moiety-containing conjugate (with PIP-Biocytin bound to it) from the blood to the tumor, with subsequent differential processing of the PIP-Biocytin and the

conjugate. Since radiolabel associated with the streptavidin conjugate component (compared to radiolabel associated with the targeting moiety) has shown increased retention in organs of metabolic processing, PIP-Biocytin associated with the streptavidin appears to be selectively retained by the tumor cells. Because radiolabel is retained at target cell sites, a greater accumulation of radioactivity at those sites results.

The AUC_{tumor}/AUC_{blood} for PIP-Biocytin is over twice that of the conjugate (4.27 compared to 1.95, where AUC means "area under the curve"). Further, the absolute AUC_{tumor} for PIP-Biocytin is nearly twice that of the conjugate (9220 compared to 4629). Consequently, an increase in radiation dose to tumor was achieved.

Example XV

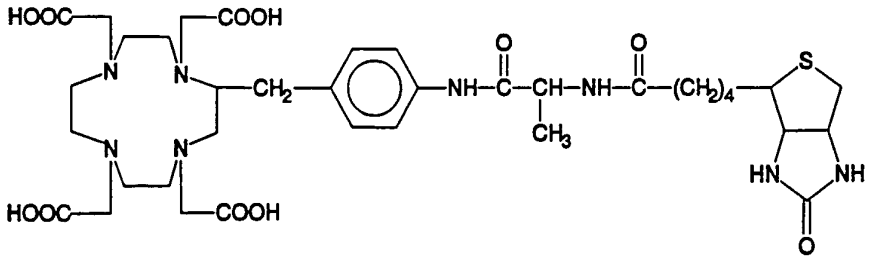
Synthesis of DOTA-Biotin Conjugates

A. Synthesis of Nitro-Benzyl-DOTA.

The synthesis of aminobenzyl-DOTA was conducted substantially in accordance with the procedure of McMurry et al., Bioconjugate Chem., 3: 108-117, 1992. The critical step in the prior art synthesis is the intermolecular cyclization between disuccinimidyl N-(tert-butoxycarbonyl)iminodiacetate and N-(2-aminoethyl)-4-nitrophenyl alaninamide to prepare 1-(tert-butoxycarbonyl)-5-(4-nitrobenzyl)-3,6,11-trioxo-1,4,7,10-tetraazacyclododecane. In other words, the critical step is the intermolecular cyclization between the bis-NHS ester and the diamine to give the cyclized dodecane. McMurry et al. conducted the cyclization step on a 140 mmol scale, dissolving each of the reagents in 100 ml DMF and adding via a syringe pump over 48 hours to a reaction pot containing 4 liters dioxane.

A 5x scale-up of the McMurry et al. procedure was not practical in terms of reaction volume, addition rate and reaction time. Process chemistry studies revealed that the reaction addition rate could be substantially increased and that the solvent volume could be greatly reduced, while still obtaining a similar yield of the desired cyclization product. Consequently on a 30 mmol scale, each of the reagents was dissolved in 500 ml DMF and added via addition funnel over 27 hours to a reaction pot containing 3 liters dioxane. The addition rate of the method employed involved a 5.18 mmol/hour addition rate and a 0.047 M reaction concentration.

B. Synthesis of a D-alanine-linked conjugate with a preserved biotin carboxy moiety. A reaction scheme to form a compound of the following formula is discussed below.



The D-alanine-linked conjugate was prepared by first coupling D-alanine (Sigma Chemical Co.) to biotin-NHS ester. The resultant biotinyl-D-alanine was then activated with 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride (EDCI) and N-hydroxysuccinimide (NHS). This NHS ester was reacted *in situ* with DOTA-aniline to give the desired product which was purified by preparative HPLC.

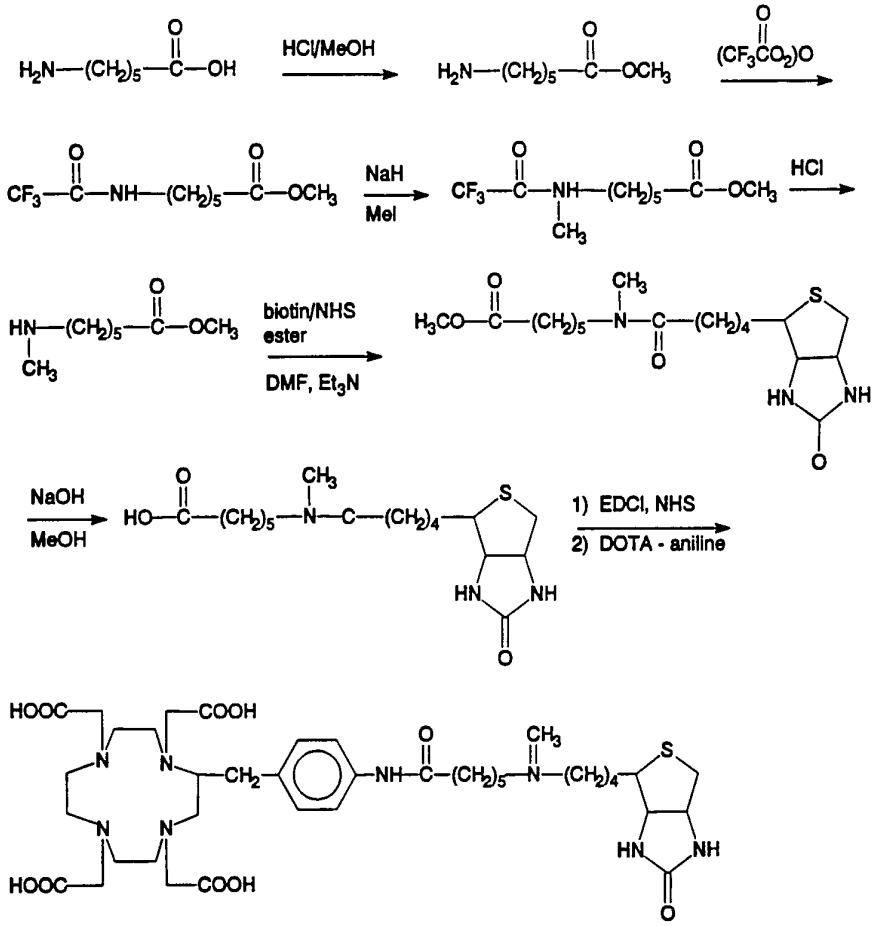
More specifically, a mixture of D-alanine (78 mg, 0.88 mmol, 1.2 equivalents), biotin-NHS ester (250 mg, 0.73 mmol, 1.0 equivalent), triethylamine (0.30 ml,

2.19 mmol, 3.0 equivalents) in DMF (4 ml) was heated at 110°C for 30 minutes. The solution was cooled to 23°C and evaporated. The product solid was acidified with glacial acetic acid and evaporated again. The product biotinyl-D-alanine, a white solid, was suspended in 40 ml of water to remove excess unreacted D-alanine, and collected by filtration. Biotinyl-D-alanine was obtained as a white solid (130 mg, 0.41 mmol) in 47% yield.

NHS (10 mg, 0.08 mmol) and EDCI (15 mg, 0.07 mmol) were added to a solution of biotinyl-D-alanine (27 mg, 0.08 mmol) in DMF (1 ml). The solution was stirred at 23°C for 60 hours, at which time TLC analysis indicated conversion of the carboxyl group to the N-hydroxy succinimidyl ester. Pyridine (0.8 ml) was added followed by DOTA-aniline (20 mg, 0.04 mmol). The mixture was heated momentarily at approximately 100°C, then cooled to 23°C and evaporated. The product, DOTA-aniline-D-alanyl-biotinamide was purified by preparative HPLC.

C. Synthesis of N-hydroxyethyl-linked conjugate. Iminodiacetic acid dimethyl ester is condensed with biotin-NHS-ester to give biotinyl dimethyl iminodiacetate. Hydrolysis with one equivalent of sodium hydroxide provides the monomethyl ester after purification from under and over hydrolysis products. Reduction of the carboxyl group with borane provides the hydroxyethyl amide. The hydroxyl group is protected with t-butyl-dimethyl-silylchloride. The methyl ester is hydrolysed, activated with EDCI and condensed with DOTA-aniline to form the final product conjugate.

D. Synthesis of N-Me-LC-DOTA-biotin. A reaction scheme is shown below.



Esterification of 6-aminocaproic acid (Sigma Chemical Co.) was carried out with methanolic HCl. Trifluoroacetylation of the amino group using trifluoroacetic anhydride gave N-6-(methylcaproyl)-trifluoroacetamide. The amide nitrogen was methylated using sodium hydride and iodomethane in tetrahydrofuran. The trifluoroacetyl protecting group was cleaved in acidic methanol to give methyl 6-methylamino-caproate hydrochloride. The amine was condensed with biotin-NHS ester to give methyl N-methyl-caproylamido-biotin. Saponification afforded the corresponding acid which was activated with EDCI and NHS and, *in situ*, condensed with DOTA-aniline to give DOTA-benzylamido-N-methyl-caproylamido-biotin.

1. Preparation of methyl 6-aminocaproate hydrochloride. Hydrogen chloride (gas) was added to a solution of 20.0 g (152 mmol) of 6-aminocaproic acid in 250 ml of methanol via rapid bubbling for 2-3 minutes. The mixture was stirred at 15-25°C for 3 hours and then concentrated to afford 27.5 g of the product as a white solid (99%):

H-NMR (DMSO) 9.35 (1 H, broad t), 3.57 (3H, s), 3.14 (2H, quartet), 2.28 (2H, t), 1.48 (4H, multiplet), and 1.23 ppm (2H, multiplet).

2. Preparation of N-6-(methylcaproyl)-trifluoroacetamide. To a solution of 20.0 g (110 mmol) of methyl 6-aminocaproate hydrochloride in 250 ml of dichloromethane was added 31.0 ml (22.2 mmol) of triethylamine. The mixture was cooled in an ice bath and trifluoroacetic anhydride (18.0 ml, 127 mmol) was added over a period of 15-20 minutes. The mixture was stirred at 0-10°C for 1 hour and concentrated. The residue was diluted with 300 ml of ethyl acetate and saturated aqueous sodium bicarbonate (3 x 100 ml). The organic phase was dried over anhydrous magnesium sulfate, filtered and concentrated to afford 26.5 g of the product as a pale yellow oil (100%):

H-NMR (DMSO) 3.57 (3H, s), 3.37 (2H, t), 3.08 (1.9H, quartet, N-CH_3), 2.93 (1.1H, s, N-CH_3), 2.30 (2H, t), 1.52 (4H, multiplet), and 1.23 ppm (2H, multiplet).

3. Preparation of methyl 6-N-methylamino-caproate hydrochloride. To a solution of 7.01 g (29.2 mmol) of N-6-(methylcaproyl)-trifluoroacetamide in 125 ml of anhydrous tetrahydrofuran was slowly added 1.75 g of 60% sodium hydride (43.8 mmol) in mineral oil. The mixture was stirred at 15-25°C for 30 minutes and then 6.2 g (43.7 mmol) of iodomethane was added. The mixture was stirred at 15-25°C for 17 hours and then filtered through celite. The solids were rinsed with 50 ml of tetrahydrofuran. The filtrates were combined

and concentrated. The residue was diluted with 150 ml of ethyl acetate and washed first with 5% aqueous sodium sulfite (2 x 100 ml) and then with 100 ml of 1 N aqueous hydrochloric acid. The organic phase was dried over anhydrous magnesium sulfate, filtered and concentrated to afford a yellow oily residue. The residue was diluted with 250 ml of methanol and then hydrogen chloride (gas) was rapidly bubbled into the mixture for 2-3 minutes. The resultant mixture was refluxed for 18 hours, cooled and concentrated. The residue was diluted with 150 ml of methanol and washed with hexane (3 x 150 ml) to remove mineral oil previously introduced with NaH. The methanol phase was concentrated to afford 4.91 g of the product as a yellow oil (86%):

H-NMR (DMSO) 8.80 (2H, broad s), 3.58 (3H, s), 2.81 (2H, multiplet), 2.48 (3H, s), 2.30 (2H, t), 1.52 (4H, multiplet), and 1.29 ppm (2H, multiplet).

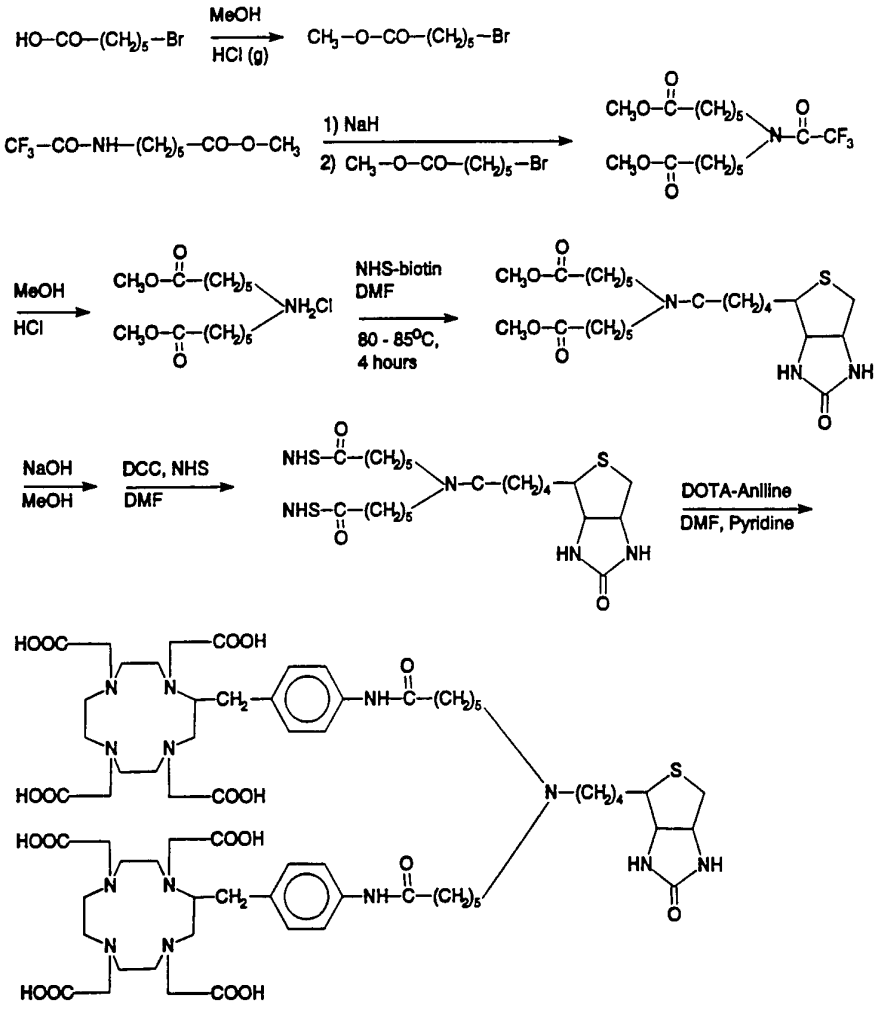
4. Preparation of methyl 6-(N-methylcaproylamido-biotin. N-hydroxysuccinimidyl biotin (398 mg, 1.16 mmol) was added to a solution of methyl 6-(N-methyl) aminocaproate hydrochloride (250 mg, 1.28 mmol) in DMF (4.0 ml) and triethylamine (0.18 ml, 1.28 mmol). The mixture was heated in an oil bath at 100°C for 10 minutes. The solution was evaporated, acidified with glacial acetic acid and evaporated again. The residue was chromatographed on a 25 mm flash chromatography column manufactured by Ace Glass packed with 50 g silica (EM Science, Gibbstown, New Jersey, particle size 0.40-0.63 mm) eluting with 15% MeOH/EtOAc. The product was obtained as a yellow oil (390 mg) in 79% yield.

5. Preparation of 6-(N-methyl-N-biotinyl) amino caproic acid. To a solution of methyl 6-(N-methyl-caproylamido-biotin (391 mg, 1.10 mmol) in methanol (2.5 ml) was added a 0.95 N NaOH solution (1.5 ml).

This solution was stirred at 23°C for 3 hours. The solution was neutralized by the addition of 1.0 M HCl (1.6 ml) and evaporated. The residue was dissolved in water, further acidified with 1.0 M HCl (0.4 ml) and evaporated. The gummy solid residue was suspended in water and agitated with a spatula until it changed into a white powder. The powder was collected by filtration with a yield of 340 mg.

6. Preparation of DOTA-benzylamido-N-methyl-caproylamido-biotin. A suspension of 6-(N-methyl-N-biotinyl)amino caproic acid (29 mg, 0.08 mmol) and N-hydroxysuccinimide (10 mg, 0.09 mmol) in DMF (0.8 ml) was heated over a heat gun for the short time necessary for the solids to dissolve. To this heated solution was added EDCI (15 mg, 0.08 mmol). The resultant solution was stirred at 23°C for 20 hours. To this stirred solution were added aminobenzyl-DOTA (20 mg, 0.04 mmol) and pyridine (0.8 ml). The mixture was heated over a heat gun for 1 minute. The product was isolated by preparative HPLC, yielding 3 mg.

E. Synthesis of a bis-DOTA conjugate with a preserved biotin carboxy group. A reaction scheme is shown below.



1. Preparation of methyl 6-bromocaproate (methyl 6-bromohexanoate). Hydrogen chloride (gas) was added to a solution of 5.01 g (25.7 mmol) of 6-bromocaproic acid in 250 ml of methanol via vigorous bubbling for 2-3 minutes. The mixture was stirred at 15-25°C for 3 hours and then concentrated to afford 4.84 g of the product as a yellow oil (90%):

H-NMR (DMSO) 3.58 (3H, s), 3.51 (2H, t), 2.29 (2H, t), 1.78 (2H, pentet), and 1.62-1.27 ppm (4H, m).

2. Preparation of N,N-bis-(6-methoxy carbonylhexyl)-amine hydrochloride. To a solution of 4.01 g (16.7 mmol) of N-(methyl 6-hexanoyl)-

trifluoroacetamide (prepared in accordance with section D.2. herein) in 125 ml of anhydrous tetrahydrofuran was added 1.0 g (25 mmol) of 60% sodium hydride in mineral oil. The mixture was stirred at 15-25°C for 1 hour and then 3.50 g (16.7 mmol) of methyl 6-bromocaproate was added and the mixture heated to reflux. The mixture was stirred at reflux for 22 hours. NMR assay of an aliquot indicated the reaction to be incomplete. Consequently, an additional 1.00 g (4.8 mmol) of methyl 6-bromocaproate was added and the mixture stirred at reflux for 26 hours. NMR assay of an aliquot indicated the reaction to be incomplete. An additional 1.0 g of methyl 6-bromocaproate was added and the mixture stirred at reflux for 24 hours. NMR assay of an aliquot indicated the reaction to be near complete. The mixture was cooled and then directly filtered through celite. The solids were rinsed with 100 ml of tetrahydrofuran. The filtrates were combined and concentrated. The residue was diluted with 100 ml of methanol and washed with hexane (3x 100 ml) to remove the mineral oil introduced with the sodium hydride. The methanol phase was treated with 6 ml of 10 N aqueous sodium hydroxide and stirred at 15-25°C for 3 hours. The mixture was concentrated. The residue was diluted with 100 ml of deionized water and acidified to pH 2 with concentrated HCl. The mixture was washed with ether (3 x 100 ml). The aqueous phase was concentrated, diluted with 200 ml of dry methanol and then hydrogen chloride gas was bubbled through the mixture for 2-3 minutes. The mixture was stirred at 15-25°C for 3 hours and then concentrated. The residue was diluted with 50 ml of dry methanol and filtered to remove inorganic salts. The filtrate was concentrated to afford 1.98 g of the product as a white solid (38%):

H-NMR (DMSO) 8.62 (2H, m) 3.58 (6H, s), 2.82 (4H, m) 2.30 (4H, t), 1.67-1.45 (8H, m) and 1.38-1.22 ppm (4H, m).

3. Preparation of N,N'-bis-(methyl 6-hexanoyl)-biotinamide. To a solution of 500 mg (1.46 mmol) of N-hydroxysuccinimidyl biotin in 15 ml of dry dimethylformamide was added 600 mg (1.94 mmol) of N,N-bis-(6-methoxy carbonylhexyl)amine hydrochloride followed by 1.0 ml of triethylamine. The mixture was stirred at 80-85°C for 3 hours and then cooled and concentrated. The residue was chromatographed on silica gel, eluting with 20% methanol/ethyl acetate, to afford 620 mg of the product as a near colorless oil (85%):

H-NMR ($CDCl_3$) 5.71 (1H, s), 5.22 (1H, s), 4.52 (1H, m), 4.33 (1H, m), 3.60 (3H, s), 3.58 (3H, s), 3.34-3.13 (5H, m), 2.92 (1H, dd), 2.75 (1H, d), 2.33 (6H, m) and 1.82-1.22 ppm (18H, m); TLC-R_f 0.39 (20:80 methanol/ ethyl acetate).

4. Preparation of N,N-bis-(6-hexanoyl)-biotinamide. To a solution of 610 mg (0.819 mmol) of N,N-bis-(methyl 6-hexanoyl)-biotinamide in 35 ml of methanol was added 5.0 ml of 1N aqueous sodium hydroxide. The mixture was stirred at 15-25°C for 4.5 hours and then concentrated. The residue was diluted with 50 ml of deionized water acidified to pH 2 with 1N aqueous hydrochloric acid at 4°C. The product, which precipitated out as a white solid, was isolated by vacuum filtration and dried under vacuum to afford 482 mg (84%):

H-NMR (DMSO) 6.42 (1H, s), 6.33 (1H, s), 4.29 (1H, m), 4.12 (1H, m), 3.29-3.04 (5H, m), 2.82 (1H, dd), 2.57 (1H, d), 2.21 (6H, m) and 1.70-1.10 ppm (18H, m).

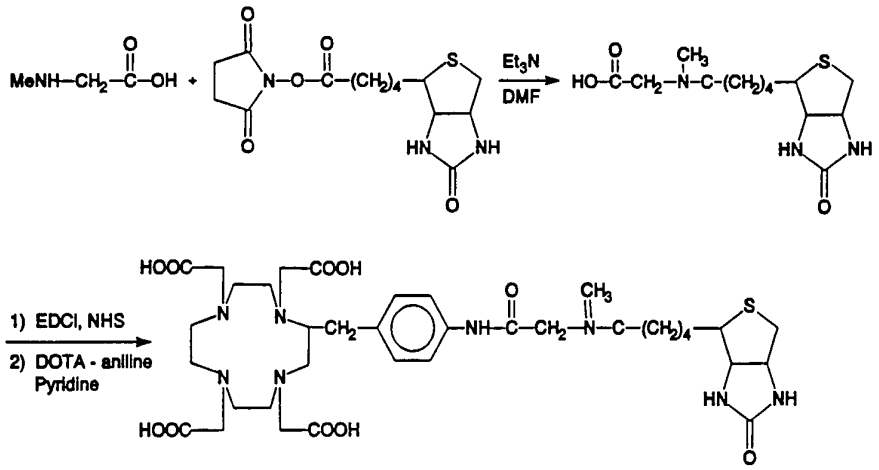
5. Preparation of N',N'-bis-(N-hydroxy-succinimidyl 6-hexanoyl)-biotinamide. To a solution of 220 mg (0.467 mmol) of N,N-bis-(6-hexanoyl)-

biotinamide in 3 ml of dry dimethylformamide was added 160 mg (1.39 mmol) of N-hydroxysuccinimide followed by 210 mg (1.02 mmol) of dicyclohexyl-carbodiimide. The mixture was stirred at 15-25°C for 17 hours and then concentrated. The residue was chromatographed on silica gel, eluting with 0.1:20:80 acetic acid/methanol/ethyl acetate, to afford 148 mg of the product as a foamy off-white solid (48%):

H-NMR (DMSO) 6.39 (1H, s), 6.32 (1H, s), 4,29 (1H, m), 4,12 (1H, m), 3.30-3.03 (5H, m), 2.81 (9H, dd and s), 2.67 (4H, m), 2.57 (1H, d), 2.25 (2H, t), 1.75-1.20 (18H, m); TLC-R_f 0.37 (0.1:20:80 acetic acid/methanol/ethyl acetate).

6. Preparation of N,N-bis-(6-hexanoylamidobenzyl-DOTA)-biotinamide. To a mixture of 15 mg of DOTA-benzylamine and 6.0 mg of N',N'-bis-(N-hydroxy-succinimidyl 6-hexanoyl)-biotinamide in 1.0 ml of dry dimethylformamide was added 0.5 ml of dry pyridine. The mixture was stirred at 45-50°C for 4.5 hours and at 15-25°C for 12 hours. The mixture was concentrated and the residue chromatographed on a 2.1 x 2.5 cm octadecylsilyl (ODS) reverse-phase preparative HPLC column eluting with a --20 minute gradient profile of 0.1:95:5 to 0.1:40:60 trifluoroacetic acid:water:acetonitrile at 13 ml/minute to afford the desired product. The retention time was 15.97 minutes using the aforementioned gradient at a flow rate of 1.0 ml/minute on a 4.6 mm x 25 cm ODS analytical HPLC column.

F. Synthesis of an N-methyl-glycine linked conjugate. A reaction scheme for this synthesis is shown below.



The N-methyl glycine-linked DOTA-biotin conjugate was prepared by an analogous method to that used to prepare D-alanine-linked DOTA-biotin conjugates. N-methyl-glycine (trivial name sarcosine, available from Sigma Chemical Co.) was condensed with biotin-NHS ester in DMF and triethylamine to obtain N-methyl glycyl-biotin. N-methyl-glycyl biotin was then activated with EDCI and NHS. The resultant NHS ester was not isolated and was condensed *in situ* with DOTA-aniline and excess pyridine. The reaction solution was heated at 60°C for 10 minutes and then evaporated. The residue was purified by preparative HPLC to give [(N-methyl-N-biotinyl)-N-glycyl]-aminobenzyl-DOTA.

1. Preparation of (N-methyl)glycyl biotin. DMF (8.0 ml) and triethylamine (0.61 ml, 4.35 mmol) were added to solids N-methyl glycine (182 mg, 2.05 mmol) and N-hydroxy-succinimidyl biotin (500 mg, 1.46 mmol). The mixture was heated for 1 hour in an oil bath at 85°C during which time the solids dissolved producing a clear and colorless solution. The solvents were then evaporated. The yellow oil residue was acidified

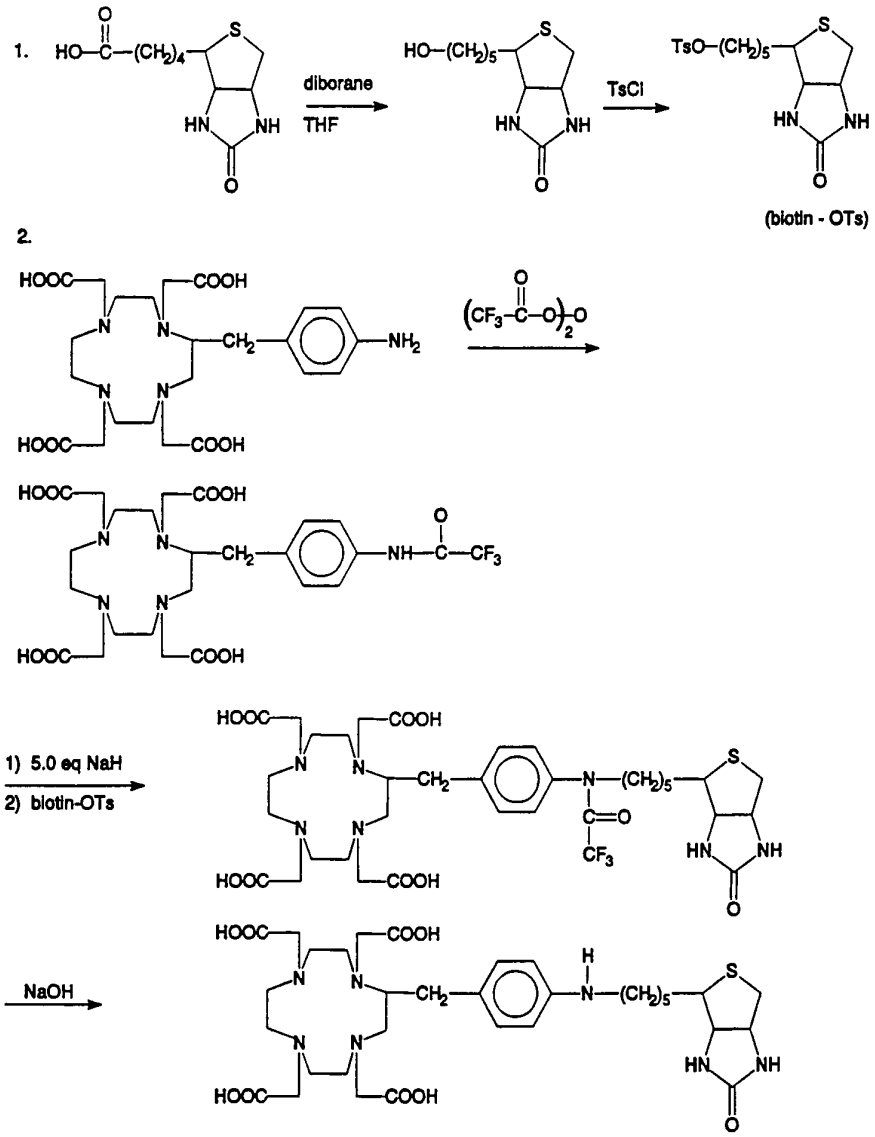
with glacial acetic acid, evaporated and chromatographed on a 27 mm column packed with 50 g silica, eluting with 30% MeOH/EtOAc 1% HOAc to give the product as a white solid (383 mg) in 66% yield.

H-NMR (DMSO): 1.18-1.25 (m, 6H, $(CH_2)_3$), 2.15, 2.35 (2 t's, 2H, CH_2CO), 2.75 (m, 2H, SCH_2), 2.80, 3.00 (2 s's, 3H, NCH_3), 3.05-3.15 (m, 1H, SCH), 3.95, 4.05 (2 s's, 2H, CH_2N), 4.15, 4.32 (2 m's, 2H, 2CHN's), 6.35 (s, NH), 6.45 (s, NH).

2. Preparation of [(N-methyl-N-biotinyl)glycyl] aminobenzyl-DOTA. N-hydroxysuccinimide (10 mg, 0.08 mmol) and EDCI (15 mg, 6.08 mmol) were added to a solution of (N-methylglycyl biotin (24 mg, 0.08 mmol) in DMF (1.0 ml). The solution was stirred at 23 'C for 64 hours. Pyridine (0.8 ml) and aminobenzyl-DOTA (20mg, 0.04 mmol) were added. The mixture was heated in an oil bath at 63°C for 10 minutes, then stirred at 23°C for 4 hours. The solution was evaporated. The residue was purified by preparative HPLC to give the product as an off white solid (8 mg, 0.01 mmol) in 27% yield.

H-NMR (D_2O): 1.30-1.80 (m, 6H), 2.40, 2.55 (2 t's, 2H, CH_2CO), 2.70-4.2 (complex multiplet), 4.35 (m, CHN), 4.55 (m, CHN), 7.30 (m, 2H, benzene hydrogens), 7.40 (m, 2H, benzene hydrogens).

G. Synthesis of a short chain amine-linked conjugate with a reduced biotin carboxy group. A two-part reaction scheme is shown on the following page.



The biotin carboxyl group is reduced with diborane in THF to give a primary alcohol. Tosylation of the alcohol with tosyl chloride in pyridine affords the primary tosylate. Aminobenzyl DOTA is acylated with trifluoroacetic anhydride in pyridine to give (N-trifluoroacetyl)aminobenzyl-DOTA. Deprotonation with 5.0 equivalents of sodium hydride followed by displacement of the biotin tosylate provides the (N-trifluoracetamido-N-descarboxylbiotinyl)aminobenzyl-DOTA. Acidic cleavage of the N-trifluoroacetamide

group with HCl(g) in methanol provides the amine-linked DOTA-biotin conjugate.

EXAMPLE XVI

Clearing Agent Evaluation Experimentation

The following experiments conducted on non-tumor-bearing mice were conducted using female BALB/c mice (20-25 g). For tumor-bearing mice experimentation, female nude mice were injected subcutaneously with LS-180 tumor cells, and, after 7 d, the mice displayed 50-100 mg tumor xenografts. The monoclonal antibody used in these experiments was NR-LU-10. When radiolabeled, the NR-LU-10-streptavidin conjugate was radiolabeled with I-125 using procedures described herein. When radiolabeled, PIP-biocytin was labeled with I-131 or I-125 using procedures described herein.

A. Utility of Asialoorosomucoid-Biotin (AO-Bt) in Reducing Circulating Radioactivity from a Subsequently Administered Radiolabeled Biotin Ligand. Mice bearing LS-180 colon tumor xenografts were injected with 200 micrograms NR-LU-10 antibody-streptavidin (MAb-StrAv) conjugate at time 0, which was allowed to prelocalize to tumor for 22 hours. At that time, 20 micrograms of AO-Bt was administered to one group of animals. Two hours later, 90 micrograms of a radioisotope-bearing, ligand-containing small molecule (PIP-biotin-dextran prepared as discussed in part B hereof) was administered to this group of mice and also to a group which had not received AO-Bt. The results of this experiment with respect to radiolabel uptake in tumor and clearance from the blood indicated that tumor-targeting of the radiolabeled biotin-containing conjugate was retained while blood clearance was enhanced, leading to an overall improvement in amount delivered to target/amount located in serum. The AUC tumor/AUC blood with clearing agent was 6.87, while AUC tumor/AUC blood without clearing agent was 4.45.

Blood clearance of the circulating MAb-StrAv conjugate was enhanced with the use of clearing agent. The clearing agent was radiolabeled in a separate group of animals and found to bind directly to tumor at very low levels (1.7 pmol/g at a dose of 488 total pmoles (0.35%ID/g), indicating that it does not significantly compromise the ability of tumor-bound MAb-StrAv to bind subsequently administered radiolabeled ligand.

B. Preparation Protocol for PIP-Biotin-Dextran. A solution of 3.0 mg biotin-dextran, lysine fixable (BDLF, available from Sigma Chemical Co., St. Louis, Missouri, 70,000 dalton molecular weight with approximately 18 biotins/molecule) in 0.3 ml PBS and 0.15 ml 1 M sodium carbonate, pH 9.25, was added to a dried residue (1.87 mCi) of N-succinimidyl p-I-125-iodobenzoate prepared in accordance with Wilbur, et al., J. Nucl. Med., 30: 216-226, 1989.

C. Dosing Optimization of AO-Bt. Tumored mice receiving StrAv-MAb as above, were injected with increasing doses of AO-Bt (0 micrograms, 20 micrograms, 50 micrograms, 100 micrograms and 200 micrograms). Tumor uptake of I-131-PIP-biocytin (5.7 micrograms, administered 2 hours after AO-Bt administration) was examined. Increasing doses of AO-Bt had no effect on tumor localization of MAb-StrAv. Data obtained 44 hours after AO-Bt administration showed the same lack of effect. This data indicates that AO-Bt dose not cross-link and internalize MAb-StrAv on the tumor surface, as had been noted for avidin administered following biotinylated antibody.

PIP-biocytin tumor localization was inhibited at higher doses of AO-Bt. This effect is most likely due to reprocessing and distribution to tumor of biotin used to derivatize AO-Bt. Optimal tumor to blood ratios (% injected dose of radiolabeled ligand/gram weight of tumor divided by % injected dose of radioligand/gram weight of blood were achieved at the

50 microgram dose of AO-Bt. Biodistributions conducted following completion of the protocols employing a 50 microgram AO-Bt dose revealed low retention of radiolabel in all non-target tissues (1.2 pmol/g in blood; 3.5 pmol/gram in tail; 1.0 pmol/g in lung; 2.2 pmol/g in liver; 1.0 pmol/g is spleen; 7.0 pmol/g in stomach; 2.7 pmol/g in kidney; and 7.7 pmol/g in intestine). With 99.3 pmol/g in tumor, these results indicate effective decoupling of the PIP-biocytin biodistribution from that of the MAb-StrAv at all sites except tumor. This decoupling occurred at all clearing agent doses in excess of 50 micrograms as well. Decreases in tumor localization of PIP-biocytin was the significant result of administering clearing agent doses in excess of 50 micrograms. In addition, the amount of PIP-biocytin in non-target tissues 44 hours after administration was identical to localization resulting from administration of PIP-biocytin alone (except for tumor, where negligible accretion was seen when PIP-biocytin was administered alone), indicating effective decoupling.

D. Further Investigation of Optimal Clearing Agent Dose. Tumored mice injected with MAb-StrAv at time 0 as above; 50 micrograms of AO-Bt at time 22 hours; and 545 microcuries of I-131-PIP-biocytin at time 25 hours. Whole body radiation was measured and compared to that of animals that had not received clearing agent. 50 micrograms of AO-Bt was efficient in allowing the injected radioactivity to clear from the animals unimpeded by binding to circulating MAb-StrAv conjugate. Tumor uptake of I-131-PIP-biocytin was preserved at the 50 microgram clearing agent dose, with AUC tumor/AUC blood of 30:1 which is approximately 15-fold better than the AUC tumor/AUC blood achieved in conventional antibody-radioisotope therapy using this model.

E. Galactose- and Biotin-Derivatization of Human Serum Albumin (HSA). HSA was evaluated because it exhibits the advantages of being both inexpensive and non-immunogenic. HSA was derivatized with varying levels of biotin (1-about 9 biotins/molecule) via analogous chemistry to that previously described with respect to AO. More specifically, to a solution of HSA available from Sigma Chemical Co. (5-10 mg/ml in PBS) was added 10% v/v 0.5 M sodium borate buffer, pH 8.5, followed by dropwise addition of a DMSO solution of NHS-LC-biotin (Sigma Chemical Co.) to the stirred solution at the desired molar offering (relative molar equivalents of reactants). The final percent DMSO in the reaction mixture should not exceed 5%. After stirring for 1 hour at room temperature, the reaction was complete. A 90% incorporation efficiency for biotin on HSA was generally observed. As a result, if 3 molar equivalences of the NHS ester of LC-biotin was introduced, about 2.7 biotins per HSA molecule were obtained. Unreacted biotin reagent was removed from the biotin-derivatized HSA using G-25 size exclusion chromatography. Alternatively, the crude material may be directly galactosylated. The same chemistry is applicable for biotinylating non-previously biotinylated dextran.

HSA-biotin was then derivatized with from 12 to 15 galactoses/molecule. Galactose derivatization of the biotinylated HSA was performed according to the procedure of Lee, et al., Biochemistry, 15: 3956, 1976. More specifically, a 0.1 M methanolic solution of cyanomethyl-2,3,4,6-tetra-O-acetyl-1-thio-D-galactopyranoside was prepared and reacted with a 10% v/v 0.1 M NaOMe in methanol for 12 hours to generate the reactive galactosyl thioimidate. The galactosylation of biotinylated HSA began by initial evaporation of the anhydrous methanol from a 300 fold molar excess of reactive thioimidate. Biotinylated

HSA in PBS, buffered with 10% v/v 0.5 M sodium borate, was added to the oily residue. After stirring at room temperature for 2 hours, the mixture was stored at 4°C for 12 hours. The galactosylated HSA-biotin was then purified by G-25 size exclusion chromatography or by buffer exchange to yield the desired product. The same chemistry is exploitable to galactosylating dextran. The incorporation efficiency of galactose on HSA is approximately 10%.

70 micrograms of Galactose-HSA-Biotin (G-HSA-B), with 12-15 galactose residues and 9 biotins, was administered to mice which had been administered 200 micrograms of StrAv-MAb or 200 microliters of PBS 24 hours earlier. Results indicated that G-HSA-B is effective in removing StrAv-MAb from circulation. Also, the pharmacokinetics of G-HSA-B is unperturbed and rapid in the presence or absence of circulating MAb-StrAv.

F. Non-Protein Clearing Agent. A commercially available form of dextran, molecular weight of 70,000 daltons, pre-derivatized with approximately 18 biotins/molecule and having an equivalent number of free primary amines was studied. The primary amine moieties were derivatized with a galactosylating reagent, substantially in accordance with the procedure therefor described above in the discussion of HSA-based clearing agents, at a level of about 9 galactoses/molecule. The molar equivalence offering ratio of galactose to HSA was about 300:1, with about one-third of the galactose being converted to active form. 40 Micrograms of galactose-dextran-biotin (GAL-DEX-BT) was then injected i.v. into one group of mice which had received 200 micrograms MAb-StrAv conjugate intravenously 24 hours earlier, while 80 micrograms of GAL-DEX-BT was injected into other such mice. GAL-DEX-BT was rapid and efficient at clearing StrAv-MAb conjugate, removing over 66% of circulating conjugate

in less than 4 hours after clearing agent administration. An equivalent effect was seen at both clearing agent doses, which correspond to 1.6 (40 micrograms) and 3.2 (80 micrograms) times the stoichiometric amount of circulating StrAv conjugate present.

G. Dose Ranging for G-HSA-B Clearing Agent. Dose ranging studies followed the following basic format:

200 micrograms MAb-StrAv conjugate administered;

24 hours later, clearing agent administered; and

2 hours later, 5.7 micrograms PIP-biocytin administered.

Dose ranging studies were performed with the G-HSA-B clearing agent, starting with a loading of 9 biotins per molecule and 12-15 galactose residues per molecule. Doses of 20, 40, 70 and 120 micrograms were administered 24 hours after a 200 microgram dose of MAb-StrAv conjugate. The clearing agent administrations were followed 2 hours later by administration of 5.7 micrograms of I-131-PIP-biocytin. Tumor uptake and blood retention of PIP-biocytin was examined 44 hours after administration thereof (46 hours after clearing agent administration). The results showed that a nadir in blood retention of PIP-biocytin was achieved by all doses greater than or equal to 40 micrograms of G-HSA-B. A clear, dose-dependent decrease in tumor binding of PIP-biocytin at each increasing dose of G-HSA-B was present, however. Since no dose-dependent effect on the localization of MAb-StrAv conjugate at the tumor was observed, this data was interpreted as being indicative of relatively higher blocking of tumor-associated MAb-StrAv conjugate by the release of biotin from catabolized clearing agent. Similar results to those described earlier for the asialoorosomucoid clearing agent regarding plots of tumor/blood ratio were found with respect to G-HSA-B,

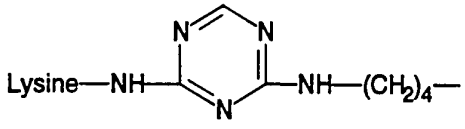
in that an optimal balance between blood clearance and tumor retention occurred around the 40 microgram dose.

Because of the relatively large molar amounts of biotin that could be released by this clearing agent at higher doses, studies were undertaken to evaluate the effect of lower levels of biotinylation on the effectiveness of the clearing agent. G-HSA-B, derivatized with either 9, 5 or 2 biotins/molecule, was able to clear MAb-StrAv conjugate from blood at equal protein doses of clearing agent. All levels of biotinylation yielded effective, rapid clearance of MAb-StrAv from blood.

Comparison of these 9-, 5-, and 2-biotin-derivatized clearing agents with a single biotin G-HSA-B clearing agent was carried out in tumored mice, employing a 60 microgram dose of each clearing agent. This experiment showed each clearing agent to be substantially equally effective in blood clearance and tumor retention of MAb-StrAv conjugate 2 hours after clearing agent administration. The G-HSA-B with a single biotin was examined for the ability to reduce binding of a subsequently administered biotinylated small molecule (PIP-biocytin) in blood, while preserving tumor binding of PIP-biocytin to prelocalized MAb-StrAv conjugate. Measured at 44 hours following PIP-biocytin administration, tumor localization of both the MAb-StrAv conjugate and PIP-biocytin was well preserved over a broad dose range of G-HSA-B with one biotin/molecule (90 to 180 micrograms). A progressive decrease in blood retention of PIP-biocytin was achieved by increasing doses of the single biotin G-HSA-B clearing agent, while tumor localization remained essentially constant, indicating that this clearing agent, with a lower level of biotinylation, is preferred. This preference arises because the single biotin G-HSA-B clearing agent is both effective at clearing MAb-StrAv

over a broader range of doses (potentially eliminating the need for patient-to-patient titration of optimal dose) and appears to release less competing biotin into the systemic circulation than the same agent having a higher biotin loading level.

Another way in which to decrease the effect of clearing agent-released biotin on active agent-biotin conjugate binding to prelocalized targeting moiety-streptavidin conjugate is to attach the protein or polymer or other primary clearing agent component to biotin using a retention linker. A retention linker has a chemical structure that is resistant to agents that cleave peptide bonds and, optionally, becomes protonated when localized to a catabolizing space, such as a lysosome. Preferred retention linkers of the present invention are short strings of D-amino acids or small molecules having both of the characteristics set forth above. An exemplary retention linker of the present invention is cyanuric chloride, which may be interposed between an epsilon amino group of a lysine of a proteinaceous primary clearing agent component and an amine moiety of a reduced and chemically altered biotin carboxy moiety (which has been discussed above) to form a compound of the structure set forth below.



When the compound shown above is catabolized in a catabolizing space, the heterocyclic ring becomes protonated. The ring protonation prevents the catabolite from exiting the lysosome. In this manner, biotin catabolites containing the heterocyclic ring are restricted to the site(s) of catabolism and,

therefore, do not compete with active-agent-biotin conjugate for prelocalized targeting moiety-streptavidin target sites.

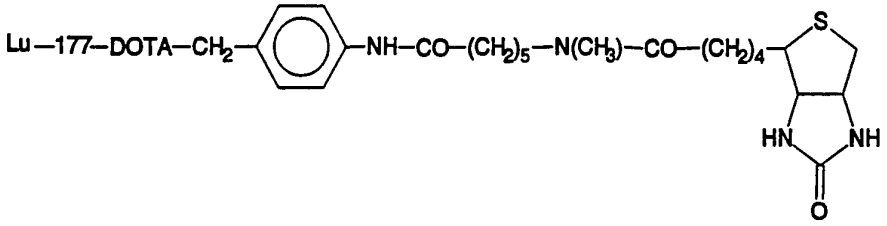
Comparisons of tumor/blood localization of radiolabeled PIP-biocytin observed in the G-HSA-B dose ranging studies showed that optimal tumor to background targeting was achieved over a broad dose range (90 to 180 micrograms), with the results providing the expectation that even larger clearing agent doses would also be effective. Another key result of the dose ranging experimentation is that G-HSA-B with an average of only 1 biotin per molecule is presumably only clearing the MAb-StrAv conjugate via the Ashwell receptor mechanism only, because too few biotins are present to cause cross-linking and aggregation of MAb-StrAv conjugates and clearing agents with such aggregates being cleared by the reticuloendothelial system.

H. Tumor Targeting Evaluation Using G-HSA-B. The protocol for this experiment was as follows:

Time 0: administer 400 micrograms MAb-StrAv conjugate;

Time 24 hours: administer 240 micrograms of G-HSA-B with one biotin and 12-15 galactoses and

Time 26 hours: administer 6 micrograms of



Lu-177 is complexed with the DOTA chelate using known techniques therefor.

Efficient delivery of the Lu-177-DOTA-biotin small molecule was observed, 20-25 % injected dose/gram of

tumor. These values are equivalent with the efficiency of the delivery of the MAb-StrAv conjugate. The AUC tumor/AUC blood obtained for this non-optimized clearing agent dose was 300% greater than that achievable by comparable direct MAb-radiolabel administration. Subsequent experimentation has resulted in AUC tumor/AUC blood over 1000% greater than that achievable by comparable conventional MAb-radiolabel administration. In addition, the HSA-based clearing agent is expected to exhibit a low degree of immunogenicity in humans.

Example XVII

Pretargeting with Lower Affinity Biotin Containing Clearing Agent

A patient presents with ovarian cancer. A monoclonal antibody (MAb) directed to an ovarian cancer cell antigen, e.g., NR-LU-10, is conjugated to streptavidin to form a MAb-streptavidin conjugate. The MAb-streptavidin conjugate is administered to the patient in an amount sufficient to substantially saturate the available antigenic sites at the target (which amount is at least sufficient to allow the capture of a therapeutically effective radiation dose at the target and which amount may be in excess of the maximum tolerated dose of conjugate administrable in a targeted, chelate-labeled molecule protocol, such as administration of monoclonal antibody-chelate-radionuclide conjugate). The MAb-streptavidin so administered is permitted to localize to target cancer cells for 24-48 hours. Next, an amount of a clearing agent consisting of human serum albumin, exposed galactose residues and 2'-thiobiotin molecules is administered in an amount sufficient to clear non-targeted MAb-streptavidin conjugate.

A biotin-radionuclide chelate conjugate of the type discussed in Example XV(F) above is radiolabeled

with Y-90 as set forth below. Carrier free $^{90}YCl_3$ (available from NEN-DuPont, Wilmington, Delaware) at 20-200 μl in 0.05 N HCl was diluted with ammonium acetate buffer (0.5M, pH 5) to a total volume of 0.4 ml. 50 μl (500 mg/ml) of ascorbic acid and 50-100 μl (10 mg/ml) of DOTA-biotin were added to the buffered $^{90}YCl_3$ solution. The mixture was incubated for one hour at 80°C. Upon completion of the incubation, 55 μl of 100 mM DTPA was added to the mixture to chelate any unbound ^{90}Y. The final preparation was diluted to 10 ml with 0.9% NaCl.

The radiolabeled DOTA-biotin conjugate is administered to the patient in a therapeutically effective dose at a time point 1-4 hours post-clearing agent administration. The biotin-radionuclide chelate conjugate localizes to targeted MAb-streptavidin or is substantially removed from the patient via the renal pathway.

Example XVIII

Pretargeting Using a Receptor Blocking Agent

A patient presents with small cell lung cancer. An amount of asialoorosomucoid sufficient to substantially saturate galactose receptors of the patient's hepatocytes is administered in a single or multiple doses. Additional administrations of asialoorosomucoid are conducted from time to time during the protocol to maintain substantial saturation of the galactose receptors for a time sufficient to permit localization of a subsequently administered monoclonal antibody-streptavidin conjugate to target cell sites, e.g., from 18-72 hours.

A monoclonal antibody (MAb) directed to a small cell lung cancer antigen, e.g., NR-LU-10, is conjugated to streptavidin to form a MAb-streptavidin conjugate. The MAb-streptavidin conjugate is administered to the patient in an amount sufficient to

substantially saturate the available antigenic sites at the target (which amount is at least sufficient to allow the capture of a therapeutically effective radiation dose at the target and which amount may be in excess of the maximum tolerated dose of conjugate administrable in a targeted, chelate-labeled molecule protocol, such as administration of monoclonal antibody-chelate-radionuclide conjugate). The MAb-streptavidin so administered is permitted to localize to target cancer cells for 20-48 hours. At this time, the MAb-streptavidin conjugate is cleared via the galactose receptors of hepatocytes, because such receptors have processed the asialoorosomucoid blocking agent.

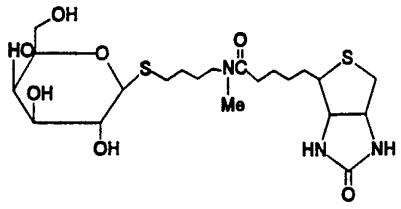
From 2-8 hours later, biotin-radionuclide chelate conjugate of the type discussed in Example XV(F) above is radiolabeled with Y-90 as set forth in Example XVII above. The radiolabeled DOTA-biotin conjugate is administered to the patient in a therapeutically effective dose. The biotin-radionuclide chelate conjugate localizes to targeted MAb-streptavidin or is substantially removed from the patient via the renal pathway.

Example XIX

In order to demonstrate the efficacy of the described small molecule clearing agents, a number of such conjugates were synthesized using biotin rather than a "low affinity" biotin analog and galactose residues. These conjugates were synthesized using different numbers of attached galactose residues. In addition, these conjugates contained either a long chain linker (LC) or the short chain linker (SC) as depicted below:

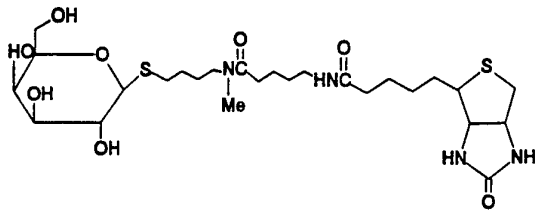
The conjugates which were synthesized are depicted below:

(Galactosyl)$_1$-SC-Biotin



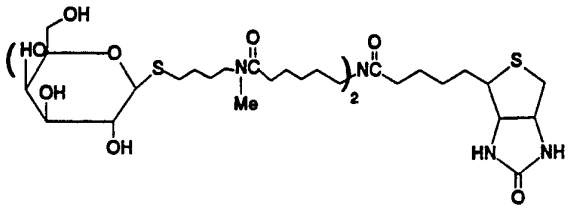
MW: 507.64

(Galactosyl)$_1$-LC-Biotin



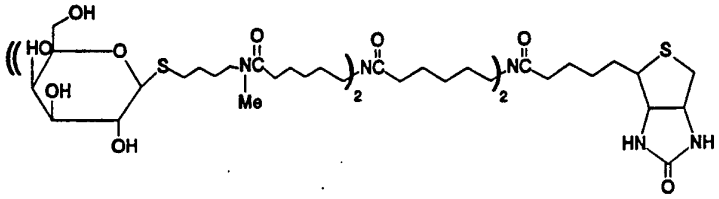
MW: 620.80

(Galactosyl)$_2$-SC-Biotin



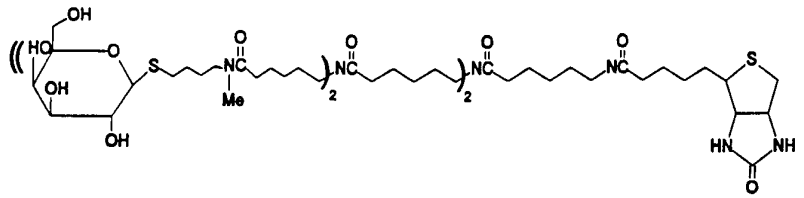
MW: 998.27

(Galactosyl)$_4$-SC-Biotin



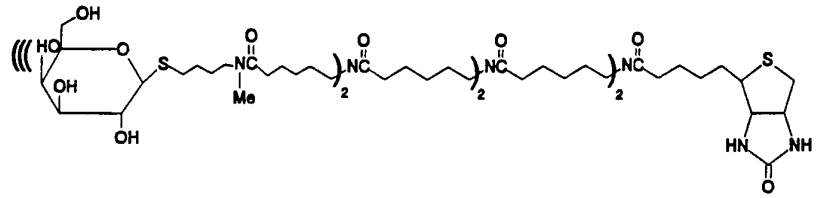
MW: 1979.63

(Galactosyl)$_4$-LC-Biotin



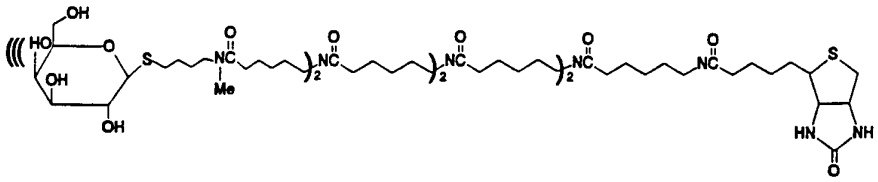
MW: 2092.79

(Galactosyl)$_8$-SC-Biotin



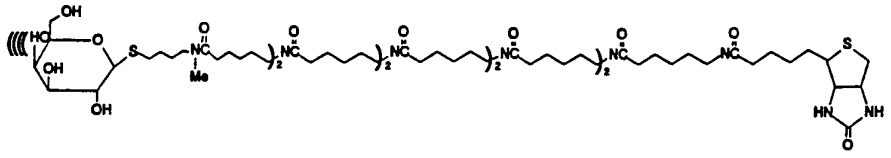
MW: 3942.26

(Galactosyl)$_8$-LC-Biotin



MW: 4055.42

(Galactosyl)$_{16}$-LC-Biotin



MW: 7981.81

These compounds were then assayed for their clearance directing activity. This was effected by first pre-binding an LU-10 streptavidin conjugate (labeled with I-125) with the clearing agent *ex vivo* and then intravenously administering the various compounds in the mouse model, and then measuring serum

levels of the conjugate. The data of these experiments are shown in Figures 12-15. This data indicates that no significant increase in clearance occurs until at least four galactose residues are attached to the biotin molecule. In addition, the data indicates that the longer linker separating the galactose cluster from the biotin molecule resulted in better clearance rates. This is consistent with the inventors' belief that the galactose cluster interferes with binding to the streptavidin conjugate or with receptor recognition of the complex if an appropriate length spacer is not used to minimize steric interactions.

The $(galactosyl)_8$-LC-biotin conjugate was also compared to galactosylated-HSA-biotin in a Balb/C mouse model for its ability to clear a I-125 LU-10-streptavidin conjugate from the circulation as a function of time. These results are shown in Figures 16 and 17. These results indicate that the $(galactosyl)_8$-LC-biotin conjugate is comparable to galactosylated-HSA-biotin in its ability to clear the streptavidin containing conjugate from the circulation. Subsequent experiments have further shown that conjugates containing 16 galactose residues provide for even better clearance than those containing 8 galactose residues.

Further experiments have been conducted in tumor bearing animals using the full pretargeting regimen. These results provided further evidence that the subject small molecule clearing agents bind to the extravascular conjugate in a limited fashion, and therefore provide for efficient clearance without adversely affecting the conjugate at the tumor. However, if further results indicate that the tumor conjugate is significantly compromised (bound by clearance agent), such problem can be substantially alleviated or prevented by the selection of

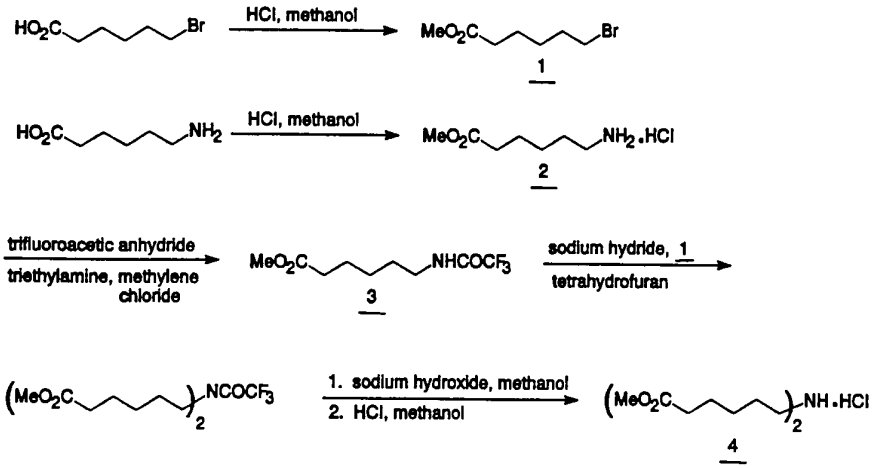
appropriate "low affinity" biotin analog containing conjugates.

Much has been reported about the binding affinity of different biotin analogs to avidin. Based on what is known in the art, the ordinary skilled artisan could readily select or use known techniques to ascertain the respective binding affinity of a particular biotin analog to either streptavidin or avidin. Examples of such analogs which may be tested include desthiobiotin, biotin, sulfone, d- and ℓ-diastereomers of biotin sulfoxide, and thiobiotin.

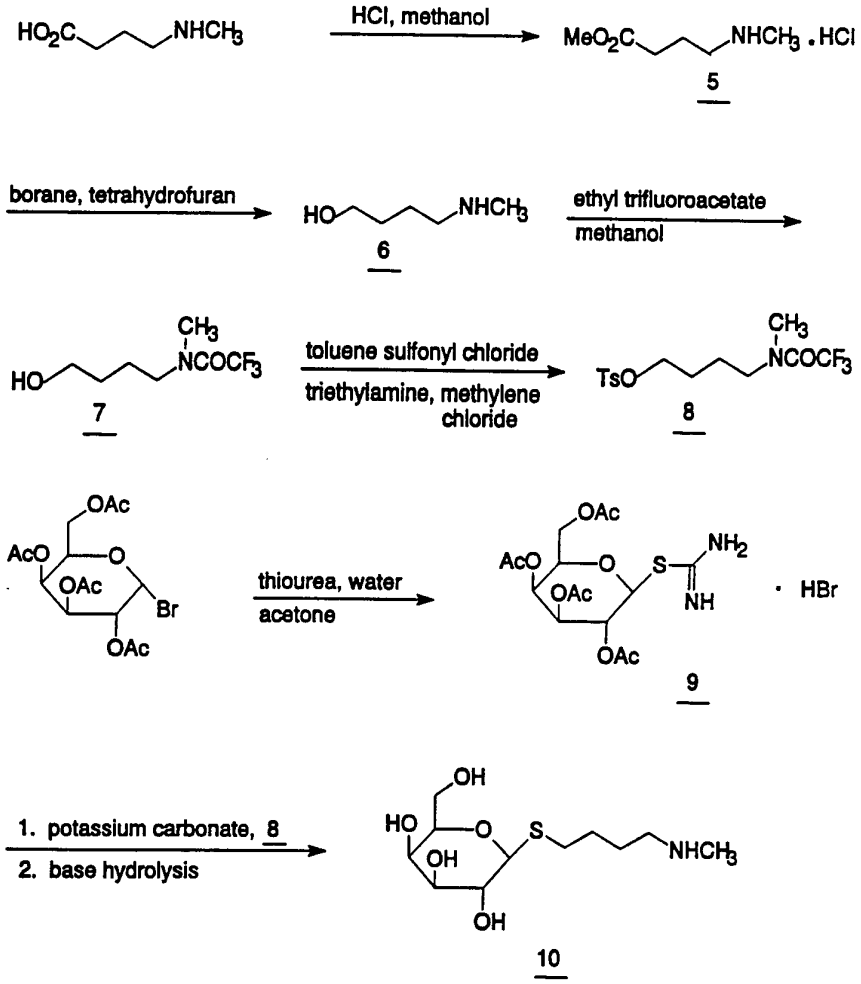
Example XX

This example describes a stepwise procedure by which an exemplary small molecular weight clearing agent, hexadeca-galactosyl biotin may be prepared. This example is exemplary of small molecule clearing agents which may be prepared according to the present invention. Additionally, this synthetic scheme is depicted schematically below:

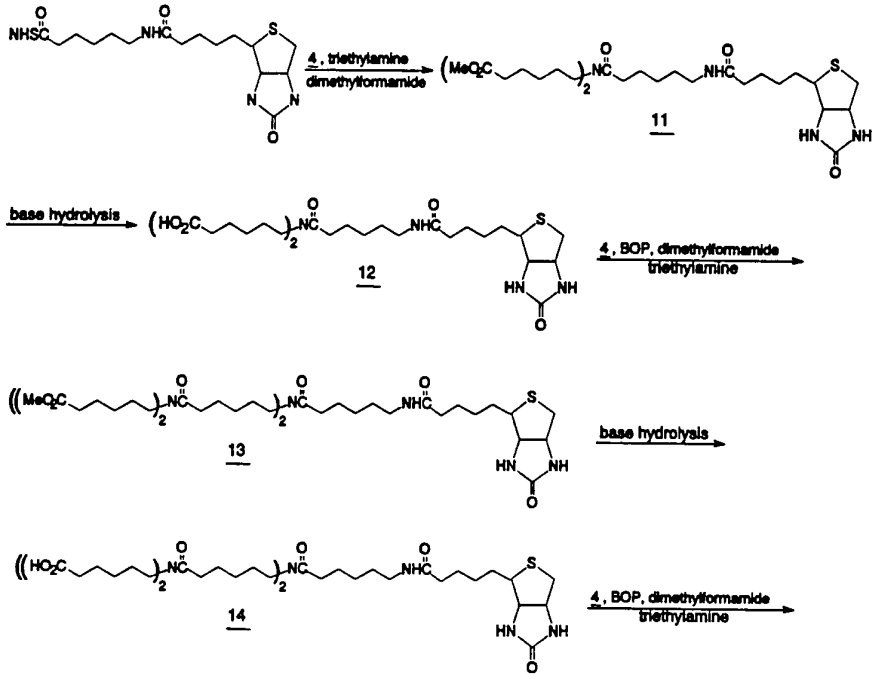
Preparation of N,N-Bis (6-methoxy carbonylhexyl)Amine Hydrochloride (4).



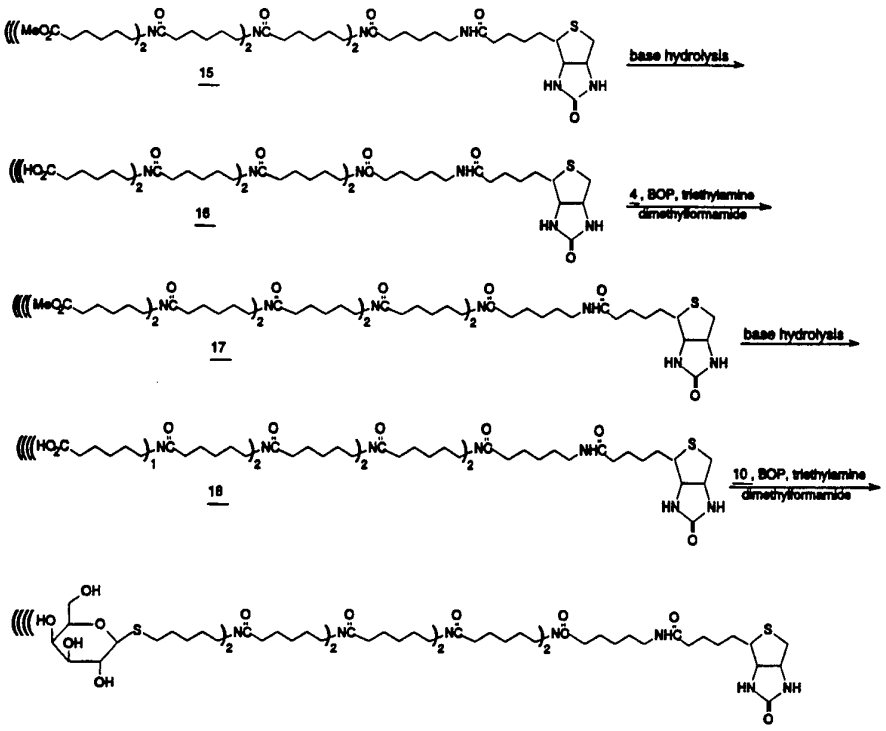
Preparation of 4-(N-Methylaminobutyl)-1-thio-β-D-galactopyranoside (10).



Preparation of Biotin Tetra Acid (14).



Preparation of Hexadeca-Galactosyl Biotin (19).



Preparation of Methyl 6-bromohexanoate (1)

To a 1 L round bottom flask, charged with 20 g (102.5 mmol) of 6-bromohexanoic acid and 500 mL of methanol was bubbled hydrogen chloride gas for 2-3 minutes. The mixture was stirred at room temperature for 4 h and then concentrated to afford 21.0 g of the product (1) as a yellow oil (99%): ^{1}H-NMR (200 MHz, d_6-DMSO); 3.57 (s, 3H), 3.51 (t, 2H), 2.30 (t, 2H); 1.78 (pentet, 2H), and 1.62-1.27 (M, 4H) ppm.

Preparation of Methyl 6-Aminohexanoate Hydrochloride (2)

To a 1 L round bottom flask, charged with 40.0 g of aminocaproic acid, was added 500 mL of methanol. Hydrogen chloride gas was bubbled through the mixture for 5 minutes and the mixture was stirred at room temperature for 5 h. The mixture was then concentrated via rotary evaporation and then under

full vacuum pump pressure (<0.1 mm Hg) to afford 55 g of the product (2) as a white solid (99%): ^{1}H-NMR (200 MHz, CD_3OD); 3.67 (s, 3H), 3.02 (t, 2H); 2.68 (s, 3H), 2.48 (t, 2H), and 2.03-1.87 (pentet, 2H) ppm.

Preparation of Methyl 6-(trifluoroacetamido)-hexanoate (3)

To a 1 L round bottom flask, charged with 25.0 g (138 mmol) of the amine hydrochloride (2) and 500 mL of methylene chloride, was added 24 mL (170 mmol) of trifluoroacetic anhydride. The mixture was cooled in an ice bath and 42 mL (301 mmol) of triethylamine was added over a 25-30 minute period. The mixture was stirred at 0°C to room temperature for 2 h and then concentrated. The residue was diluted with 150 mL of diethyl ether and 150 mL of petroleum ether and the resulting solution was washed first with 1 N aqueous HCl (3 x 150 mL) and then with saturated aqueous sodium bicarbonate (3 x 150 mL) . The organic phase was dried over magnesium sulfate, filtered and concentrated to give 32.9 g of the product (3) as a pale yellow oil (99%): ^{1}H-NMR (200 MHz, d_6-DMSO); 9.39 (m, 1H), 3.57 (s, 3H), 3.14 (q, 2H), 2.29 (t, 2H) 1.60-1.38 (m, 4H), and 1.32-1.19 (m, 2H) ppm.

Preparation of N,N-Bis (6-methoxy carbonylhexyl)amine Hydrochloride (4)

To a 500 mL dry round bottom flask, charged with 12.0 g (50.0 mmol) of the secondary amide (3) and 250 mL of dry tetrahydrofuran, was added 2.2 g (55 mmol, 1.1 equiv) of 60% sodium hydride. The mixture was stirred at room temperature for 30 minutes and then 10.25 g (49.0 mmol, 0.98 equiv) of the alkyl bromide (1) was added. The mixture was stirred at reflux for 3 h. An additional 5.80 g (27.7 mmol, 0.55 equiv) of (1) was added and the mixture was stirred at reflux for 70 h. The mixture was cooled, diluted with 150 mL

of 1 N aq HCl and then extracted with ethyl acetate (3 x 100 mL). The organic extracts were combined, dried over magnesium sulfate, filtered and concentrated. The residue was diluted with 200 mL of methanol and then treated with 30 mL of 10 N aqueous sodium hydroxide. The mixture was stirred at room temperature for 18 h and then concentrated. The residue was diluted with 200 mL of deionized water and acidified to pH = 1-2 with 37% concentrated HCl. The solution was washed with diethyl ether (3 x 100 mL). The aqueous phase was concentrated. The residue was diluted with 200 mL of methanol and reconcentrated. The subsequent residue was diluted with 250 mL of methanol and HCl gas was bubbled through the mixture for 2-3 minutes and stirred at room temperature for 3 h. The mixture was concentrated. The residue was diluted with 300 mL of methanol and filtered to remove inorganic salts. The filtrate was treated with 3 g of activated charcoal, filtered through Celite and concentrated. The residue, an off-white solid, was recrystallized from 100 mL of 2-propanol to afford 7.0 g of the product (4) as a white solid. Concentration of the filtrate and further recrystallization of the residue yielded an additional 1.65 g of product (4) for a total of 8.65 g (56%): ^{1}H-NMR (200 MHz, d_6-DMSO); 3.57 (s, 6H), 2.90-2.73 (m, 4H), 2.30 (t, 4H), 1.67-1.44 (m, 8H), and 1.37-1.20 (m, 4H) ppm.

Preparation of Methyl 4-Methylaminobutyrate Hydrochloride (5)

To a 1 L round bottom flask, charged with 30.0 g (195 mmol) of 4-methylaminobutyric acid and 500 mL of methanol was bubbled HCl gas for 1-2 minutes. The mixture was stirred at room temperature for 3-4 h and then concentrated to afford 32.5 g of the product (5) as a foamy, off-white solid (99%): ^{1}H-NMR- (200 MHz,

CD_3OD); 3.67 (s, 3H), 3.03 (t, 2H), 2.68 (s, 3H), 2.48 (t, 2H), and 2.03-1.87 (pentet, 2H) ppm.

Preparation of 4-Methylaminobutanol (6)

To a 1 L round bottom flask, charged with 32.5 g (194 mmol) of the ester (5), was added 500 mL of 1 M borane in tetrahydrofuran over a 1 h period at 0°C. After the addition was complete, the mixture was refluxed for 20 h, cooled to 0°C and the excess borane was destroyed by careful addition of 100 mL of methanol. After all the methanol was added, the mixture was stirred at room temperature for 1 h and then concentrated. The residue was diluted with 400 mL of methanol and then HCl gas was bubbled into the solution for 5 minutes. The mixture was refluxed for 16 h. The mixture was cooled, concentrated and then diluted with 250 mL of deionized water. The product was initially free based by addition of 10 N aq sodium hydroxide, to a pH of 9-9.5, and then by addition of 70 g of AG 1 X-8 anion exchange resin (hydroxide form) and allowing the solution to stir for 2 h. The resin was filtered off and washed with 150 mL of deionized water. The aqueous filtrates were combined and concentrated. The residue was diluted with 200 mL of 2-propanol and filtered. The collected solids were rinsed with 100 mL of 2-propanol. The organic filtrates were combined and concentrated. The residue was distilled under reduced pressure to afford 12.85 g of the product (6) as a colorless oil (bp 68°C at 0.1-0.2 mm Hg; 640%): ^{1}H-NMR (200 MHz, D20); 3.52 (t, 2H), 2.56 (tg 2H) 3 2.31 (s, 3H), and 1.65-1.43 (m, 4H) PPM.

Preparation of 4-(N-Methyl-trifluoracetamido)-1-butanol (7)

To a 250 mL round bottom flask, charged with 10.0 g (96.9 mmol) of the amine (6) in 100 mL of dry methanol, was added 17.5 mL (147 mmol) of ethyl trifluoroacetate. The mixture was stirred at room temperature for 24 h and then concentrated to afford 18.55 g of the product (7) as a near colorless oil (96%): ^{1}H-NMR (200 MHz, D_2O); 3.63 and 3.50 (2t's, 4H), 3.20 and 3.05 (d and s, 3H), 1.82-1.47 (m, 4H) ppm.

Preparation of 1-(p-Toluenesulfonyloxy)-4-(N-Methyl-trifluoroacetamido)butane (8)

To a 1 L dry round bottom flask, charged with 17.0 g (85.4 mmol) of the alcohol (7) in 400 mL of methylene chloride, was added 17.1 g (89.7 mmol, 1.05 equiv) of toluenesulfenyl chloride followed by 30 mL (213 mmol, 2.5 equiv) of triethylamine at 0°C over a 10 minute period. The mixture was stirred at 0°C to room temperature for 15 h and then washed with 5% v/v aqueous HCl (3 x 200 mL). The organic phase was dried over magnesium sulfate, filtered and concentrated. The residue was chromatographed on silica gel, eluting with 50:50 hexane/methylene chloride and then with methylene chloride, to give 25.1 g of the product (8) as a pale yellow oil (83%): ^{1}H-NMR (200 MHz, $CDCl_3$); 7.80 (d, 2H), 7.37 (d, 2H), 4.07 (m, 2H), 3.41 (m, 2H), 3.09 and 2.98 (q and s, 3H), 2.45 (s, 3H), and 1.68 (m, 4H) ppm: TLC (methylene chloride) R_f = 0.31.

Preparation of 1-S-(2,3,4,6-tetra-0-acetyl-ß-D-galactopyranosyl)-2-thiopseudourea hydrobromide (9)

To a 250 mL round bottom flask, charged with 5.08 g (60.8 mmol, 1.09 equiv) of thiourea and 35 mL of acetone, was added 25.0 g (66.7 mmol) of tetra-acetyl-α-D-galactopyranosyl bromide. The mixture was

stirred at reflux for 15-20 minutes and then cooled on ice. The mixture was filtered into a Buchner funnel and rinsed with 25 mL of ice cold acetone. The solids were treated with 50 mL of acetone, refluxed for 15 minutes, cooled on ice and filtered. The solids were rinsed with 25 mL of cold acetone, air dried and then dried under vacuum to give 22.6 g of the product (9) as a white solid (76%): ^{1}H-NMR (200 MHz, d_6-DMSO); 9.4-9.0 (broad d, 4H), 5.63 (d, 1H) 5.38 (d, 1H), 5.23 (dd, 1H) 5.09 (t, 1H), 4.40 (t, 1H), 4.10 (dd, 1H); 4.04 (dd, 1H), 2.13 (s, 3H), 2.08 (s, 3H), 2.00 (s, 3H), and 1.93 (s, 3H) ppm.

Preparation of 4-(N-Methylaminobutyl)-1-thio-ß-D-galactopyranoside (10)

To a 500 mL round bottom flask charged with 20.7 g (42.5 mmol, 1.07 equiv) of the thiopseudourea hydrobromide (9) in 70 mL of deionized water, was added 6.4 g (46.3 mmol, 1.16 equiv) of potassium carbonate and 4.7 g (45.2 mmol, 1.13 equiv) of sodium bisulfite followed immediately by 14.1 g (39.9 mmol, 1.0 equiv) of the tosylate in 70 mL of acetone. The mixture was stirred at room temperature for 16 h. The mixture was then diluted with 50 mL of brine and extracted with ethyl acetate (3 x 200 mL). The organic extracts were combined, dried over magnesium sulfate, filtered and concentrated. The residue was chromatographed on silica gel, eluting first with 75% methylene chloride/hexane, followed by methylene chloride, then with 2% methanol/methylene chloride and finally with 10% methanol/methylene chloride. Fractions containing alkylation product with, different degrees of acetylation, were combined and concentrated. The residue was diluted with 250 mL of methanol and 150 mL of deionized water and treated with 1.10 g of AG-1 X-8 resir (hydroxide form; 2.6 m equiv/g dry weight). The mixture was stirred at room

temperature for 18 h. The mixture was filtered and the resin was rinsed with methanol (2 x 150 mL). The filtrates were combined and concentrated to afford 6.1 g of the product (10); (54%): ^{1}H-NMR (200 MHz, D_2O); 4.38 (d, 1H), 3.88 (d, 1H), 3.69-3.41 (m, 5H), 2.82-2.64 (m, 4H), 2.43 (s, 3H), and 1.68-1.57 (m, 4H) ppm.

Preparation of Biotin Bis-Methyl Ester (11)

To a 50 mL round bottom flask, charged with 1.00 g (3.23 mmol 1.13 equiv) of amine hydrochloride 4 and 1.30 g (2.86 mmol) of caproamidobiotin NHS-ester and 10 mL of dry dimethylformamide, was added 1.5 mL (10.6 mmol) of triethylamine. The mixture was stirred at 85°C for 2 h and then concentrated via reduced pressure rotary evaporation. The residue was chromatographed on silica gel, eluting with 75:25:0.05 ethyl acetate/methanol/acetic acid, to afford 1.63 g of the product (11) as a white foamy solid (93%): ^{1}H-NMR (200 MHz, d_6-DMSO); 7.72 (t, 1H)2 6.41 (s, 1H), 6.34 (s, 1H), 4.29 (m, 1H), 4.11 (m, 1H), 3.57 (s, 6H), 3.23-2.91 (m, 7H) 2.81 (dd3 1H), 2.55 (d, 1H), 2.35-2.13 (m, 6H), 2.03 (t, 2H) 1.65-1.10 (m, 24H) ppm: TLC; R_f = 0.58 (75:25:01 ethylacetate/methanol/acetic acid).

Preparation of Biotin Bis-Acid (12)

To a 200 mL round bottom flask, charged with 1.61 g (2.63 mmol) of the bis-methylester (11) and 50 mL of methanol, was added 5 mL of 3 N aq sodium hydroxide. The mixture was stirred at 40°C for 3 h and then concentrated via reduced pressure rotary evaporation. The residue was diluted with 50 mL of deionized water and then 3 N aq HCl was added until a pH 1-2 was attained. The mixture was again concentrated. The residue was chromatographed on C-18 reverse phase silica gel, eluting first with 20:80:0.1

acetonitrile/water/trifluoroacetic acid and then with 50:50:0.1 acetonitrile/water/trifluoroacetic acid. The fractions containing product (2) were combined and concentrated. The residue was diluted with 40 mL of water and 20 mL of acetonitrile. The solution was frozen (-70°C) and lyophilized to afford 1.42 g of the product (12) as a fluffy white solid (92%): ^{1}H-NMR (200 MHz, d_6-DMSO); 7.72 (t, 1H), 6.61 (broad s, 2H), 4.29 (m, 1H), 4.11 (m, 1H), 3.35-2.93 (m, 7H), 2.81 (dd, 1H), 2.55 (d, 1H), 2.28-2.12 (m, 6H), 2.03 (t, 2H) and 1.68-1.10 (m, 24H) ppm: TLC; R_f = 0.30 (50:50:0.1 acetonitrile/water/trifluoroacetic acid).

Preparation of Biotin Tetra-Methyl Ester (13)

To a 50 mL round bottom flask, charged with 350 mg (0.599 mmol) of the biotin bisacid (12), 402 mg (1.30 mmol, 2.16 equiv) of amine hydrochloride 4 and 10 mL of dry dimethylformamide, was added 556 mg (1.26 mmol, 2.10 equiv) of benzotriazol-lyloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) and 500 AL (3.54 mmol, 5.91 equiv) of triethylamine. The mixture was stirred at room temperature for 2 h and then concentrated via reduced pressure rotary evaporation. The residue was chromatographed on C-18 reverse phase silica gel, eluting first with 50:50 methanol/water and then with 85:15 methanol/water, to afford 618 mg of the product (13) as a foamy white solid (95%): ^{1}H-NMR 9200 MHz, d_6-DMSO); 7.71 (t, 1H), 6.41 (broad s, 2H), 4.29 (m, 1H), 4.11 (m, 1H), 3.57 (s, 12H), 3.25-2.,91 (m, 15H), 2.81 (dd,1H), 2.55 (d, 1H), 2.35-2.12 (m, 14H), 2.02 (t, 2H), and 1.65-1.10 (m, 48H) ppm: TLC; R_f = 0.48 (85:15 methanol/water).

Preparation of Biotin Tetra-Acid (14)

To a 50 mL round bottom flask, charged with 350 mg (0.319 mmol) of tetra-ester 13 and 15 mL of methanol, was added 5 mL of 1 N aq sodium hydroxide and 5 mL of deionized water. The mixture was stirred at room temperature for 14 h and then concentrated via reduced pressure rotary evaporation. The residue was diluted with 15 mL of deionized water, acidified to pH 1-2 by addition of 6 N aq HCl and then reconcentrated. The residue was chromatographed on C-18 reverse phase silica gel, eluting first with 50:50 methanol/water and then with 70:30 methanol/water. The fractions containing the product (1-4) were combined and concentrated. The residue was diluted with 10 mL water and 8 mL of acetonitrile. The solution was frozen (-70°C) and lyophilized to afford 262 mg of the product (14) as a fluffy white solid (79%): ^{1}H-NMR (200 MHz, d_6-DMSO); 7.71 (t, 1H), 6.41 (s, 1H), 6.34 (s, 1H), 4.29 (m, 1H), 4.11 (m, 1H), 3.25-2.93 (m, 15H), 2.81 (dd, 1H), 2.55 (d, 1H), 2.31-2.10 (m, 14H), 2.02 (t, 2H), and 1.63-1.09 (m, 48H) ppm: TLC; R_f = 0.45 (70:30 methanol/water.

Preparation of Biotin Octa-Methyl Ester (15)

To a 25 mL round bottom flask, charged with 220 mg (0.710 mmol, 4.93 equiv) of amine hydrochloride 4, 150 mg (0.144 mmol) of biotin tetra-acid 14 and 5 mL of dry dimethylformamide, was added 300 mg (0.678 mmol, 4.71 equiv) of BOP followed by 500 μL of dry triethylamine (3.54 mmol, 24.0 equiv). The mixture was stirred at room temperature for 3 h and then concentrated via reduced pressure rotary evaporation. The residue was chromatographed on C-18 reverse phase silica gel, eluting first with 60:40 methanol/water and then with 90:10 methanol/water, to afford 246 mg of the product (L5) as a foamy white solid (83%): ^{1}H-NMR (200 MHz, d_6-DMSO); 7.71 (s 1H), 6.41 (s, 1H),

6.34 (s, 1H), 4.29 (m, 1H), 4.11 (m, 1H), 3.57 (s, 24H), 3.25-2.91 (m, 31H), 2.81 (dd, 1H), 2.55 (d, 1H), 2.32-2.12 (m, 30H), 2.02 (t; 2H), and 1.65-1.08 (m, 96H) ppm: TLC; R_f = 0.42 (90:10 methanol/water).

Preparation of Biotin Octa-Acid (16)

To a 50 mL round bottom flask, charged with 235 mg (0.114 mmol) of biotin octamethyl ester (15) and 10 mL of methanol, was added 5 mL of 1 N aq sodium hydroxide and 5 mL of deionized water. The mixture was stirred at room temperature for 14 h and then concentrated via reduced pressure rotary evaporation. The residue was diluted with 10 mL of deionized water, acidified to pH 1-2 with 6 N aq HCl and reconcentrated. The residue was chromatographed on C-18 reverse phase silica gel, eluting first with 50:50 methanol/water and then with 75:25 methanol/water. The fraction containing the product (16) were combined and concentrated. The residue was diluted with 20 mL of 1:1 acetonitrile/water. The solution was frozen (-70°C) and lyophilized to give 202 mg of the product (L6) as a fluffy white solid (91%): ^{1}H-NMR (200 MHz, d_6-DMSO); 7.71 (t, 1H), 6.41 (s, 1H), 6.34 (s, 1H), 4.29 (ml 1H), 4.11 (m, 1H), 3.25-2.91 (m, 31H), 2.81 (dd, 1H), 2.55 (d, 1H), 2.31-2.10 (m, 30H), 2.03 (t, 2H), and 1.65-1.05 (m, 96H) ppm: TLC; R_f = 0.51 (75:25 methanol/water).

Preparation of Biotin Hexadeca-Methyl Ester (17)

To a 25 mL round bottom flask, charged with 154 mg (0.497 mmol, 10.0 equiv) of amine hydrochloride (4), 97 mg (0.0497 mmol) of biotin octa-methyl ester (16), and 5 mL of dry dimethylformamide, was added 202 mg (0.457 mmol, 9.2 equiv) of BOP followed by 500 μL (3.54 mmol, 71.2 equiv) of triethylamine. The mixture was stirred at room temperature for 8 h and then concentrated via reduced pressure rotary evaporation.

The residue was chromatographed on silica gel, eluting first with 70:30 methanol/water and then with 95:5 methanol/water, to afford 149 mg of the product (17) as a foamy white solid (75%): ^{1}H-NMR (200 MHz, D_6-DMSO); 7.71 (t, 1H), 6.41 (s, 1H), 6.34 (s, 1H), 4.29 (m, 1H), 4.12 (m, 1H), 3.57 (s, 48H), 3.25-2.92 (m, 63H), 2.81 (dd3 1H), 2.55 (d, 1H), 2.35-2.11 (m, 62H), 2.01 (t, 2H), and 1.65-1.08 (m, 192H) ppm: TLC; R_f = 0.31 (95:5 methanol/water).

Preparation of Biotin Hexadecyl-Acid (18)

To a 50 mL round bottom flask, charged with 141 mg (0.0353 mmol) of biotin hexadecyl-methyl ester 17 and 15 mL of methanol, was added 8 mL of 1 N aqueous sodium hydroxide and 5 mL of deionized water. The mixture was stirred at room temperature for 14 h and then concentrated via reduced pressure rotary evaporation. The residue was diluted with 15 mL of deionized water, acidified to pH 1-2 with 1 N aqueous HCl and then reconcentrated. The residue was chromatographed on C-18 reverse phase silica gel, eluting first with 60:40 methanol/water and then with 85:15 methanol/water. The fraction containing the product (18) were combined and concentrated. The residue was diluted in 20 mL of 1:1 acetonitrile/water. The solution was frozen (-70°C) and lyophilized to afford 130 mg of the product (18) as a fluffy white solid: (75%): ^{1}H-NMR (200 MHz, D_6-DMSO); 7.71 (s 1H), 6.41 (s, 1H), 6.34 (s, 1H), 4.29 (m, 1H), 4.11 (m, 1H), 3.26-2.92 (m, 63H), 2.81 (dd, 1H), 2.55 (d, 1H), 2.35-2.10 (m, 62H), 2.01 (t, 2H), 1.65-1.09 (m, 192H) ppm: TLC; R_f 0.64 (85:15 methanol/water).

Preparation of Hexadeca-Galactosyl Biotin (19)

To a 25 mL round bottom flask, charged with 125 mg (0.0332 mmol) of biotin hexadeca-acid (18), 179 mg

(0.636 mmol, 19.2 equiv) of galactose-amine 10, and 4 mL of dry dimethylfurmamide, was added 264 mg (0.597 mmol, 18.0 equiv) of BOP followed by 400 mL (2.87 mmol, 86.5 equiv) of dry triethylamine. The mixture was stirred at room temperature for 17 h and then concentrated via reduced pressure rotary evaporation. The residue was chromatographed on C-18 reverse phase silica gel, eluting first with 60:40 methanol/water and then with 75:25 methanol/water. The fractions containing the product (19) were combined, concentrated and rechromatographed on C-18 reverse phase silica gel, eluting first with 40:60:0.1 acetonitrile/water/trifluoroacetic acid and then with 50:50:0.1 acetonitrile/water/trifluoroacetic acid. The fractions containing the product (19) were again combined and concentrated. The residue was dissolved in 20 mL of water. The solution was frozen (-70°C) and lyophilized to afford 173 mg at the product as a fluffy white solid (65%): ^{1}H-NMR (200 MHz, D_2O); 4.52 (m, 1H), 4.37 (d, 17H), 3.90 (d, 16H), 3.70-3.42 (m, 80H), 3.41-3.05 (m, 97H), 2.98-2.82 (2s and m, 49H), 2.80-2.49 (m, 33H), 2.44-2.11 (m, 62H), 1.75-1.10 (m, 256H) ppm: TLC; R, = 0.53 (75:25 methanol/water).

Example XXI

This example demonstrates the *in vivo* efficacy of the subject small molecule clearing agents containing galactose residues and biotin and specifically the $(gal)_{16}$-BT clearing agent for providing for clearance of conjugates during therapeutic pretargeting methods. The protocol of these experiments comprised the evaluation of biodistribution of ^{111}In-DOTA-biotin at 1 μg dose from 2 - 120 hr in SW-1222 tumored nude mice. Four hundred μg of LU-10/SA was administered I.V., and 46 μg of $(GAL)_{16}$-BT was administered 24 hr later. At 27 hr post-MAb/SA, ^{111}In-DOTA-biotin was administered.

The above biodistribution protocol was repeated for a saturating 15 μg dose of ^{111}In-DOTA-biotin in SW-1222 tumored nude mice at the 2 hr post-DOTA-BT time-point only.

The first protocol conducted at the 1 μg biotin dose demonstrated that the pretargeting process works well with the $(GAL)_{16}$-BT clearing agent. Normal tissue backgrounds were low and tumor uptake and retention pharmacokinetics were also good. These results although not as good as the best attained so far, are typical for this SW-1222 colon carcinoma xenograft and are consistent with the observed results with those obtained with gal-HSA-BT clearing agents.

The second protocol conducted at the saturating 15 μg biotin dose further demonstrated that the $(GAL)_{16}$-BT clearing agent does not accrete heavily in tumor at the 2 hr post-DOTA-BT time point. A mole ratio of 2.65:1 DOTA-BT:MAb/SA was attained indicating that although not the ideal 4:1 biotins/streptavidin attained with BT-HSA-gal, the $(GAL)_{16}$-BT did not compromise tumor appreciably by localizing to pretargeted conjugate.

However, it should be noted that the $(GAL)_{16}$-BT clearing agent used does not have a stabilized biotin linkage. Therefore it may release BT quickly post-hepatic processing potentially blocking some of the prelocalized SA by 3 hr when the ^{111}In-DOTA-biotin is administered.

Accordingly, the results of these experiments indicate that small molecule clearing agents containing biotin and galactose are highly effective. It is believed that because $(GAL)_{16}$-BT weighs only 8,000 daltons, it distributes into a larger volume of distribution than the BT-HSA-gal clearing agent (m.w. greater than 66,000 daltons) and is apparently able to bind and clear more conjugate from vascular and extravascular space. It was initially hypothesized

that the smaller compounds might reach the tumor and compromise biotin binding there. However, this experiment demonstrates that $(GAL)_{16}$-BT surprisingly does not strongly compromise prelocalized biotin binding sites by immediate uptake. Thus, in spite of its small size, the $(GAL)_{16}$-BT is apparently being effectively cleared by dual processes of renal excretion and hepatic uptake via the Ashwell receptor. It is additionally hypothesized that the $(GAL)_{16}$-BT clustered galactose sugars may bind more strongly to the Ashwell receptor than the gal-HSA-BT clearing agent because the array of galactose on $(GAL)_{16}$-BT may be better matched to the structure of the Ashwell receptor.

Example XXII

Experiments were designed and executed to evaluate a particular small molecule biotin-galactose construct: $(gal)_{16}$-biotin (structure 1 shown in Fig. 18). BALB/c female mice (20-25 g) were injected i.v. with 120 μg of LU-10/streptavidin conjugate with I-125 and blood was serially collected from n=3 mice. The clearance of conjugate from the blood was measured (control, Fig. 19). Separate groups of mice were injected with either 120 or 12 μg of radiolabeled which had been precomplexed with $(gal)_{16}$-biotin by mixing the biotin analog at a 20-fold molar excess with the antibody conjugate, and purifying the excess small molecule from the protein by size-exclusion chromatography. As shown in Fig. 19, both doses of precomplexed conjugate showed extremely rapid clearance from the blood, relative to the antibody conjugate control.

Having shown that pre-complexed material could clear rapidly and efficiently from the blood, experiments were conducted to measure the effectiveness of various doses of $(gal)_{16}$-biotin to

form rapidly clearing complexes *in vivo*. Mice received 400 μg of I-125 LU-10/streptavidin (LU-10/SA) i.v., and approximately 22 hours later, received $(gal)_{16}$-biotin i.v. at doses of 100, 50 or 10 : 1 molar excess to circulating LU-10/SA. Fig. 20 shows the blood clearance of conjugate in each group. While it is apparent that each dose was effective at clearing conjugate, the most effective dose (both kinetic and absolute) was the 10 : 1 (45 μg) dose. Fig. 21 shows an expansion of the time frame from administration of the clearing agent to about four hours later. For the larger doses, there is apparently some saturation of the liver receptor, for both doses show a plateau in conjugate clearance for about an hour after administration of $(gal)_{16}$-biotin. These doses are probably sufficiently high to achieve competing levels of non-conjugate bound $(gal)_{16}$-biotin at the liver, which preclude all but the first fraction of complexed conjugate from being removed from the blood. After this plateau period, clearing of conjugate is still slow and eventually less complete than that achieved with the lower (45 μg) dose of $(gal)_{16}$-biotin (approximately 10% conjugate levels remained versus only 2% in the lower dose group). This version of the $(gal)_{16}$-biotin construct was not stabilized to potential biotinidase-mediated cleavage of the biotin portion of the small molecule. While the stability of this construct has yet to be measured, it is possible that the release of biotin from $(gal)_{16}$-biotin was high enough at the higher doses (456 and 228 μg) that a significant portion of circulating conjugate became blocked with this released biotin, and was therefore not cleared via galactose-mediated hepatic uptake. Evident in all groups is a lack of "rebound" or gradual increase in blood levels of circulating conjugate following disruption of equilibrium between vascular and

extravascular concentrations of conjugate. This provides strong evidence that small molecule clearing agents to extravasate into extravascular fluid and that conjugate which is complexed extravascularly clears very rapidly when it passes back into the vascular compartment.

Further experimentation in the same animal model compared $(gal)_{35}$HSA-$(biotin)_2$ to decreasing doses of $(gal)_{16}$-biotin as *in vivo* clearing agents. As shown in Fig. 22, a 46 μg dose of $(gal)_{16}$-biotin was found to be optimal and more effective than the previously optimized dose of $(gal)_{35}$-HSA-$(biotin)_2$. Lower (12 and 23 μg) and higher (228 μg) doses of $(gal)_{16}$-biotin were less efficient at removing circulating conjugate, and the lower doses showed a significant rebound of levels, indicating that incomplete complexation with circulating conjugate had occurred.

Having shown that effective clearing could be achieved with the appropriate dose of $(gal)_{16}$-biotin, studies were undertaken in tumored nude mice to evaluate the potential blockade of tumor-associated conjugate by the small molecule $(gal)_{16}$-biotin construct. Mice bearing either SW-1222 (colon) tumor xenografts or SHT-1 (SCLC) tumor xenografts were pretargeted with LU-10/SA and, 22 hours later, received 46 μg of $(gal)_{16}$-biotin. After 2 hours ^{90}Y-DOTA-biotin was administered and its uptake and retention in tumor and non-target tissues was evaluated by sacrifice and tissue counting for radioactivity 2 hours after administration. Comparison of the biodistributions are shown in Fig. 23, as compared to historical controls utilizing $(gal)_{35}$-HSA-$(biotin)_2$ as the clearing agent. Tumor targeting was slightly lower in the high antigen-expressing colon tumor but was slightly higher in the low antigen-expressing SCLC tumor. Given the normal variability in such experiments, tumor uptake was

assessed as roughly equivalent to that achieved with the HSA clearing agent. A surprising result considering the potential for target uptake by the small molecule $(gal)_{16}$-biotin, Non-target organ uptake was comparable in all tissues except liver, where animals receiving $(gal)_{16}$-biotin showed slightly higher levels. The historical experimental controls were done allowing a 3 hour period to elapse between $(gal)_{35}$-HSA-$(biotin)_2$ administration and injection of DOTA-biotin. When a 3 hour period was allowed for $(gal)_{16}$-biotin (Fig. 24), liver levels were lower and equivalent to those seen with the HSA clearing agent (~1% ID/g).

Experiments were also carried out using I-125 labeled LU-10/SA In-111 labeled DOTA-biotin to assess the relative stoichiometry of these materials at the tumor when $(gal)_{16}$-biotin was used as a clearing agent. Previous studies with $(gal)_{35}$-HSA-$(biotin)_2$ had shown that an expected 4:1 ratio of DOTA-Biotin to LU-10/SA could be achieved at the tumor with the optimized dose of this clearing agent. When a similar protocol was used with $(gal)_{16}$-biotin, the ratio of DOTA-Biotin to LU-10/SA was only 2.65 (Fig. 25). This indicated that, indeed, some filling of tumor-associated streptavidin had occurred, though it is still unclear whether the material responsible for this blocking was the $(gal)_{16}$-biotin or merely biotin released from this construct. Experiments to assess the nature of this blockade are underway.

In summary, $(gal)_{16}$-biotin has proven to be a more effective construct for clearing the circulation (both vascular and extravascular spaces) of LU-10/SA conjugate. Despite the apparent blockade of some tumor sites by either $(gal)_{16}$-biotin or biotin released by this agent, efficient tumor targeting can still be achieved using this agent. Stabilization of the linkage between galactosyl residues and biotin may

yield a construct which will not compromise any tumor-associated streptavidin.

Kits containing one or more of the components described above are also contemplated. For instance, radiohalogenated biotin may be provided in a sterile container for use in pretargeting procedures. A chelate-biotin conjugate provided in a sterile container is suitable for radiometallation by the consumer; such kits would be particularly amenable for use in pretargeting protocols. Alternatively, radiohalogenated biotin and a chelate-biotin conjugate may be vialed in a non-sterile condition for use as a research reagent.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

WHAT IS CLAIMED IS:

1. An article of manufacture comprising a package having a label and containing a clearing agent suitable for substantially clearing from a mammalian recipient's circulation a previously administered targeting moiety-ligand or targeting moiety-anti-ligand conjugate, wherein the clearing agent comprises:

a ligand or anti-ligand component which binds with high affinity to previously administered conjugate; and

a clearance-directing component;

wherein the clearing agent, upon administration to the recipient, binds to circulating targeting moiety-ligand or targeting moiety-anti-ligand conjugate and directs the clearance thereof via a liver receptor-based mechanism;

and wherein the label identifies the ligand or the anti-ligand component and the clearance-directing component of the clearing agent.

2. An article of manufacture of claim 1 wherein the package or the label further identifies a targeting moiety-ligand or targeting moiety-anti-ligand conjugate suitable for use with the clearing agent.

3. An article of manufacture of claim 1 wherein the package or the label indicates that the clearing agent is limited to investigational use or indicates an indication for which the clearing agent has been approved for use in humans.

4. An article of manufacture of claim 3 wherein the indication is small cell lung cancer, and the

targeting moiety binds to an antigen associated with small cell lung cancer.

5. An article of manufacture of claim 4 wherein the antigen is the NR-LU-10 antigen.

6. An article of manufacture of claim 1 wherein the clearing agent is contained within a vial.

7. An article of manufacture of claim 6 wherein the clearing agent is vialed in a sterile, pyrogen-free environment.

8. A clearing agent for increasing blood clearance or decreasing *in vivo* non-target binding capability of a circulating targeting moiety-ligand or targeting moiety-anti-ligand conjugate in a mammalian recipient, wherein the clearing agent comprises:

a binding moiety comprising a lower affinity ligand or anti-ligand complementary to the ligand-anti-ligand pair member of the circulating conjugate and capable of associating with the circulating conjugate; and

a clearance-directing moiety bound to, complexed with or otherwise associated with the binding moiety, and wherein, upon becoming associated with the circulating conjugate, the clearing agent enhances the clearance of the circulating conjugate from the blood or diminishes *in vivo* binding of the circulating conjugate for a subsequently administered active agent conjugate.

9. A clearing agent of claim 8 wherein the clearance-directing moiety is recognized by a hepatocyte receptor.

10. A clearing agent of claim 9 wherein the clearance-directing moiety bears an exposed galactose or mannose residue or is capable of derivitization to provide such an exposed residue.

11. A clearing agent of claim 8 wherein the clearance-directing moiety exhibits physical properties that limit access of the clearance-directing moiety to target sites.

12. A clearing agent according to claim 8 wherein the binding moiety is a lower affinity ligand.

13. A clearing agent according to claim 12 wherein the binding moiety is a lower affinity biotin, capable of binding with avidin or streptavidin.

14. A clearing agent according to claim 13 wherein the lower affinity form of biotin is selected from the group consisting of 2'-thiobiotin; 1'-N-methoxycarbonyl-biotin; 3'-N-methoxycarbonylbiotin; 1-oxy-biotin; 1-oxy-2'-thiobiotin; 1-sulfoxide-biotin; 1-sulfoxide-2'-thiobiotin; 1-sulfone-biotin; 1-sulfone-2'-thio-biotin; desthiobiotin; dl-desthiobiotin methyl ester; dl-desthiobiotinol; D-4-n-hexyl-imidazolidone; L-4-n-hexylimidazolidone; dl-4-n-butyl-imidazolidone; dl-4-n-propylimidazolidone; dl-4-ethyl-imidazolidone; dl-4-methylimidazolidone; imidazolidone; dl-4,5-dimethylimidazolidone; meso-4,5-dimethylimidazolidone; dl-norleucine hydantoin; D-4-n-hexyl-2-thiono-imidazolidine; d-4-n-hexyl-2-imino-imidazolidine; D-4-n-hexyl-oxazolidone; D-5-n-hexyloxazolidone; [5-(3,4-diamino-thiophan-2-yl] pentanoic acid; and lipoic acid.

15. A clearing agent according to claim 13 wherein the lower affinity form of biotin is selected

from the group consisting of 2'-thiobiotin; 1'-N-methoxycarbonyl-biotin; 3'-N-methoxycarbonylbiotin; 1-oxy-biotin; 1-oxy-2'-thiobiotin; 1-sulfoxide-biotin; 1-sulfoxide-2'-thiobiotin; 1-sulfone-biotin; 1-sulfone-2'-thio-biotin; desthiobiotin; dl-desthiobiotin methyl ester; dl-desthiobiotinol; D-4-n-hexyl-imidazolidone; and L-4-n-hexylimidazolidone.

16. A clearing agent according to claim 8 wherein the clearance-directing moiety is proteinaceous.

17. A clearing agent according to claim 8 wherein the clearance-directing moiety comprises human serum albumin, non-immunogenic serum soluble protein, polyglutamate, polylysine, polyarginine, polyaspartate, IgM or IgG.

18. A method of increasing active agent localization at a target cell site of a mammalian recipient, which comprises:

administering to the recipient a receptor blocking agent in an amount sufficient to substantially block a subpopulation of hepatocyte receptors;

administering to the recipient a first conjugate comprising a targeting moiety, a hepatocyte receptor recognizing agent, and a member of a ligand-anti-ligand binding pair; and

subsequently administering to the recipient a second conjugate comprising an active agent and a ligand/anti-ligand binding pair member, wherein the second conjugate binding pair member is complementary to that of the first conjugate.

19. A method of claim 18 wherein the hepatocyte receptor to be blocked is selected from the group consisting of Ashwell receptor, mannose/N-

acetylglucosamine receptor and mannose 6-phosphate receptor.

20. The method of claim 18 wherein the targeting moiety is a monoclonal antibody or an antigen-recognizing fragment thereof which is reactive with an antigen recognized by the antibody NR-LU-10.

21. A method of delivering an active agent to a targeted *in vivo* site comprising:

(i) administering to a recipient a first conjugate comprising a targeting moiety and a member of a ligand/anti-ligand binding pair; and

(ii) thereafter administering to the recipient a second conjugate comprising an active agent and a ligand/anti-ligand binding pair member, wherein the second conjugate is complementary to the first conjugate, and wherein the ligand and anti-ligand are selected from the group consisting of S-peptide and derivatives and analogs thereof, S-protein and derivatives and analogs thereof, head activator (HA) peptide and derivatives and analogs thereof, cystatin-C, and cathepsin B.

22. The method of claim 21 wherein said method additionally includes an additional step comprising the administration of a clearing agent capable of directing the clearance of circulating first conjugate which does or does not contain a member of the ligand or anti-ligand binding pair or a lower binding affinity derivative thereof.

23. The method of claim 21 wherein the ligand and anti-ligand are both the head activator peptide or a derivative or analog thereof which exhibits autoaffinity.

24. The method of claim 21 wherein the targeting protein is an antibody or antibody fragment and at least one head activator peptide is fused or inserted into the antibody or antibody fragment such that it does not adversely affect antigen binding.

25. The method of claim 24 wherein said antibody or antibody fragment is a Fab fragment, $(Fab)'_2$ fragment, Fv, single chain antibody, chimeric antibody, bispecific antibody or a humanized antibody or a multimer thereof.

26. The method of claim 25 wherein multimerization of said antibody fragment is effected by the incorporation of head activator peptide sequences in the antibody fragment sequence which are comprised in the multimeric protein.

27. The method of claim 25 wherein the antibody is an Fv or dimer thereof, wherein the heavy and light variable regions are fused by head activator peptide sequences.

28. The method of claim 22 wherein the clearing agent comprises a ligand or anti-ligand selected from S-peptide and derivatives and analogs thereof, S-protein and derivatives thereof, head activator peptide, cystatin-C, cathepsin-B, which is directly or directly attached to a clearance directing moiety or moieties.

29. The method of claim 28 wherein said clearance directing moiety or moieties direct clearance via hepatocyte receptors.

30. The method of claim 29 wherein said clearance directing moiety or moieties comprises a hexose-based or non-hexose based moiety.

31. The method of claim 30 wherein the hexose is selected from galactose, glucose, mannose, mannose 6-phosphate, N-acetylglucosamine, pentamannosyl-phosphate, N-galactosamine, thioglycosides of galactose, D-galactosides, galactosamine, N-acetyl-galactosamine and D-glucosides.

32. The method of claim 31 wherein the hexose is galactose.

33. The method of claim 30 wherein the hexose-based moiety is a galactosylated protein.

34. A recombinant antibody molecule or fragment wherein said antibody or antibody fragment contains at least one head activator peptide sequence to facilitate domain attachment or dimerization.

35. The recombinant antibody or antibody fragment of claim 34 which is selected from the group consisting of single chain antibodies, Fab fragments, Fv's, bispecific antibodies, chimeric antibodies, humanized antibodies, and dimers or multimers thereof which bind antigen.

36. A small molecule clearing agent which is capable of effectively clearing a previously, concurrently or subsequently administered conjugate containing a ligand or anti-ligand containing conjugates from the circulation wherein said small molecular weight clearing agent comprises:

(i) a small molecular weight ligand or anti-ligand or low affinity derivative thereof; which has been directly or indirectly attached to

(ii) one or more hexose moieties selected from the group consisting of galactose, glucose, mannose, mannose 6-phosphate, N-acetylglucosamine, N-acetylgalactosamine, thioglycosides of galactose, D-galactosides, N-acetylgalactosamine, D-glucosides; and mixtures thereof.

37. The small molecule clearing agent of claim 36 wherein said hexose is galactose.

38. The small molecule clearing agent of claim 36 wherein the ligand or anti-ligand is selected from biotin, S-peptide, head activator peptide, and low affinity derivatives and analogs thereof.

39. The small molecule clearing agent of claim 38 wherein the ligand is biotin or a low affinity analog thereof.

40. The small molecule clearing agent of claim 39 wherein the hexose is galactose.

41. The small molecule clearing agent of claim 40 wherein at least some of the galactose residues are preferably separated by a distance of at least 25 A^0.

42. The small molecule clearing agent of claim 40 wherein the number of hexose residues range from about 3 to 32.

43. The small molecule clearing agent of claim 42 wherein the number of hexose residues is 16 or 32.

44. The small molecule clearing agent of claim 39 wherein the low affinity biotin analog is selected from the group consisting of 2'-thiobiotin, 1'-N-methoxycarbonyl-biotin, 3'-N-methoxycarbonylbiotin, 1-oxy-biotin, 1-oxy-2'-thiobiotin, biotin-d-sulfoxide, biotin-ℓ-sulfoxide, d- and ℓ-sulfoxide-2'-thiobiotin, biotin sulfone, 2'-thiobiotin sulfone, d-desthiobiotin, ℓ-desthiobiotin, d-desthiobiotinol, ℓ-desthiobiotinol, D-4-n-hexyl-imidazolidone, L-4-n-hexylimidazolidone, d-4-ethyl-imidazolidone, d-4-methyl-imidazolidone, ℓ-4-ethyl-imidazolidone, ℓ-4-methyl-imidazolidone, imidazolidone, ℓ-4,5-dimethylimidazolidone, d-4,5-dimethylimidazolidone, meso-4,5-dimethylimidazolidone, d-norleucine hydantoin, ℓ-norleucine hydantoin, D-4-n-hexyl-2-thiono-imidazolidone, ℓ-4-n-hexyl-2-thioimidazolidone, ℓ-4-n-hexyl-2-iminoimidazolidine, ℓ-4-n-hexyloxazolidone, d-4-n-hexyl-2-imino-imidazolidine, D-4-n-hexyl-oxazolidone, ℓ-5-n-hexyl-oxazolidone, ℓ-5-n-hexyloxazolidone, D-5-n-hexyloxazolidone, [5-(3,4-diamino-thiophan-2-yl] pentanoic acid, lipoic acid, d-4-n-butylimidazolidene, l-4-n-butylimidazolidene, d-4-n-propylimidazolidone, l-4-n-propylimidazolidone or derivatives thereof.

45. The small molecule clearing agent of claim 39 wherein the biotin or a low affinity biotin analog is linked to galactose residues by a linker of a sufficient length to allow for steric effects between the galactose and avidin or streptavidin.

46. An improved biotinylated, galactosylated protein clearing agent wherein the improvement comprises the attachment of the biotin or a biotin analog to the protein via a linker which is resistant to cleavage during clearance and catabolism and/or

which substantially prevents the release of biotin or biotin analogs into the circulation.

47. The clearing agent of claim 46 wherein the linker comprises amino acid sequences, D-amino acids, sugars, and highly charged or polar groups.

48. The clearing agent of claim 47 wherein the linker comprises a tertiary amide linker.

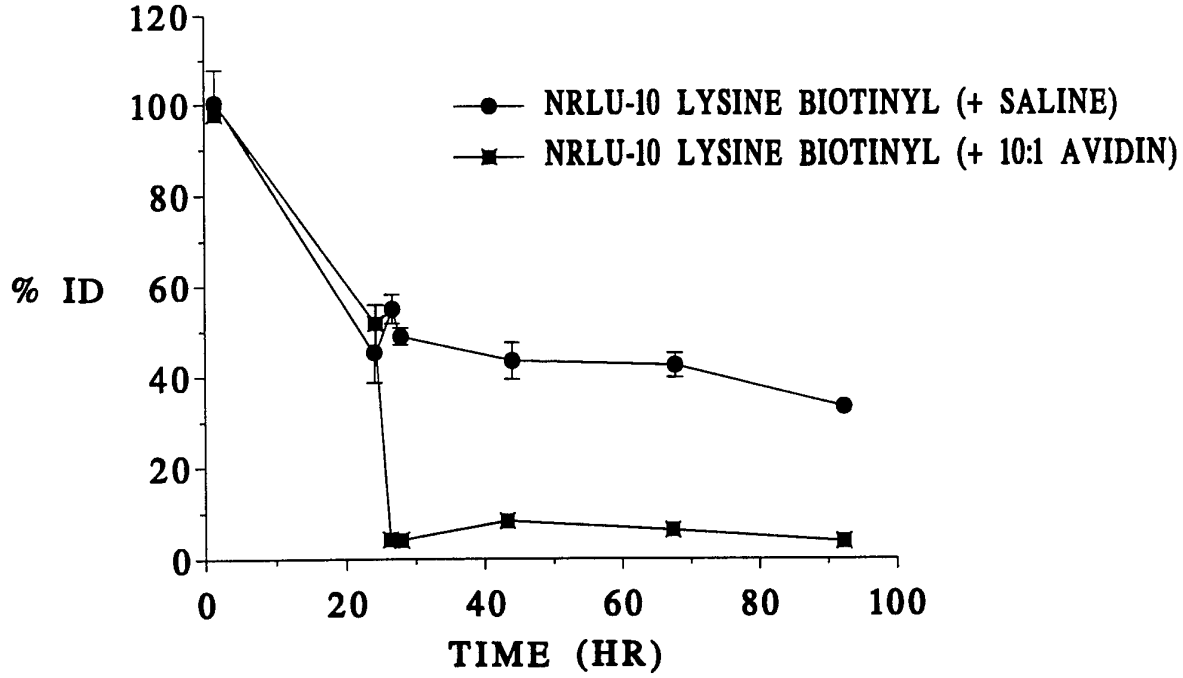

120
100
80
60
40
20
0
% ID
0
20
40
60
80
100
TIME (HR)
NRLU-10 LYSINE BIOTINYL (+ SALINE)
NRLU-10 LYSINE BIOTINYL (+ 10:1 AVIDIN)

Fig. 1

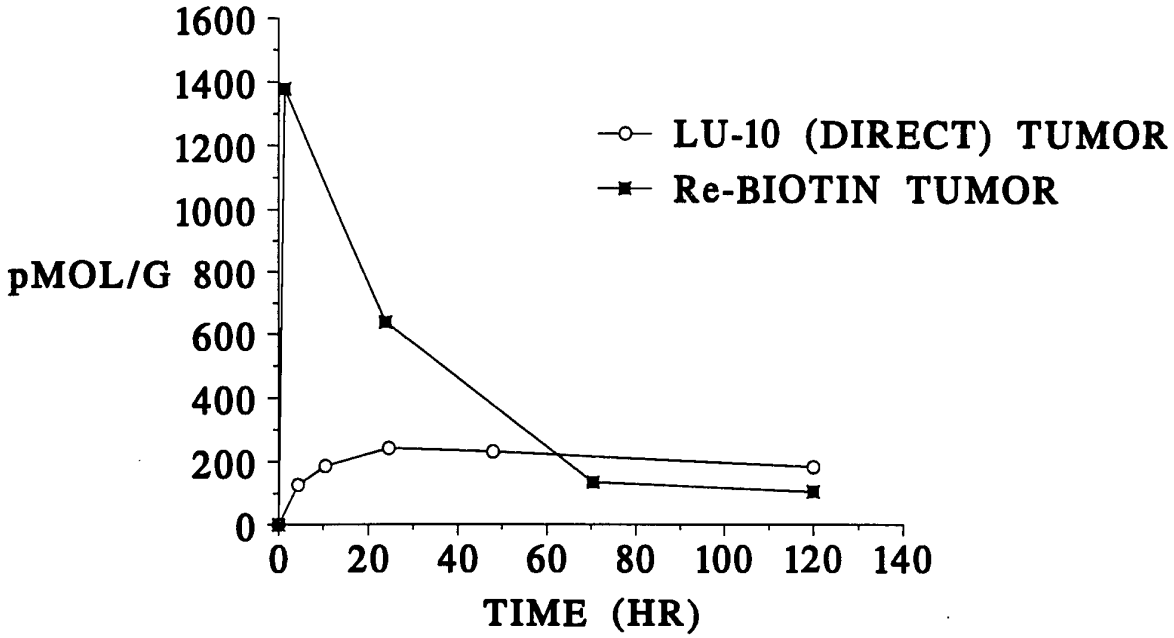

1600
1400
1200
1000
pMOL/G 800
600
400
200
0
0 20 40 60 80 100 120 140
TIME (HR)
LU-10 (DIRECT) TUMOR
Re-BIOTIN TUMOR

Fig. 2

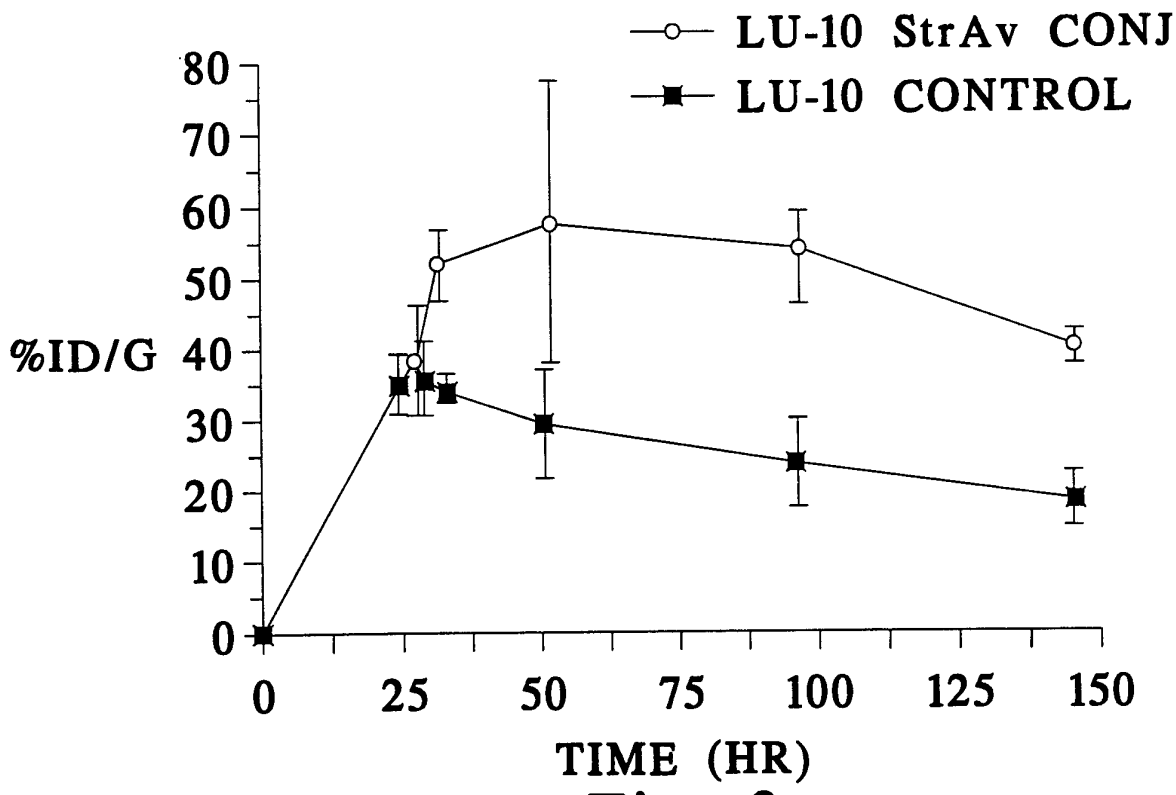

LU-10 StrAv CONJ
LU-10 CONTROL
%ID/G
80
70
60
50
40
30
20
10
0
0
25
50
75
100
125
150
TIME (HR)

Fig. 3

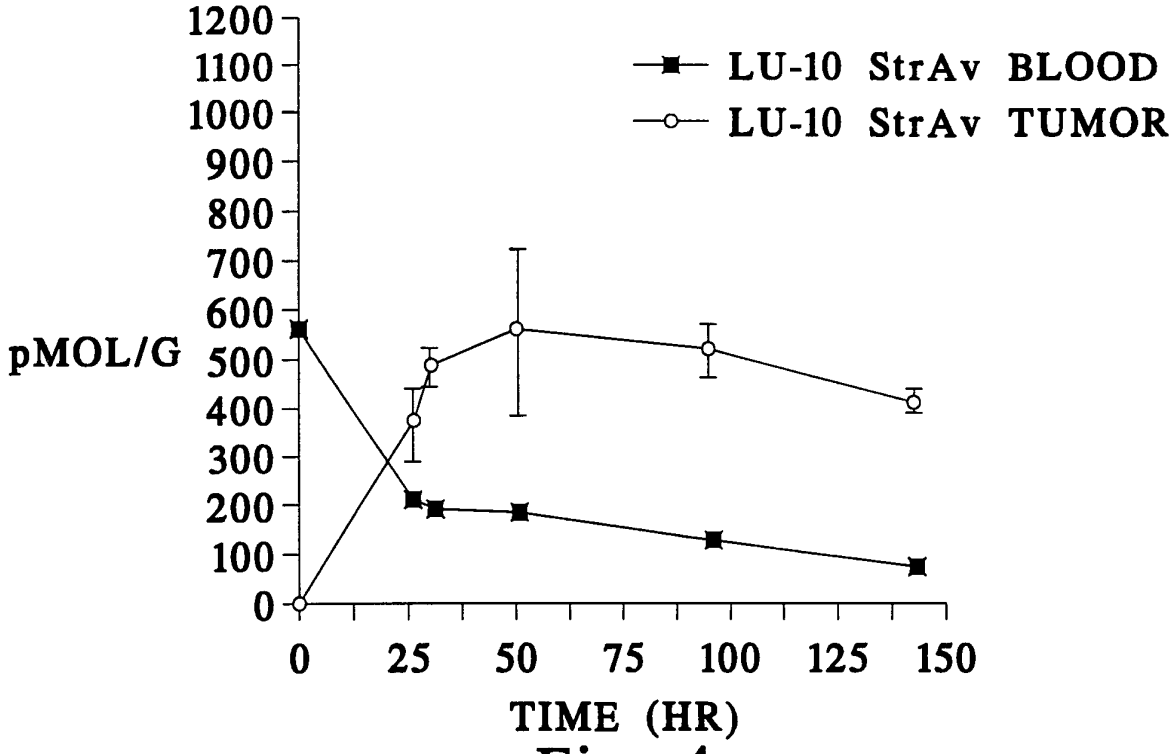

1200
1100
1000
900
800
700
600
500
400
300
200
100
0
pMOL/G
LU-10 StrAv BLOOD
LU-10 StrAv TUMOR
0
25
50
75
100
125
150
TIME (HR)

Fig. 4

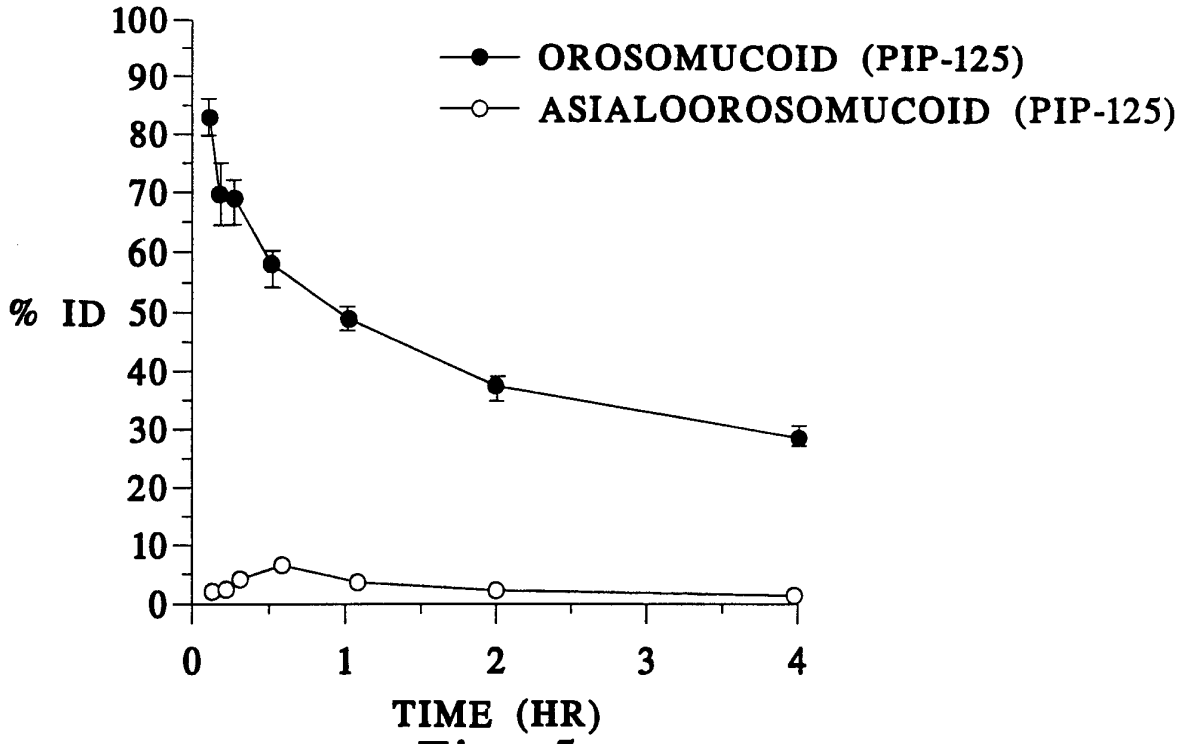

100
90
80
70
60
% ID 50
40
30
20
10
0
0
1
2
3
4
TIME (HR)
OROSOMUCOID (PIP-125)
ASIALOOROSOMUCOID (PIP-125)

Fig. 5

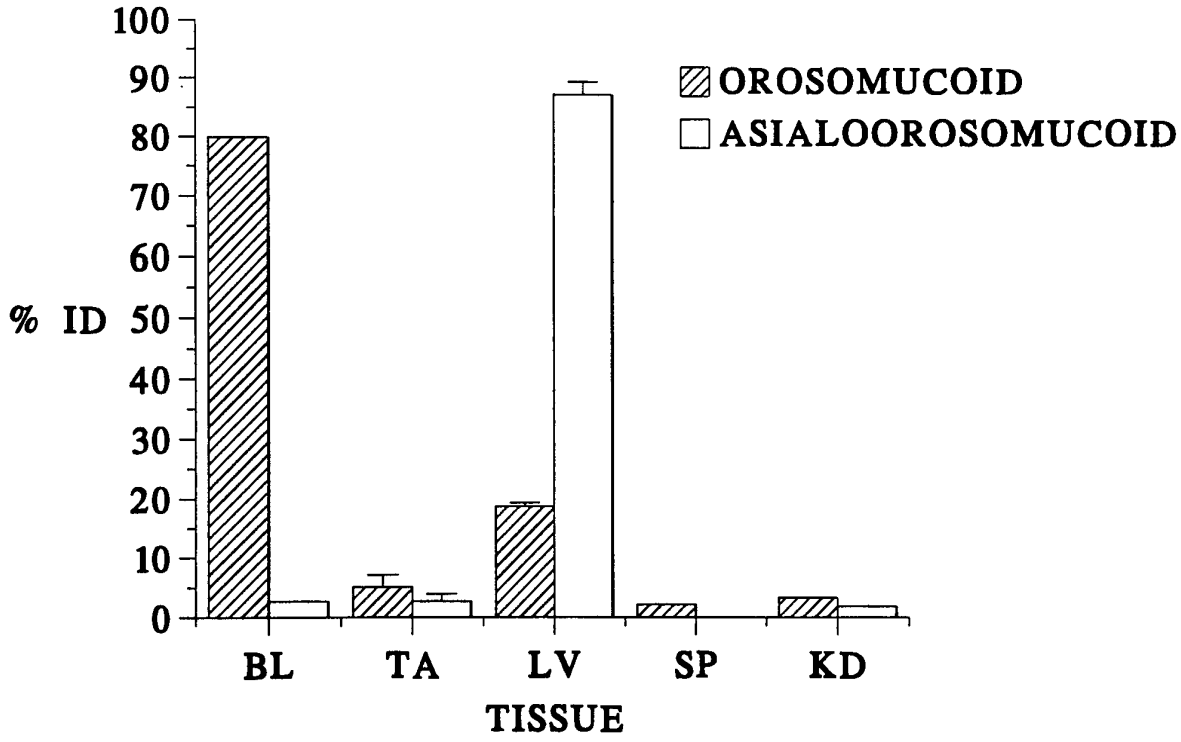

100
90
80
70
60
% ID 50
40
30
20
10
0
OROSOMUCOID
ASIALOOROSOMUCOID
BL
TA
LV
SP
KD
TISSUE

Fig. 6

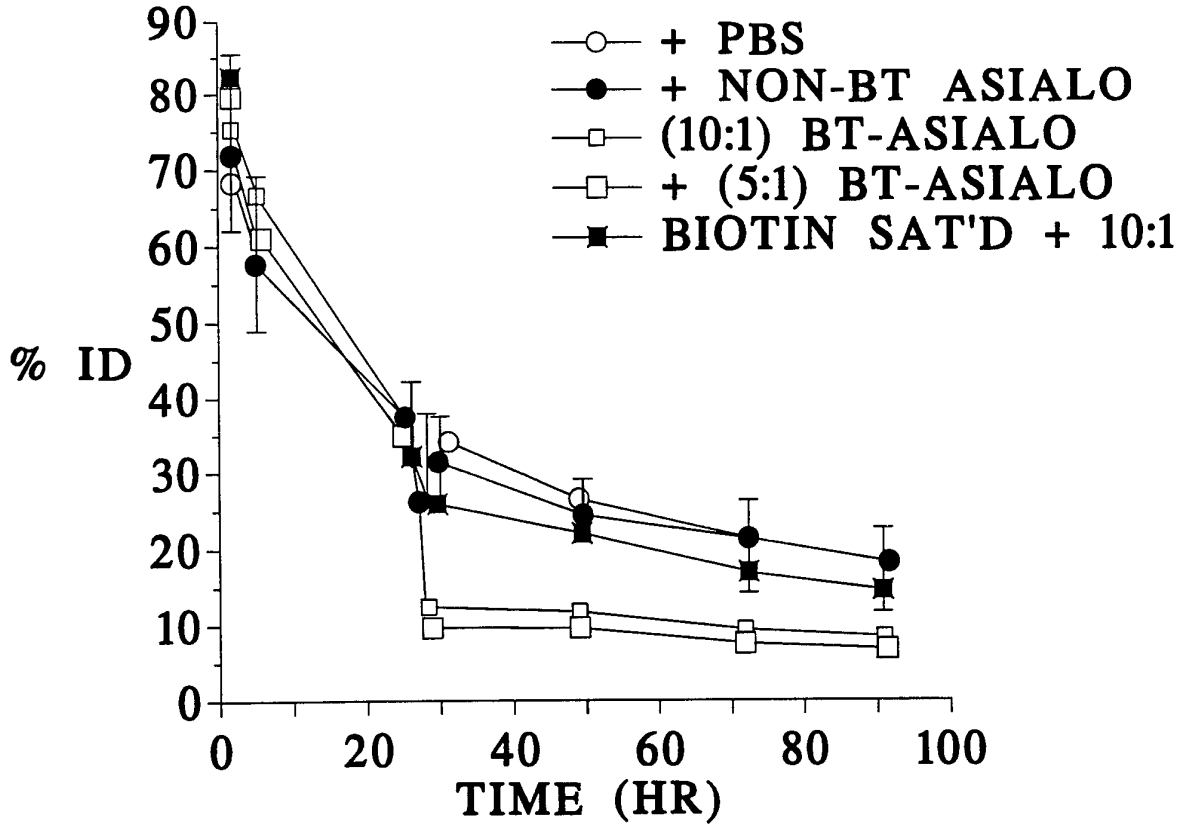

90
80
70
60
50
40
30
20
10
0
% ID
0
20
40
60
80
100
TIME (HR)
+ PBS
+ NON-BT ASIALO
(10:1) BT-ASIALO
+ (5:1) BT-ASIALO
BIOTIN SAT'D + 10:1

Fig. 7

%ID

	+PBS	SD	+NON-BT	SD	+10:1	SD	+5:1	SD	BT-SAT'D	SD
BLOOD	31.05	5.08	29.94	1.35	8.54	0.91	7.03	0.18	24.58	0.68
TAIL	2.43	0.70	1.80	0.09	1.46	0.09	1.76	0.04	1.96	0.40
LUNG	1.47	0.26	1.09	0.22	0.54	0.10	0.48	0.07	0.76	0.01
LIVER	5.42	0.69	4.66	0.36	9.60	1.20	9.11	0.41	6.76	0.06
SPLEEN	0.25	0.05	0.34	0.03	0.17	0.03	0.18	0.00	0.38	0.02
STOMACH	0.28	0.02	0.33	0.03	0.53	0.34	0.49	0.00	0.29	0.04
KIDNEY	1.72	0.24	1.38	0.08	2.76	0.00	3.28	0.32	1.58	0.08
INTESTINE	3.40	0.73	3.44	0.10	4.22	0.02	6.62	0.14	2.83	0.13
	46.02		42.98		27.83		28.95		39.13	
	Group 1		Group 2		Group 3		Group 4		Group 5	

Fig. 8

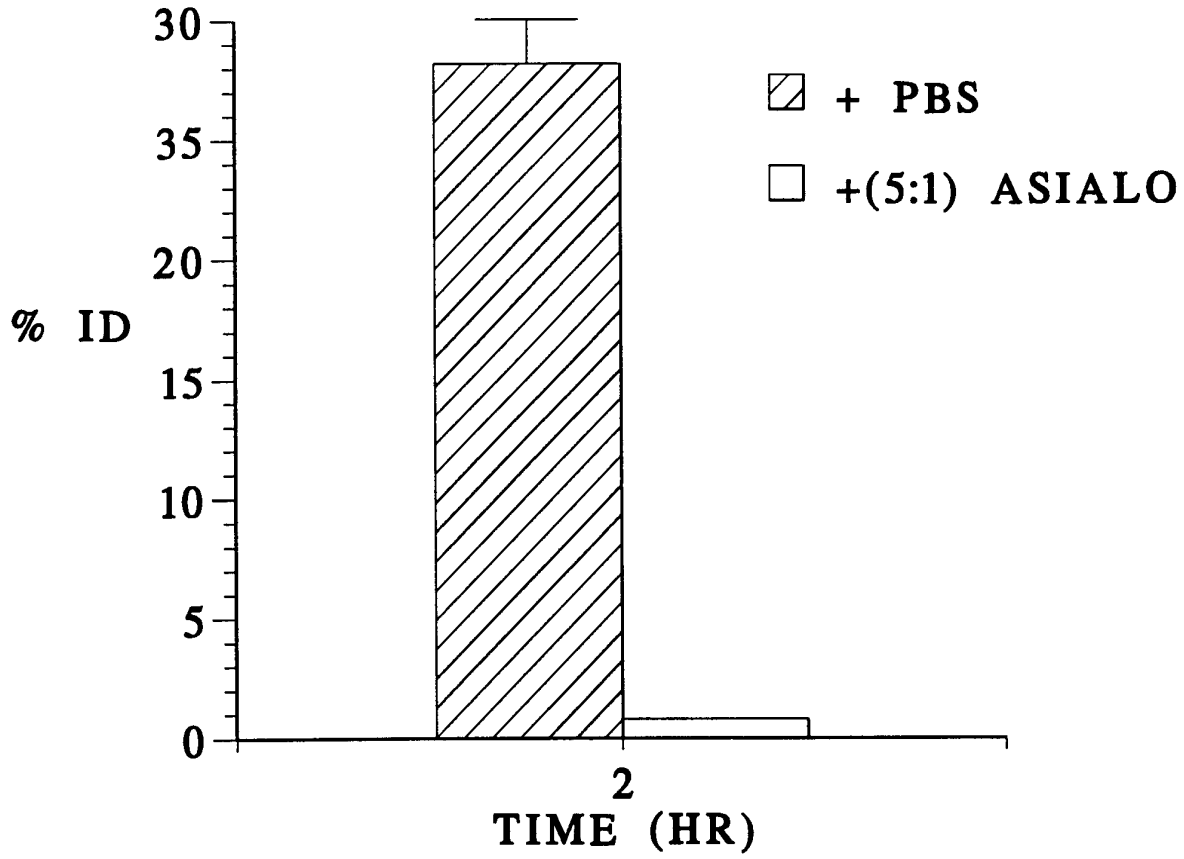


Fig. 9

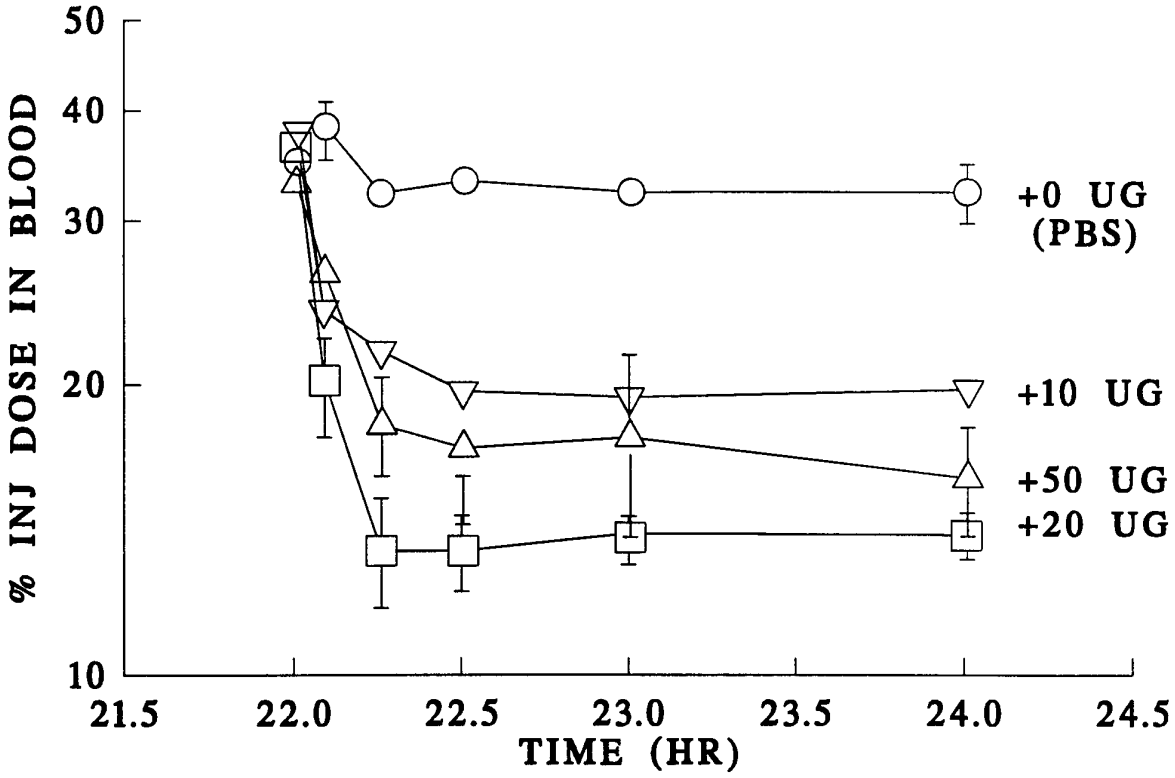

50
40
30
20
10
% INJ DOSE IN BLOOD
21.5
22.0
22.5
23.0
23.5
24.0
24.5
TIME (HR)
+0 UG
(PBS)
+10 UG
+50 UG
+20 UG

Fig. 10

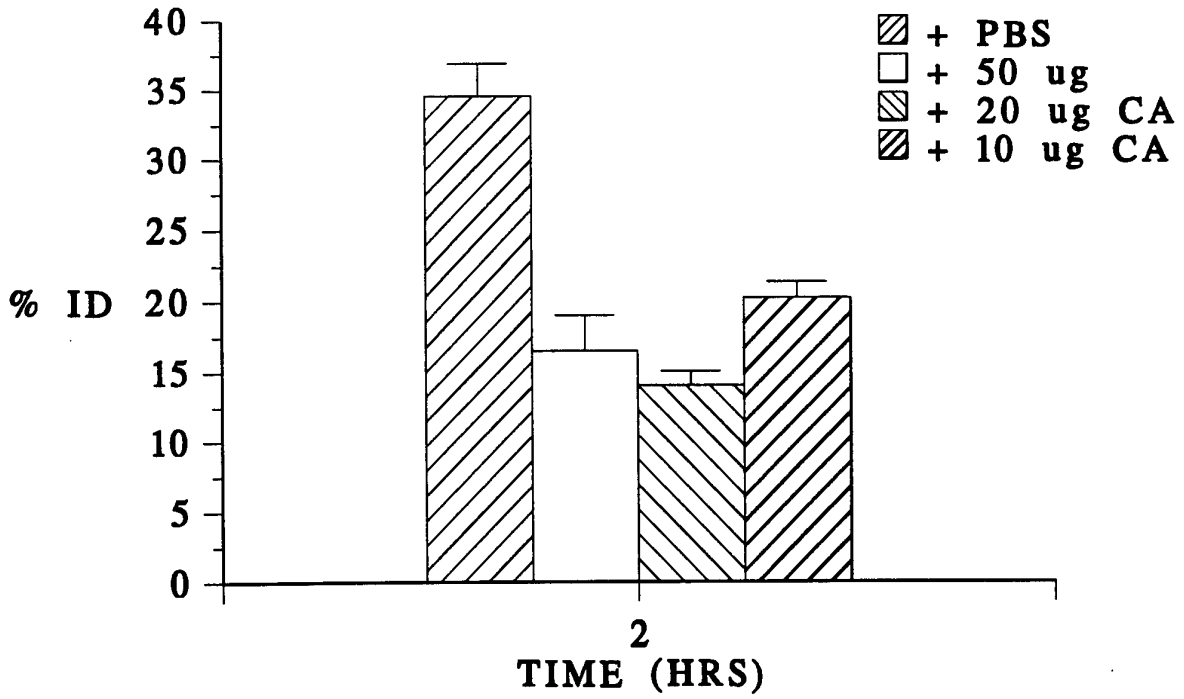


Fig. 11A

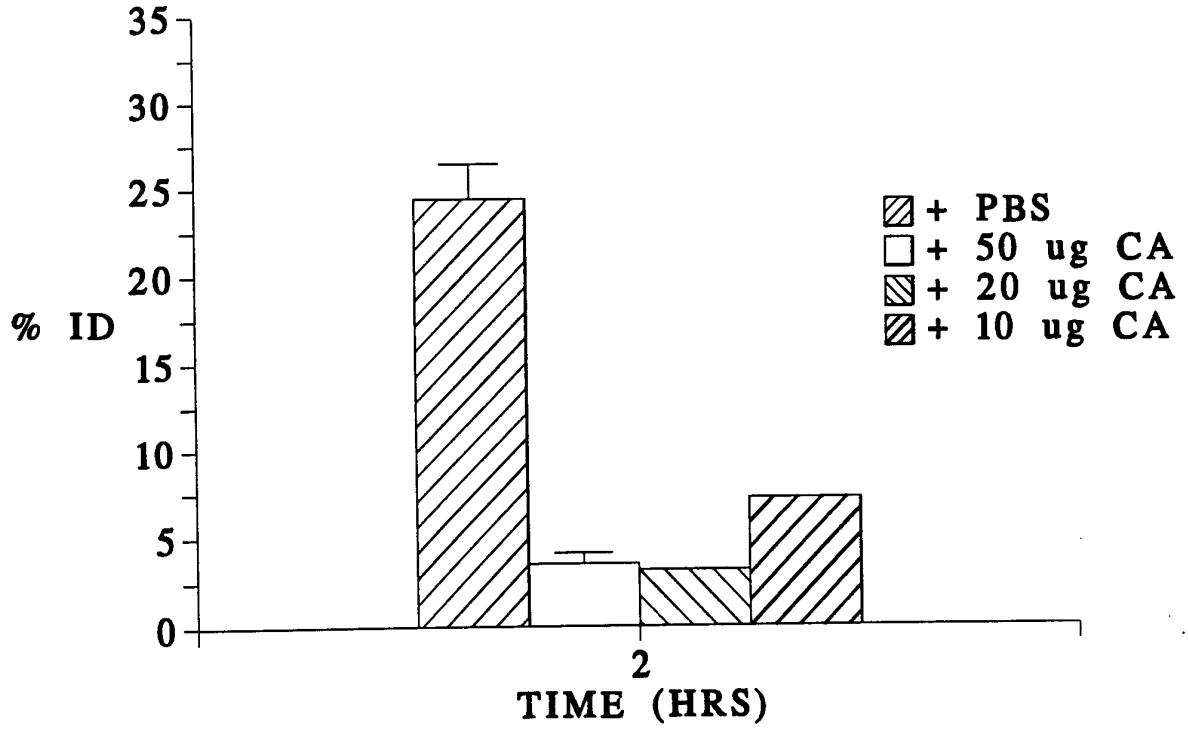


Fig. 11B

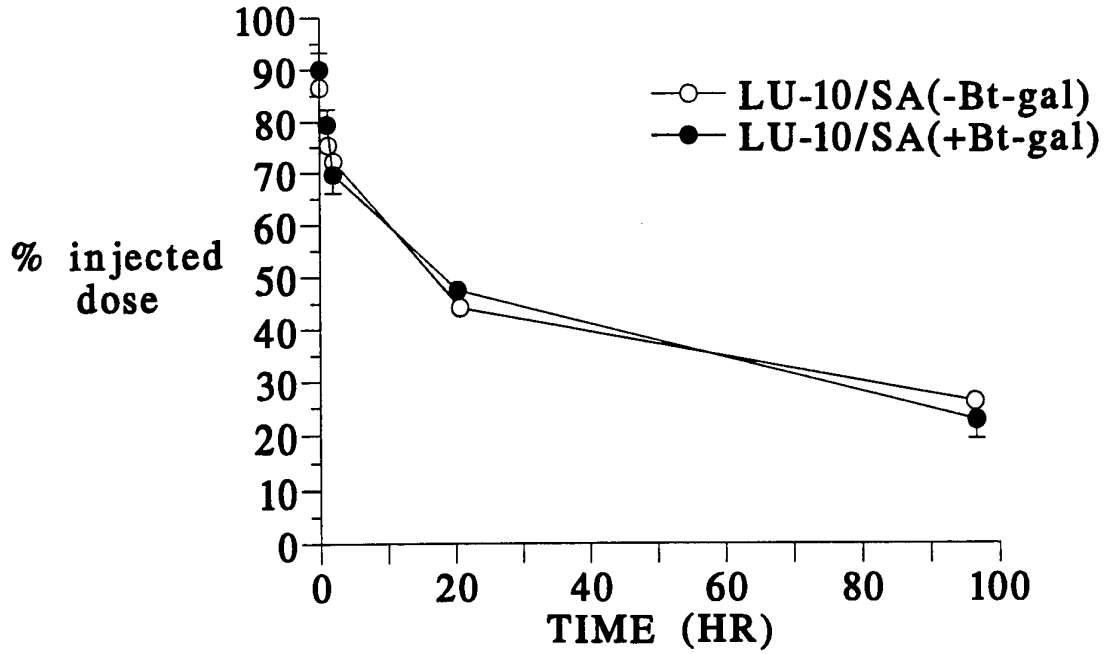

100
90
80
70
60
50
40
30
20
10
0
% injected dose
0
20
40
60
80
100
TIME (HR)
LU-10/SA(-Bt-gal)
LU-10/SA(+Bt-gal)

Fig. 12

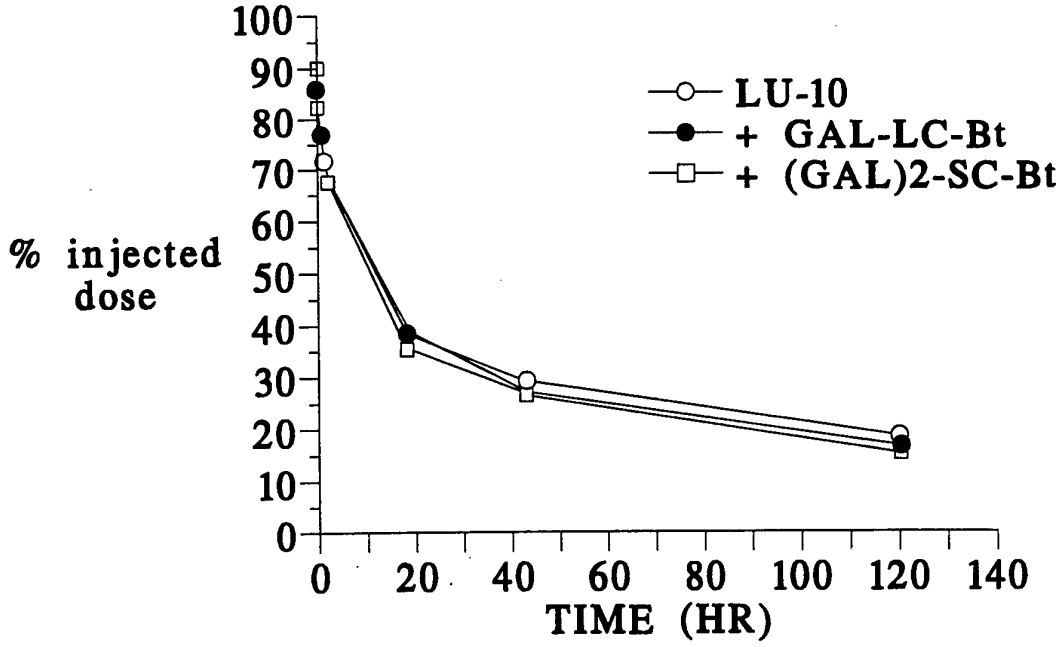

100
90
80
70
60
50
40
30
20
10
0
% injected dose
0
20
40
60
80
100
120
140
TIME (HR)
LU-10
+ GAL-LC-Bt
+ (GAL)2-SC-Bt

Fig. 13

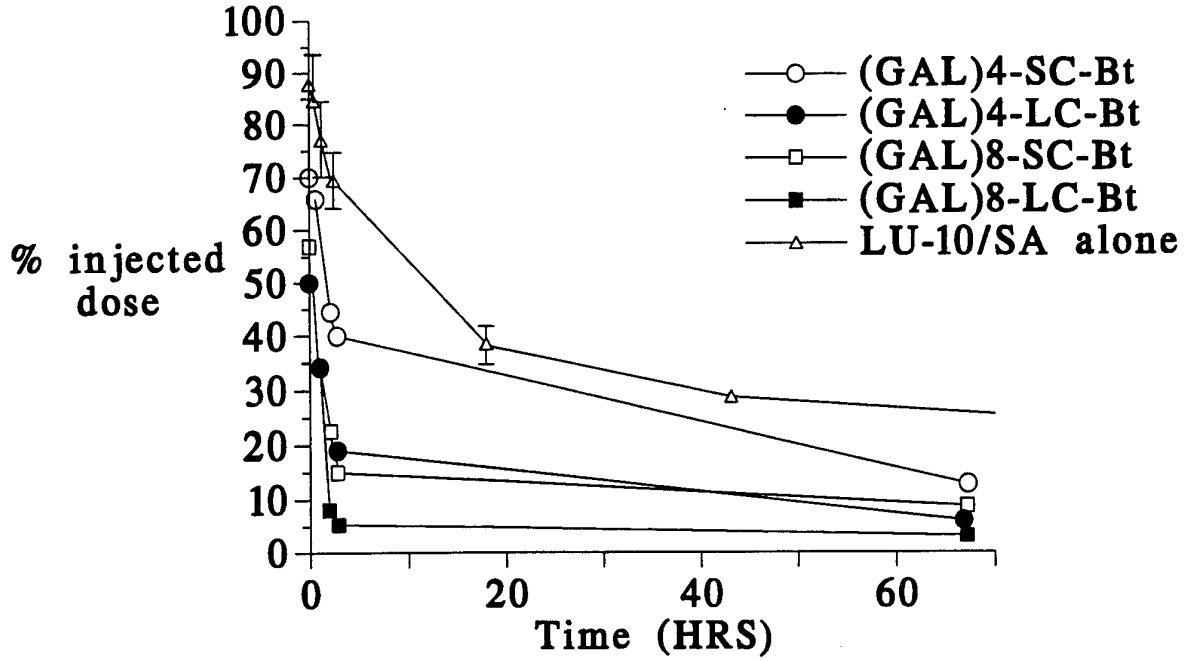

% injected dose
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
Time (HRS)
(GAL)4-SC-Bt
(GAL)4-LC-Bt
(GAL)8-SC-Bt
(GAL)8-LC-Bt
LU-10/SA alone

Fig. 14

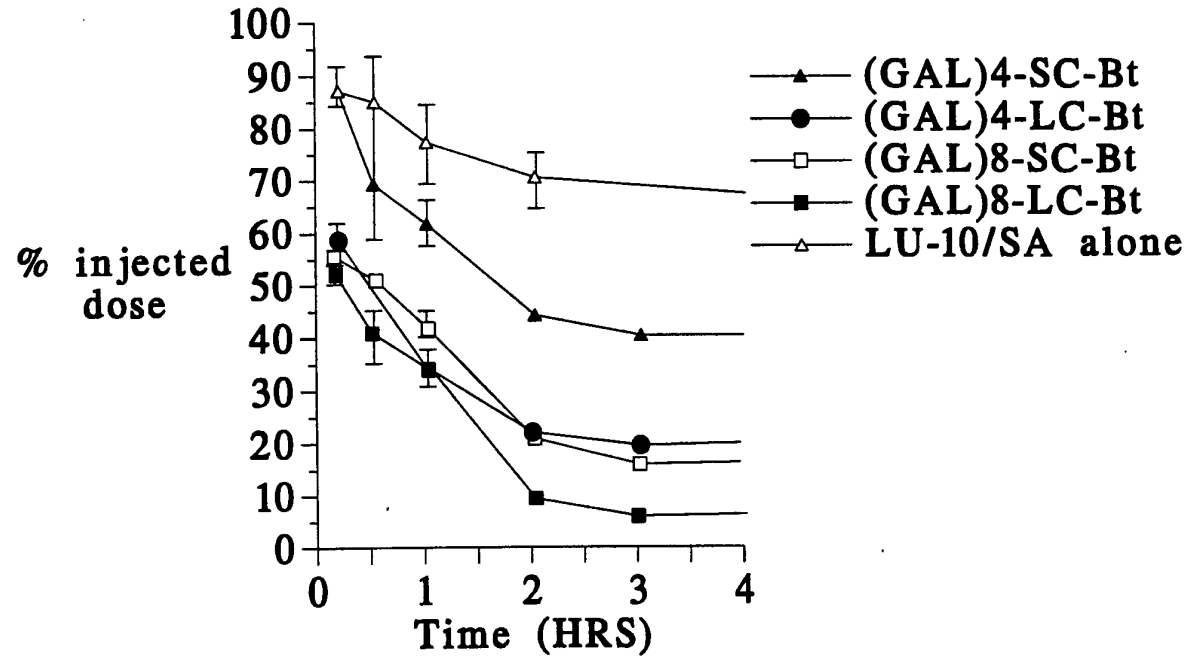

% injected dose
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
Time (HRS)
(GAL)4-SC-Bt
(GAL)4-LC-Bt
(GAL)8-SC-Bt
(GAL)8-LC-Bt
LU-10/SA alone

Fig. 15

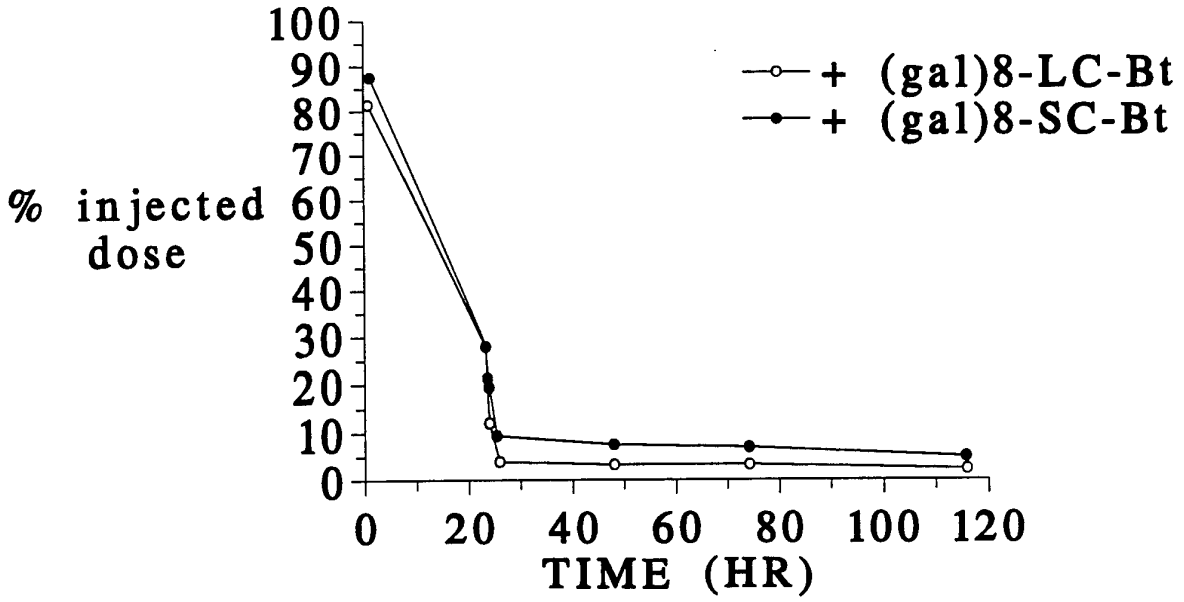

100
90
80
70
60
50
40
30
20
10
0
% injected dose
0 20 40 60 80 100 120
TIME (HR)
+ (gal)8-LC-Bt
+ (gal)8-SC-Bt

Fig. 16

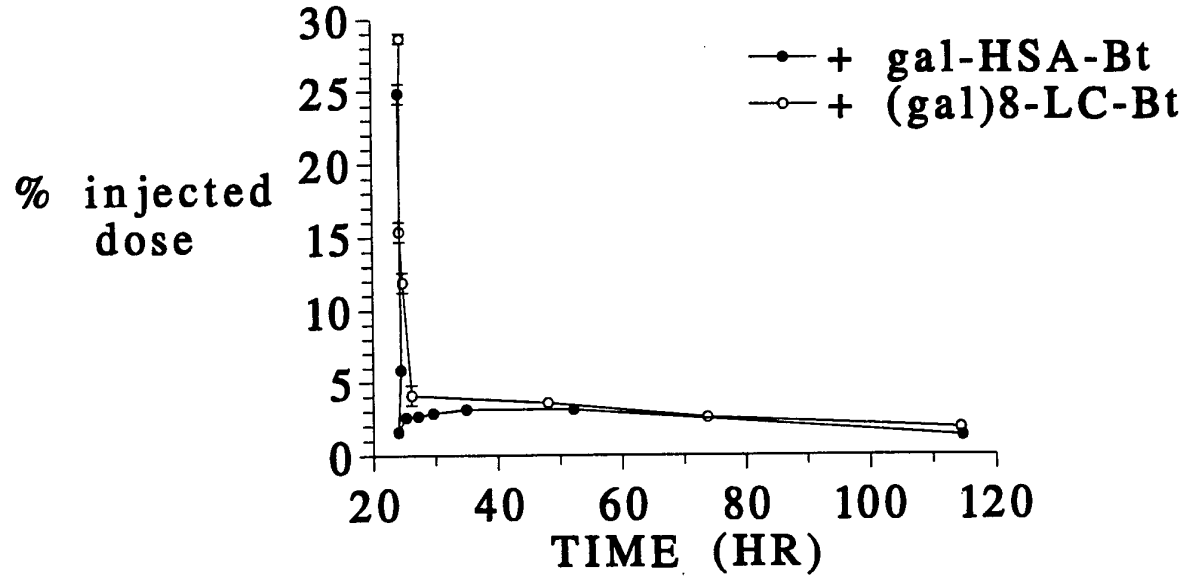

30
25
20
15
10
5
0
% injected dose
20 40 60 80 100 120
TIME (HR)
+ gal-HSA-Bt
+ (gal)8-LC-Bt

Fig. 17

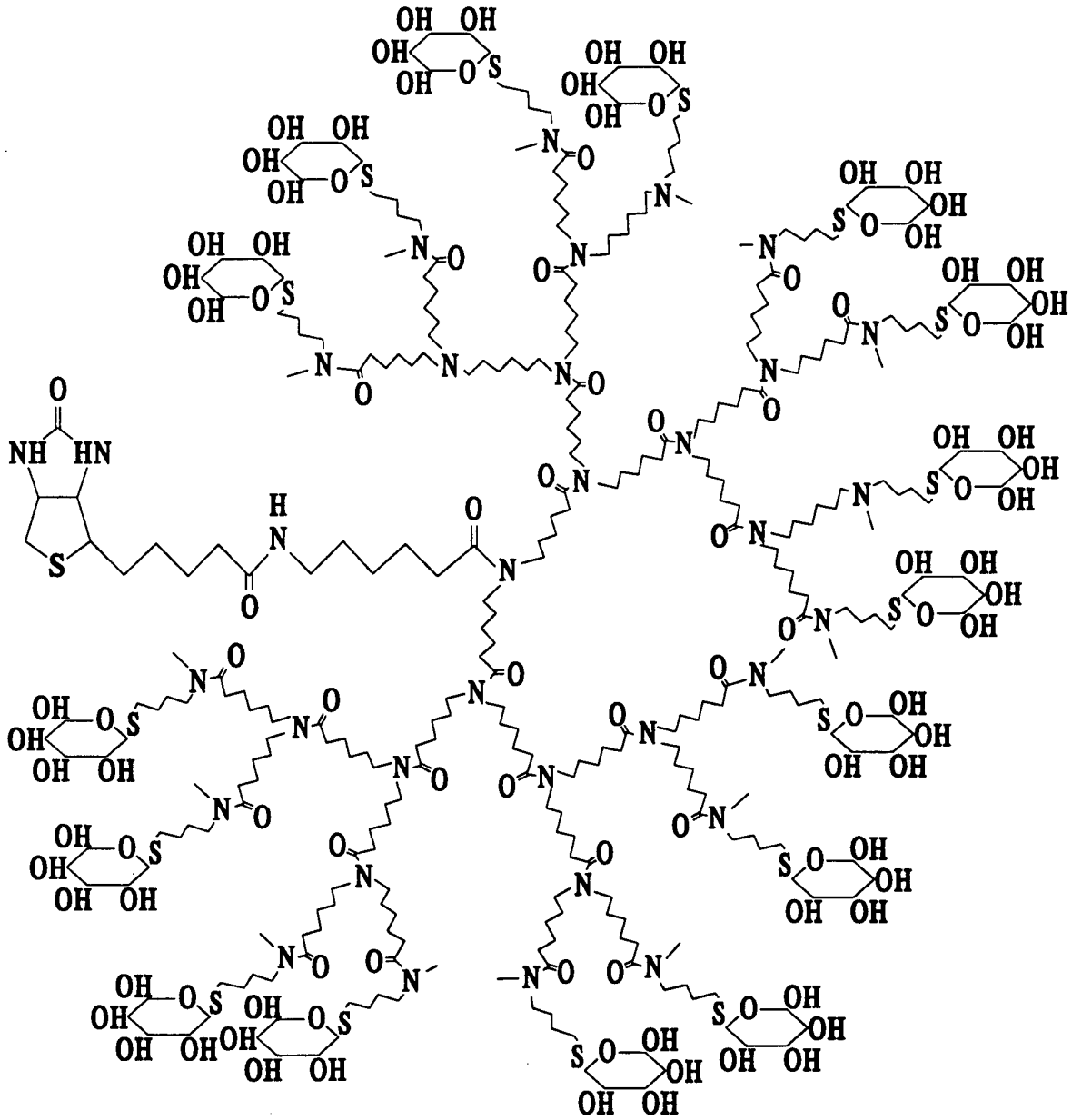


Fig. 18

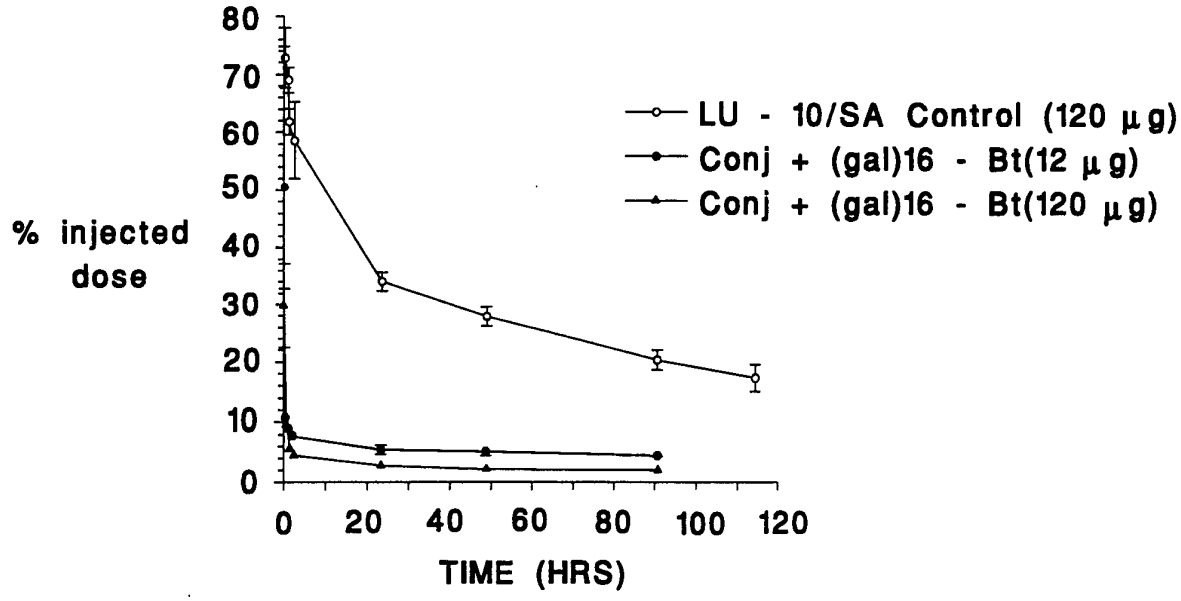


Fig. 19A

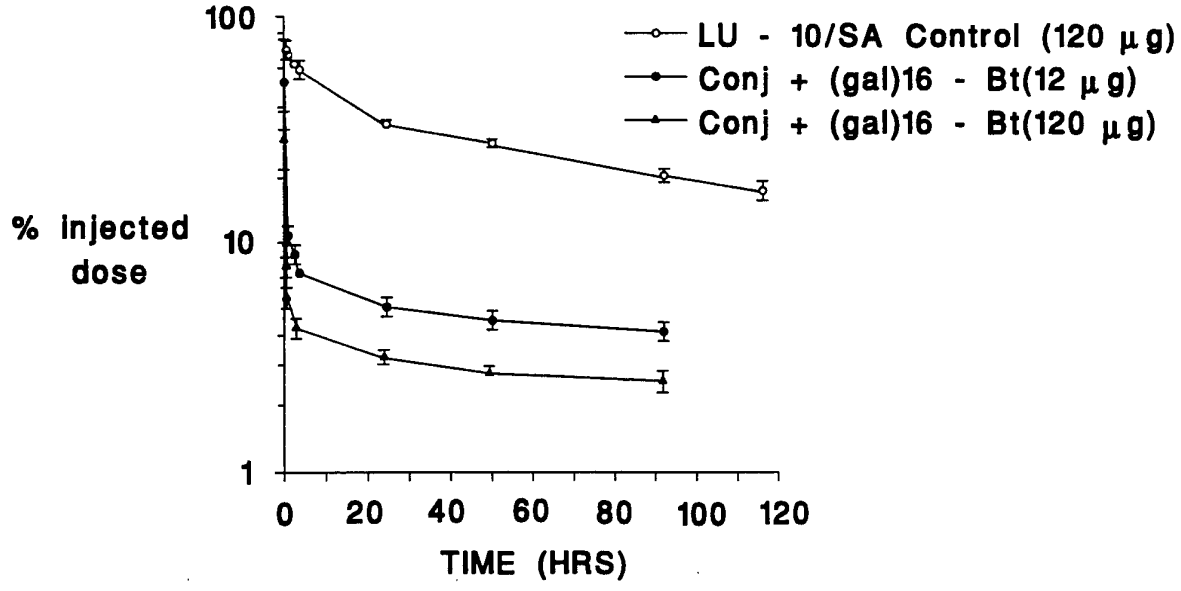
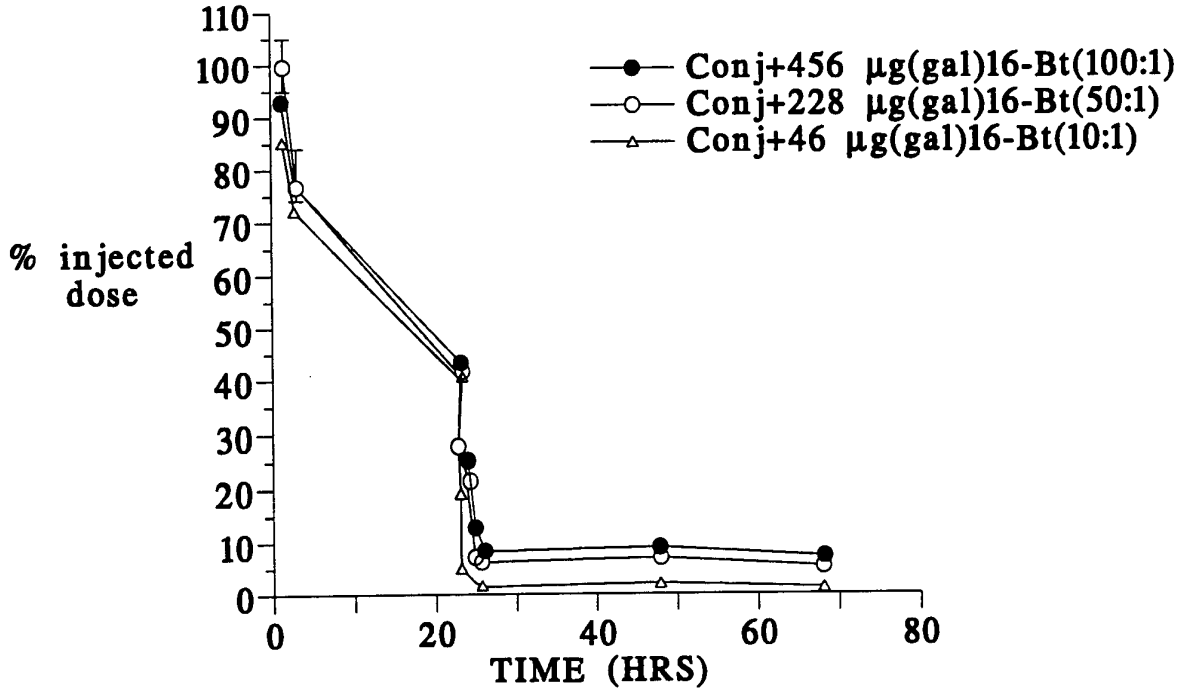


Fig. 19B


110
100
90
80
70
60
50
40
30
20
10
0
% injected dose
0
20
40
60
80
TIME (HRS)
Conj+456 µg(gal)16-Bt(100:1)
Conj+228 µg(gal)16-Bt(50:1)
Conj+46 µg(gal)16-Bt(10:1)

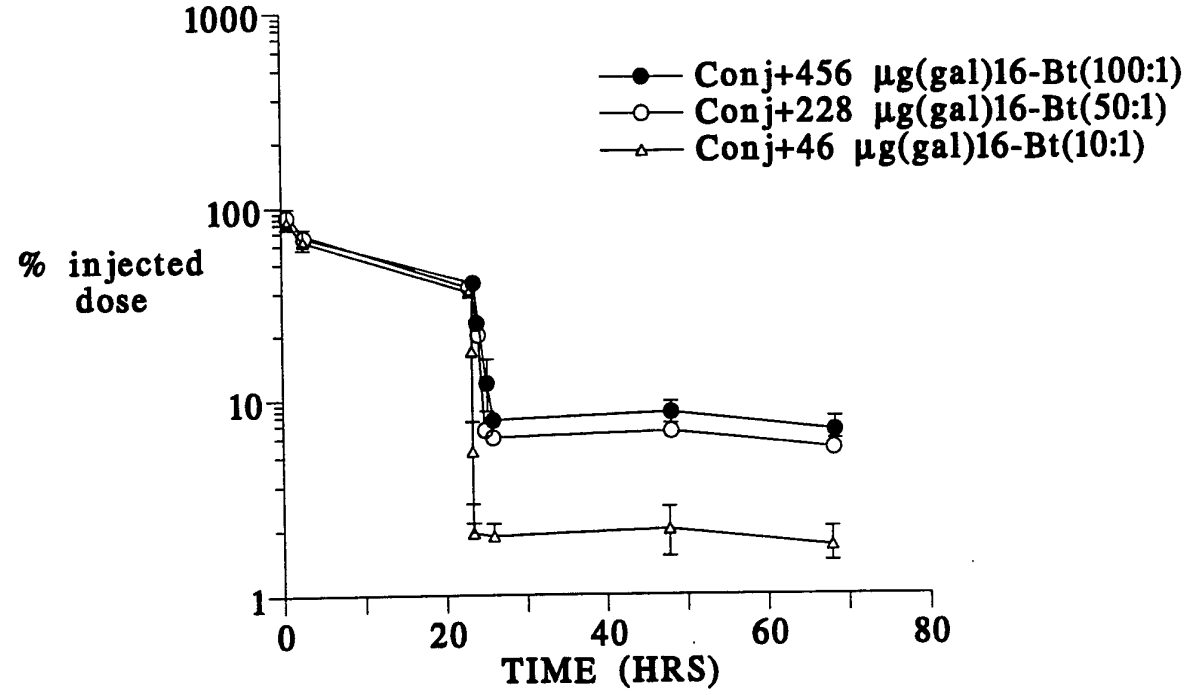

1000
100
10
1
% injected dose
0
20
40
60
80
TIME (HRS)
Conj+456 µg(gal)16-Bt(100:1)
Conj+228 µg(gal)16-Bt(50:1)
Conj+46 µg(gal)16-Bt(10:1)

Fig. 20

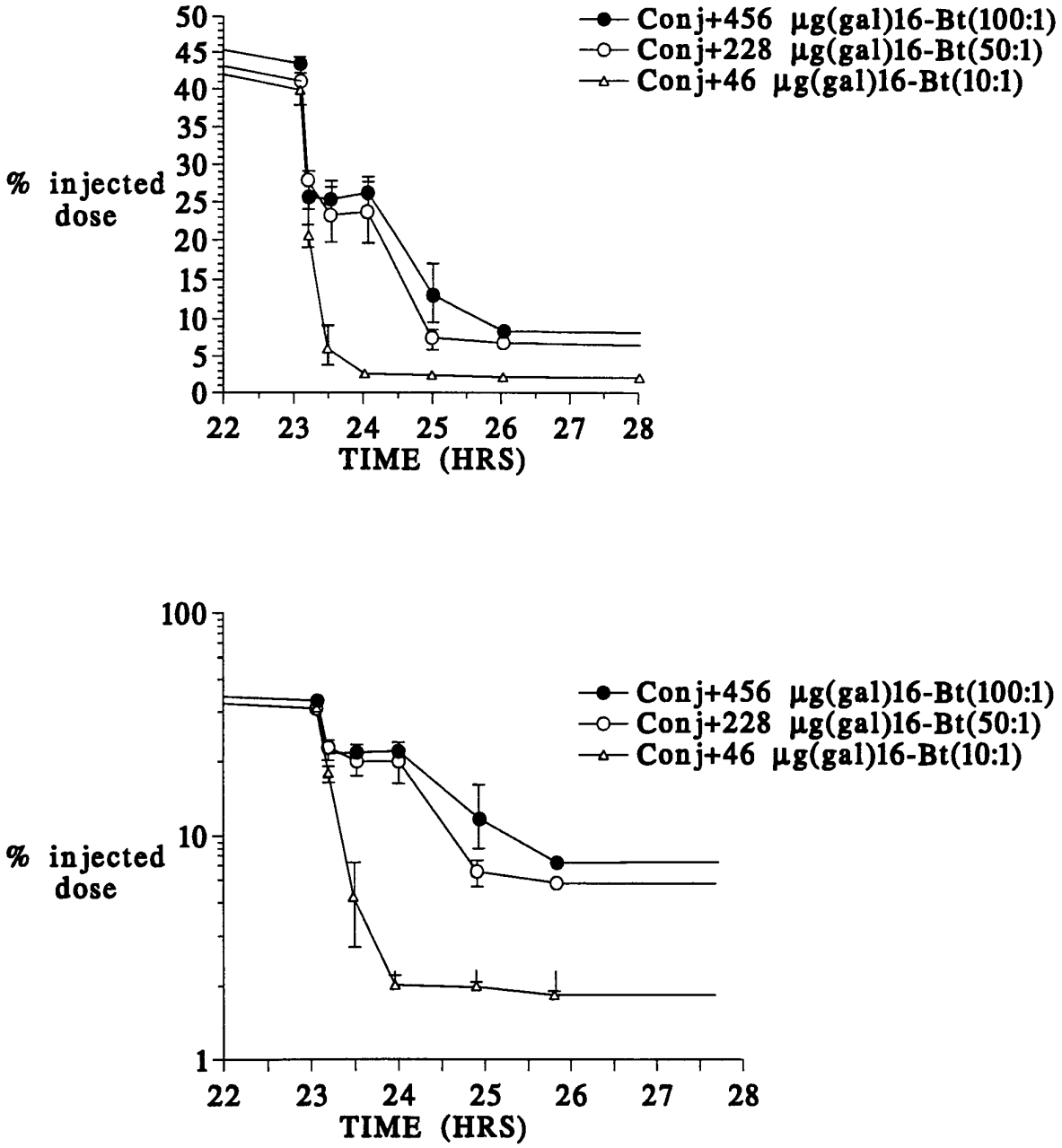

Conj+456 µg(gal)16-Bt(100:1)
Conj+228 µg(gal)16-Bt(50:1)
Conj+46 µg(gal)16-Bt(10:1)
% injected dose
50
45
40
35
30
25
20
15
10
5
0
22
23
24
25
26
27
28
TIME (HRS)
Conj+456 µg(gal)16-Bt(100:1)
Conj+228 µg(gal)16-Bt(50:1)
Conj+46 µg(gal)16-Bt(10:1)
% injected dose
100
10
1
22
23
24
25
26
27
28
TIME (HRS)

Fig. 21

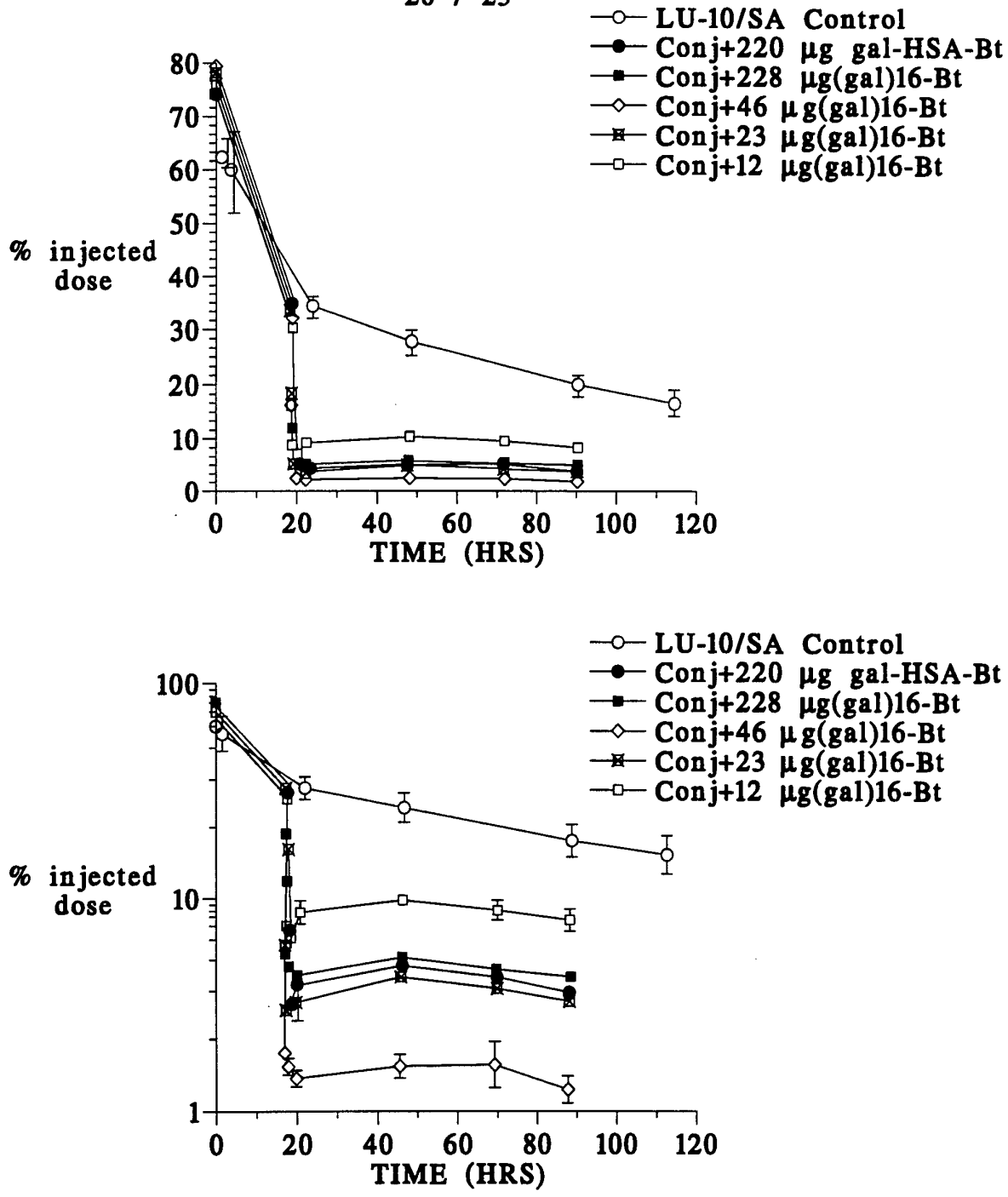

LU-10/SA Control
Conj+220 µg gal-HSA-Bt
Conj+228 µg(gal)16-Bt
Conj+46 µg(gal)16-Bt
Conj+23 µg(gal)16-Bt
Conj+12 µg(gal)16-Bt
% injected dose
TIME (HRS)
0
10
20
30
40
50
60
70
80
100
120

Fig. 22A

TIME	#1	#2	%ID AVG	STD	
0.17	65.29	77.82	71.55	6.27	
18.00	28.41	29.90	29.15	0.75	(PRE-CA)
18.17	15.63	14.65	15.14	0.49	
18.50	10.44	10.31	10.38	0.06	
19.00	2.07	2.36	2.21	0.14	
20.00	2.81	2.89	2.85	0.04	
22.00	3.90	3.57	3.74	0.16	
46.00	4.77	4.72	4.74	0.03	
72.00	4.12	4.35	4.23	0.12	
96.00	3.63	3.46	3.55	0.09	

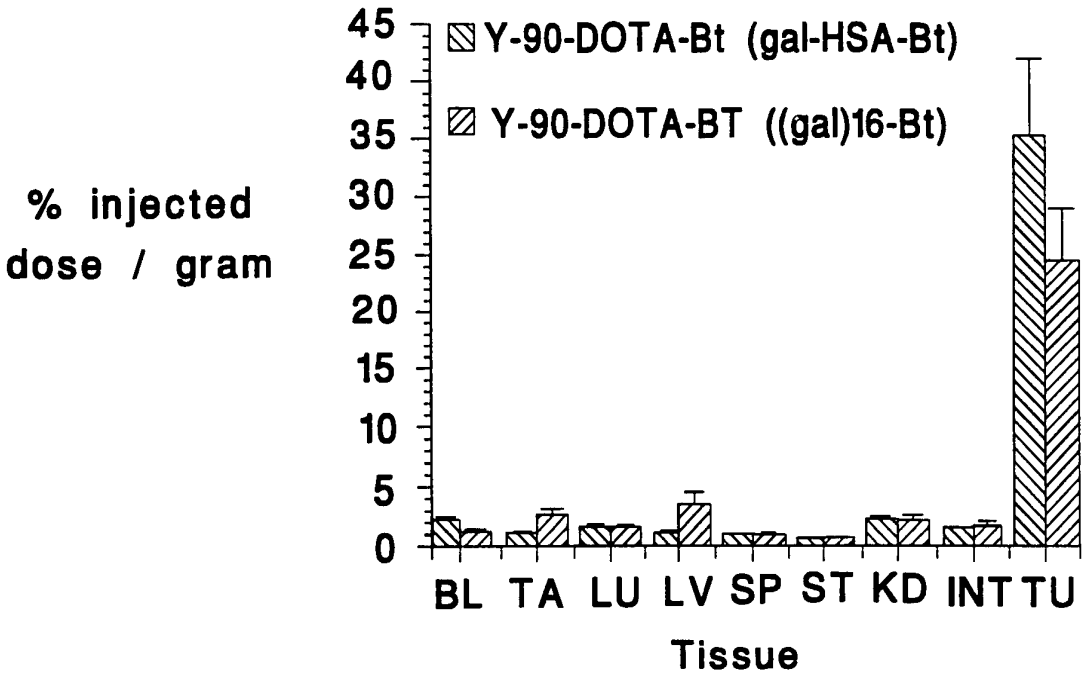
TIME	#3	#4	%ID AVG	STD	
0.17	76.74	77.17	76.96	0.22	
18.00	30.59	31.50	31.04	0.46	(PRE-CA)
18.17	19.93	17.50	18.71	1.21	
18.50	17.24	17.13	17.19	0.05	
19.00	15.95	16.51	16.23	0.28	
20.00	3.97	4.34	4.16	0.19	
22.00	4.37	4.13	4.25	0.12	
46.00	5.24	5.11	5.18	0.06	
72.00	4.72	4.49	4.61	0.11	
96.00	4.39	4.10	4.25	0.14	

TIME	#5	#6	%ID AVG	STD	
0.17	80.79	77.36	79.08	1.72	
18.00	34.86	33.47	34.16	0.69	(PRE-CA)
18.17	16.52	16.01	16.27	0.26	
18.50	0.00	4.43	4.43	0.00	
19.00	1.81	1.80	1.81	0.01	
20.00	1.52	1.57	1.54	0.03	
22.00	1.34	1.43	1.38	0.05	
46.00	1.68	1.50	1.59	0.09	
72.00	1.82	1.41	1.62	0.20	
96.00	1.35	1.14	1.25	0.10	

TIME	#7	#8	%ID AVG	STD	
0.17	71.62	76.06	73.84	2.22	
18.00	31.56	34.42	32.99	1.43	(PRE-CA)
18.17	11.16	9.57	10.37	0.79	
18.50	2.69	2.26	2.47	0.21	
19.00	2.46	2.51	2.48	0.02	
20.00	2.86	2.99	2.92	0.07	
22.00	2.65	3.62	3.14	0.49	
46.00	3.94	4.51	4.23	0.28	
72.00	3.48	3.88	3.68	0.20	
96.00	3.12	3.37	3.24	0.13	

TIME	#9	#10	%ID AVG	STD	
0.17	77.36	70.36	73.86	3.50	
18.00	30.57	28.06	29.32	1.26	(PRE-CA)
18.17	7.39	6.87	7.13	0.26	
18.50	5.00	4.45	4.72	0.28	
19.00	5.62	4.73	5.17	0.44	
20.00	6.95	6.01	6.48	0.47	
22.00	9.37	7.66	8.52	0.86	
46.00	10.16	9.42	9.79	0.37	
72.00	9.54	8.27	8.90	0.64	
96.00	8.78	7.53	8.16	0.62	

Fig. 22B


% injected dose / gram
45
40
35
30
25
20
15
10
5
0
Y-90-DOTA-Bt (gal-HSA-Bt)
Y-90-DOTA-BT ((gal)16-Bt)
BL TA LU LV SP ST KD INT TU
Tissue

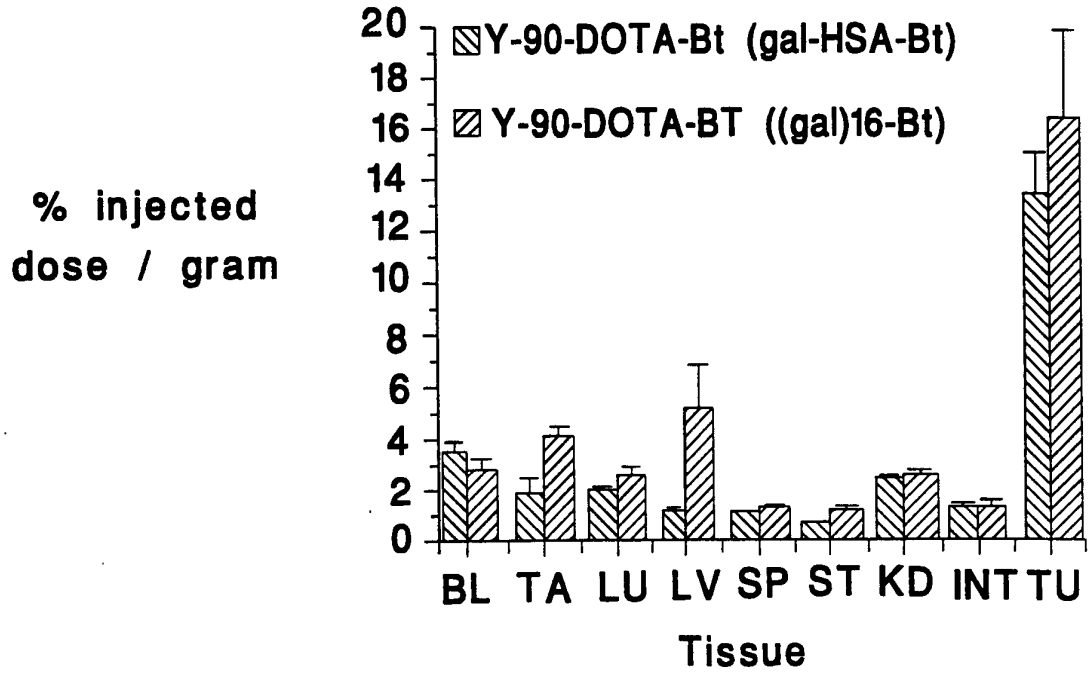

% injected dose / gram
20
18
16
14
12
10
8
6
4
2
0
Y-90-DOTA-Bt (gal-HSA-Bt)
Y-90-DOTA-BT ((gal)16-Bt)
BL TA LU LV SP ST KD INT TU
Tissue

Fig. 23

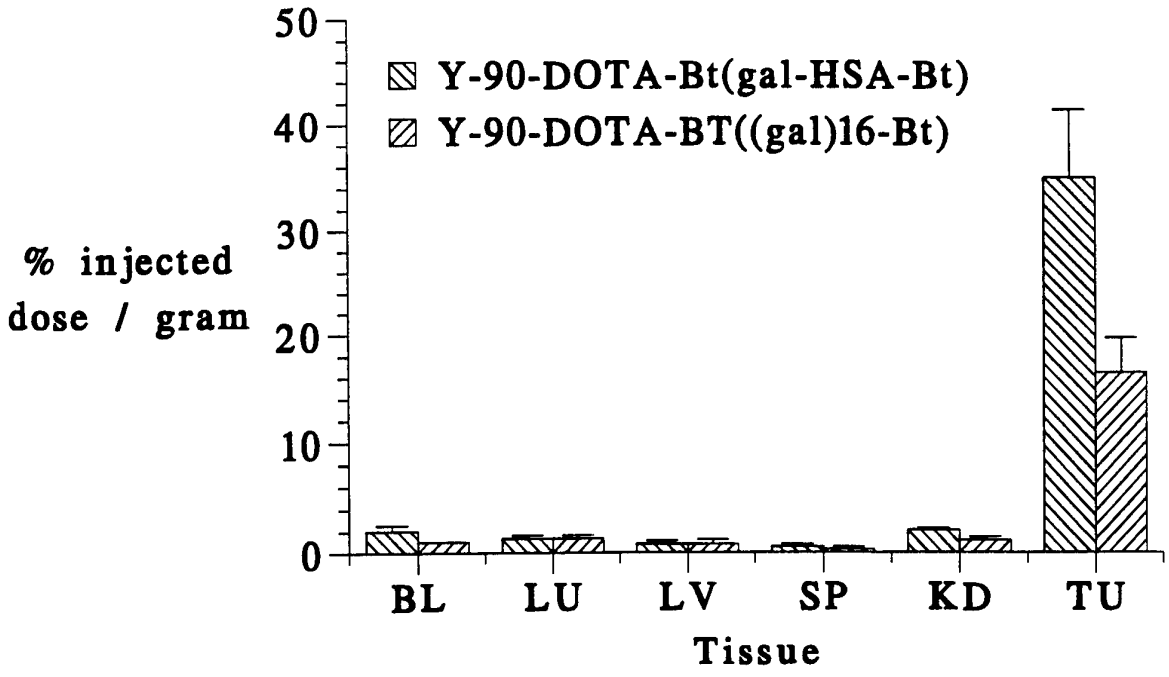

50
40
30
20
10
0
% injected dose / gram
Y-90-DOTA-Bt(gal-HSA-Bt)
Y-90-DOTA-BT((gal)16-Bt)
BL
LU
LV
SP
KD
TU
Tissue

Fig. 24

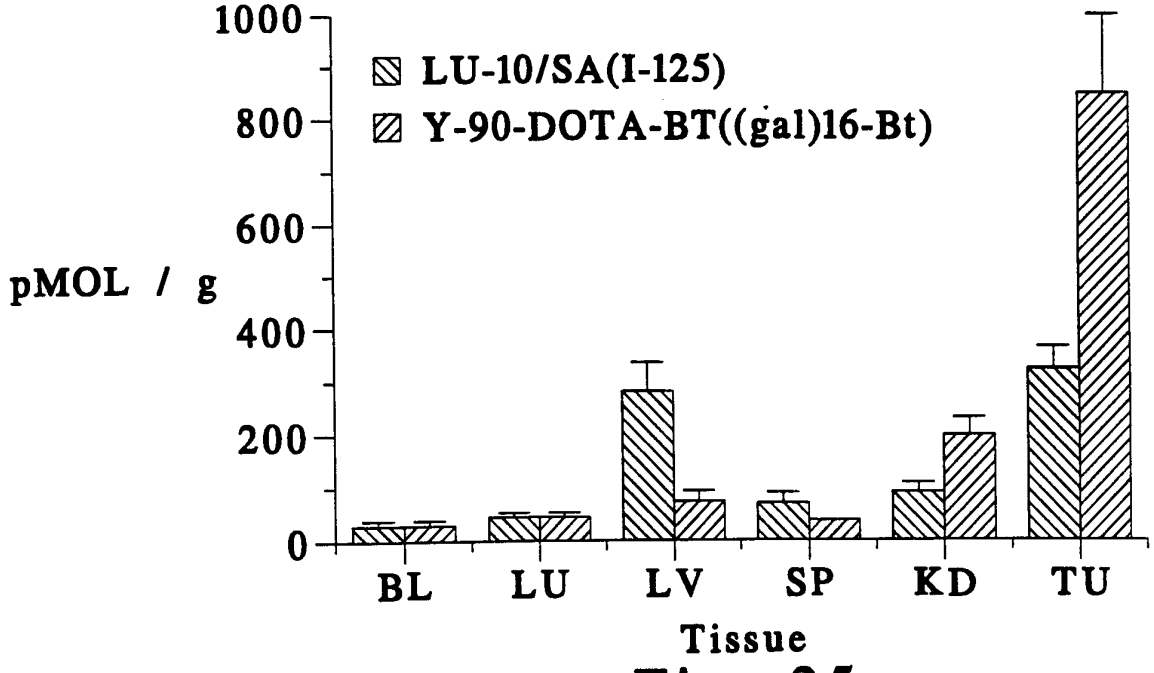

1000
800
600
400
200
0
pMOL / g
LU-10/SA(I-125)
Y-90-DOTA-BT((gal)16-Bt)
BL
LU
LV
SP
KD
TU
Tissue

Fig. 25

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/14172

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :424/179.1, 85.8; 530/362, 363, 402, 410, 388.8; 548/303
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/179.1, 85.8; 530/362, 363, 402, 410, 388.8; 548/303

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS/DIALOG EMBASE, MEDLINE, LIFESCI, BIOSIS, WPI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP, A, 0 251 494 (GOODWIN ET AL.) 07 JANUARY 1988, SEE ENTIRE DOCUMENT.	1-48
Y	EP, A, 0 496 074 (PAGANELLI ET AL.), 29 JULY 1992, SEE ENTIRE DOCUMENT.	1-48
Y	WO 89/10140 (BAGSHAWE ET AL.), 02 NOVEMBER 1989, SEE ENTIRE DOCUMENT.	1-48
Y	J. NATL. CANC. INSTIT., VOLUME 79, NO. 4, ISSUED 01 OCTOBER 1987, M.J. MATTES, "BIODISTRIBUTION OF ANTIBODIES AFTER INTRAPERITONEAL OR INTRAVENOUS INJECTION AND EFFECT OF CARBOHYDRATE MODIFICATIONS", PAGES 855-863, SEE ENTIRE DOCUMENT.	1-48

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 14 FEBRUARY 1995	Date of mailing of the international search report 06 MAR 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer CHRISTOPHER EISENSCHENK Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HEPATOLOGY, VOL. 6, NO. 7, ISSUED 1986, P.J. VAN DER SLUIS ET AL., "DRUG TARGETING TO THE LIVER WITH LACTOSYLATED ALBUMINS: DOES THE GLYCOPROTEIN TARGET THE DRUG OR IS THE DRUG TARGETING THE GLYCOPROTEIN?", PAGES 723-728, SEE ENTIRE DOCUMENT.	1-48
Y	J. BIOL. CHEM., VOL. 266, NO.5, ISSUED 15 FEBRUARY 1991, R.W. JANSEN ET AL., "HEPATIC ENDOCYTOSIS OF VARIOUS TYPES OF MANNOSE-TERMINATED ALBUMINS", PAGES 3343-3348, SEE ENTIRE DOCUMENT.	1-48
Y	NUCL. MED. COMM., VOL. 12, ISSUED 1991, G. PAGANELLI ET AL., "MONOCLONAL ANTIBODY PRETARGETTING TECHNIQUES FOR TUMOR LOCALIZATION: THE AVIDIN-BIOTIN SYSTEM", PAGES 211-234, SEE ENTIRE DOCUMENT.	1-48

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/14172

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C07K 14/52, 14/525, 14/53, 14/535, 14/54, 14/555, 14/76, 14/765, 16/00, 16/30, 103:00; C07D 495/04;