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(54) Titre : THERAPIE PAR CAPTURE DES NEUTRONS DE BORE A BASE DE TECHNIQUES DE PRECIBLAGE
(54) Title: BORON NEUTRON CAPTURE THERAPY USING PRE-TARGETING METHODS

(57) Abrégé/Abstract:
The present invention provides a method for targeting boron atoms to tumor cells in a patient. The method includes the steps of: (A) administering a targeting composition comprising a conjugate of: (i) at least one first antibody or antigen-binding antibody fragment which selectively binds to an antigen produced by or associated with the tumor cells and present at the tumor cells, and (ii) at least one second antibody or antibody fragment which specifically binds to a hapten on a boron compound; (B) optionally, a clearing composition; (C) said boron compound; and (D) optionally, a second clearing composition. The method may further comprise the step of irradiating the boron atoms of the boron compound, thereby effecting BNCT of the tumor cells. Compositions and kits for carrying out the method also are provided.

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(54) Title: BORON NEUTRON CAPTURE THERAPY USING PRE-TARGETING METHODS

(57) Abstract

The present invention provides a method for targeting boron atoms to tumor cells in a patient. The method includes the steps of: (A) administering a targeting composition comprising a conjugate of: (i) at least one first antibody or antigen–binding antibody fragment which selectively binds to an antigen produced by or associated with the tumor cells and present at the tumor cells, and (ii) at least one second antibody or antibody fragment which specifically binds to a hapten on a boron compound; (B) optionally, a clearing composition; (C) said boron compound; and (D) optionally, a second clearing composition. The method may further comprise the step of irradiating the boron atoms of the boron compound, thereby effecting BNCT of the tumor cells. Compositions and kits for carrying out the method also are provided.

BORON NEUTRON CAPTURE THERAPY USING PRE-TARGETING METHODS

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to improved methods for targeting boron atoms to tumor cells for effecting boron neutron capture therapy (BNCT). BNCT is a binary system designed to deliver ionizing radiation to tumor cells by neutron irradiation of tumor-localized boron-10 atoms. In the present invention, the cancer cells are pre-targeted, for example, with a multivalent antibody conjugate wherein at least one antibody or antibody fragment specifically targets tumor cells and at least one antibody or antibody fragment specifically binds to a boron compound. Then, the boron compound is administered and is bound by the multivalent antibody conjugate localized at the cancer site. The localized boron may then be irradiated, thereby effecting treatment of the tumor cells.

Description of Related Art

BORON NEUTRON CAPTURE THERAPY

Boron neutron capture therapy (BNCT) is based on the nuclear reaction which occurs when a stable isotope, B-10 (present in 19.8% natural abundance), is irradiated with thermal neutrons to produce an alpha particle and a Li-7 nucleus. These particles have a path length of about one cell diameter, resulting in high linear energy transfer. Just a few of the short-range 1.7 MeV alpha particles produced in this nuclear reaction are sufficient to target the cell nucleus and destroy it. Barth *et al.*, *Cancer*, 70: 2995-3007 (1992). Since the $^{10}B(n,\alpha)^{7}Li$ reaction will occur, and thereby produce significant biological

effect, only when there is a sufficient fluence (number) of thermal neutrons and a critical amount of B-10 localized around or within the malignant cell, the radiation produced is localized. The neutron capture cross section of B-10 far exceeds that of nitrogen and hydrogen found in tissues, which also can undergo capture reactions, (relative numbers: 1 for N-14, 5.3 for H-1, and 11560 for B-10), so that once a high concentration differential of B-10 is achieved between normal and malignant cells, only the latter will be affected upon neutron irradiation. This is the scientific basis for boron neutron capture therapy. Barth *et al.*, *supra*; Barth *et al. Cancer Res.*, 50: 1061-70 (1990); Perks *et al.*, *Brit. J. Radiol.*, 61: 1115-26 (1988).

Nuclear reactors are the source of neutrons for BNCT. Thermal neutron beams with energies in the range of 0.023 eV, used in early experiments for treating brain tumors, are easily attenuated by tissues, and are poorly penetrating. More recent advances with neutrons of intermediate energy (epithermal neutrons, 1-10,000 eV energy) have led to the consensus for its use in planned clinical trials in the US and Europe. Alam *et al.*, *J. Med. Chem.*, 32: 2326-30 (1989). Fast neutrons with a probable energy of 0.75 MeV are of little use in BNCT.

Original calculations estimated that a boron concentration of 35-50 μg per gram of tumor, or 10^9 B-10 atoms per tumor cell, would be necessary to sustain a cell-killing nuclear reaction with thermal neutron fluences of 10^{12}-10^{13} $n.cm^{-2}$. Fairchild *et al.*, *Int. J. Radiat. Oncol. Biol. Phys.*, 11: 831 (1985). These calculations were based on uniformly distributed boron, as seen with non-specific boronated compounds. For antibody-based boron agents, assuming saturation of all surface antigens on the tumor cell, this level of boron requirement translates to about 1000 atoms per antibody molecule. However, more recent Monte Carlo calculations led to the analysis that for a non-internalizing antibody, boron loading could be as low as 300 atoms per MAb molecule. Kalend *et al.*, *Med. Phys.*, 18: 662 (1991); Zamenhof *et al.*, *J. Nat'l Cancer Inst.*, 84: 1290-91 (1992).

This was based on the following rationale: for tumor cells exhibiting a nucleus-to-cell volume ratio of 0.5 and an effective cell diameter of 10 μm, three B-10 fissions on the cell surface would produce at least one heavy particle trajectory into the nucleus. Assuming saturation of antigen sites on the cell surface, it was deduced that under these conditions just 300 atoms per antibody molecule would suffice to bring about the three fission reactions on the tumor cell surface. The present invention describes a method which can attach a 20-fold greater number of boron atoms per MAb than these prior methods entailed.

Historically, BNCT was first employed for the treatment of glioblastoma (a fatal form of brain tumor) and other brain tumors at a time when tumor specific substances were almost unknown. Hatanaka *et al.*, in BORON NEUTRON CAPTURE THERAPY FOR TUMORS, pp.349-78 (Nishimura Co., 1986). One of the first boronated compounds employed, a sulfhydryl-containing boron substance called sodium borocaptate or BSH ($Na_2,B_{12}H_{11}SH$), crosses the blood-brain barrier to localize in brain, and this has been the anatomical basis for neutron capture therapy of brain tumors. Clinical trials have been carried out, or are scheduled, for the treatment of gliomas in Japan, the US and Europe. Barth *et al.*, *Cancer, supra*. Problems with previous inorganic boron therapy methods was that the boron reached both targeted and non-target areas. Accordingly, when the boron was irradiated, healthy cells as well as cancerous cells were destroyed.

The BNCT concept has been extended to other cancers, spurred on by the discovery of a number of tumor-localizing substances, including tumor-targeting monoclonal antibodies. For instance, boronated amino acids such as p-boronophenylalanine accumulated in melanoma cells. The potential of using boronated monoclonal antibodies directed against cell surface antigens, such as CEA, for BNCT of cancers has been demonstrated. Ichihashi *et al.*, *J. Invest. Dermatol.*, 78: 215-18 (1982); Goldenberg *et al.*, *P.N.A.S., USA*, 81:560-63 (1984); Mizusawa *et al. P.N.A.S., USA*, 79: 3011-14 (1982); Barth *et al.*, *Hybridoma*, 5(supp. 1): 543-5540 (1986); Ranadive *et al. Nucl. Med. Biol.*, 20:

663-68 (1993). However, heavily boronated antibodies failed to target tumor *in vivo* in animal models. Alam *et al.*, *supra;* Barth *et al.*, *Bioconjugate Chem.*, 5: 58-66 (1994).

Success with BNCT of cancer requires methods for localizing a high concentration of boron-10 at tumor sites, while leaving non-target organs essentially boron-free. Non-antibody boronated compounds which accumulate in tumor preferentially, but not specifically, have the disadvantage that tumor-to-blood and tumor-to-organ ratios are often less than ideal, with the result that damage to normal organs could occur during irradiation with neutron beams.

In the case of antibodies, the perceived need to load the same with 1000 boron atoms per antibody molecule has led to the design of a variety of heavily boronated antibodies using, for instance, polylysine, dendrimer or dextran as intermediate carriers of boron clusters. Alam *et al.*, *supra*; Barth *et al.*, *Bioconjugate Chem., supra*. Although in many instances some antigen-binding was found to be retained *in vitro*, these boronated conjugates predominantly localized in liver with little accretion in tumor in *in vivo* animal tumor models.

Thus, there is need for a method of targeting boron atoms to tumor cells that is able to deliver a large amount of boron atoms to tumor sites, while leaving non-cancerous sites relatively boron-free.

PRE-TARGETING

The concept of pre-targeting for *in vivo* imaging application was proposed by Hnatowich *et al.*, *J. Nucl. Med.*, 28: 1294-1302 (1987), and was later examined from a theoretical viewpoint. Van Osdol *et al.*, J. Nucl. Med., 34: 1552-64 (1993). Pre-targeting has been recently reported to have resulted in very encouraging preclinical results with yttrium-90 radioimmunotherapy. Axworthy *et al.*, *J. Immunother.*, 16: 158 (1994). U.S. Patent No. 5,525,338, U.S. Patent No. 5,482,698, U.S. Patent No. 5,736,119 and international publication No. WO 96/40245 also disclose various pre-targeting methods.

Therapy requires a high absolute accretion of the therapeutic agent at the cancer site, as well as a reasonably long duration of uptake and binding. High background levels of non-targeting antibody have long been recognized as a major impediment to high target: background ratios being achieved. To overcome this impediment, various methods have been developed, such as those described in Hansen *et al.*, U.S. Patent No. 3,927,193 and Goldenberg, U.S. Patent. Nos. 4,331,647, 4,348,376, 4,361,544, 4,468,457, 4,444,744, 4,460,459, 4,460,561, 4,624,846 and 4,818,709.

Pre-targeting methods using biotin/avidin approaches are described, for example, in Hnatowich *et al.*, *J. Nucl. Med.* 28: 1294, 1987; Oehr *et al.*, *J. Nucl. Med.* 29: 728, 1988; Klibanov *et al.*, *J. Nucl. Med.* 29: 1951, 1988; Sinitsyn *et al.*, *J. Nucl. Med.* 30: 66, 1989; Kalofonos *et al.*, *J. Nucl. Med.* 31: 1791, 1990; Schechter *et al.*, *Int. J. Cancer* 48: 167, 1991; Paganelli *et al.*, *Cancer Res.* 51: 5960, 1991; Paganelli *et al.*, *Nucl. Med. Commun.* 12: 211, 1991; Stickney *et al.*, *Cancer Res.* 51: 6650, 1991; and Yuan *et al.*, *Cancer Res.* 51: 3119, 1991.

Pre-targeting a target site with a targeting protein, such as an antibody or an antibody fragment, conjugated to an antibody or antibody fragment specific for the second conjugate also has been described. U.S. Patent Nos. 5,274,076, 4,863,713 and 5,256,395.

These methods involve pre-targeting a target site, such as a tumor or lesion, with a targeting protein, such as an antibody or antibody fragment, conjugated to one member of a binding pair, such as biotin, avidin, or a second antibody, whereby the antibody conjugate localizes at the target site. Then, a conjugate of a detection or therapeutic agent, such as a radioisotope, and the complementary member of the binding pair, such as avidin, biotin, or a corresponding hapten is administered. The binding affinity between the members of the binding pair causes the second conjugate to localize at the target site, where the first conjugate already is bound.

In some of these methods, an intermediate clearing or localizing step is used. In this case, the first conjugate comprises one member of the binding pair (for example, biotin), the clearing and localizing agent may comprise the other member of the binding pair (for example, avidin), and the second conjugate comprises the same member of the binding pair as the first (for example, biotin). Other clearing agents, such as antibodies, also have been described.

There is a need for a method of targeting boron atoms to tumor cells that obtains high tumor:non-tumor ratios of the boron atoms, and that delivers sufficient amounts of boron atoms to tumor sites in an efficient manner. Compositions suitable for use in such a method also are needed.

SUMMARY OF THE INVENTION

It is therefore an object of the present invention to provide a method for targeting boron atoms to tumor cells that overcomes the previous problems of maintaining a high tumor:non-tumor ratio of boron-10 atoms, and for delivering sufficient amounts of boron-10 atoms to tumor sites efficiently, and to provide compositions for use in this method.

In accomplishing these and other objects of the invention, there is provided, in accordance with one aspect of the present invention, a method for targeting boron atoms to tumor cells in a patient, comprising the steps of: (A) administering to the patient a targeting composition comprising a conjugate of (i) at least one first antibody or antigen-binding antibody fragment which selectively binds to an antigen produced by or associated with the tumor cells and present at the tumor cells, and (ii) at least one second antibody or antibody fragment which specifically binds to a hapten on a boron compound, and allowing the conjugate to localize at the tumor cells; (B) optionally, administering to the patient a first clearing composition, and allowing the clearing composition to clear non-localized conjugate from circulation; (C) administering to the patient the boron compound and allowing the boron compound to localize at the tumor cells; (D) optionally, administering to the

patient a second clearing composition, and allowing the clearing composition to clear non-localized boron compound from circulation.

The method may further comprise the step of irradiating the boron atoms of the boron compound localized at the tumor cells, thereby effecting BNCT of the tumor cells.

In one embodiment of the present invention, the boron compound is radiolabeled with a detectable label, in which case the method may further comprise the step of detecting the detectable label of the boron compound. In accordance with one aspect of this embodiment, the boron atoms of the boron compound localized at the tumor cells are irradiated after the detectable label is detected.

In accordance with another aspect of the present invention there is provided a sterile, injectable composition for human use comprising a composition for use in targeting boron atoms to tumor cells, comprising a conjugate of (i) at least one first antibody or antigen-binding antibody fragment which selectively binds to an antigen produced by or associated with the tumor cells and present at the tumor cells, and (ii) at least one second antibody or antibody fragment which specifically binds to a hapten on a boron compound.

In accordance with another aspect of the present invention, there is provided a kit suitable for use in a method for targeting boron atoms to tumor cells in a patient, the kit comprising: (A) a sterile, injectable preparation of a targeting composition comprising a conjugate of (i) at least one first antibody or antigen-binding antibody fragment which selectively binds to an antigen produced by or associated with the tumor cells and present at the tumor cells, and (ii) at least one second antibody or antibody fragment which specifically binds to a hapten on a boron compound; (B) optionally, a first clearing composition; (C) a boron compound; and (D) optionally, a second clearing composition.

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According to one aspect of the present invention, there is provided use, for targeting boron atoms to tumor cells in a patient, of: (A) a conjugate of (i) at least one first antibody or antigen-binding antibody fragment which selectively binds to an antigen produced by or associated with the tumor cells and present at the tumor cells, and (ii) at least one second antibody or antibody fragment which specifically binds to a non-boron containing hapten on a carrier portion of a boron-carrier compound wherein said conjugate is adapted to localize at said tumor cells upon administration to said patient of said conjugate and said boron-carrier compound.

According to another aspect of the present invention, there is provided a sterile conjugate adapted for human injection for use in targeting boron atoms to tumor cells, comprising a conjugate of (i) at least one first antibody or antigen-binding antibody fragment which selectively binds to an antigen produced by or associated with the tumor cells and present at the tumor cells, and (ii) at least one second antibody or antibody fragment which specifically binds to a non-boron containing hapten on a carrier portion of a boron-carrier compound.

According to still another aspect of the present invention, there is provided a kit comprising in separate containers adapted for injection: (A) a sterile conjugate of (i) at least one first antibody or antigen-binding antibody fragment which selectively binds to an antigen produced by or associated with the tumor cells and present at the tumor cells, and (ii) at least one second antibody or antibody fragment which specifically binds to a non-boron containing hapten on a carrier portion of a boron-carrier compound; (B) optionally, a first clearing composition; (C) said boron-carrier compound; (D) optionally, a second

clearing composition; and instructions for use in a method for targeting boron atoms to tumor cells in a patient.

Additional objects and advantages of the invention are set forth in part in the description that follows, and in part will be obvious from the description, or

may be learned by practice of the invention. The objects and advantages may be realized and obtained by means of the processes and compositions particularly pointed out in the appended claims.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is an illustrative representation of the preparation of an embodiment of biotin-dextran-boron compound.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention overcomes the aforementioned problems with antibody-targeted BNCT by decoupling the antibody and boron delivery steps by using, for example, a two- or three-step pre-targeting procedure. While prior methods of antibody-targeted BNCT involve loading about 1500 boron-10 atoms onto a single molecule of antibody, the present invention does not load the targeting antibody with boron, thereby eliminating the problems associated with boron-mAb hyper-substitution.

In accordance with one aspect of the present invention, a high concentration of boron is specifically localized at the tumor cells by pre-targeting the tumor cells with a monoclonal, multispecific antibody conjugate. At least one first antibody or antibody fragment of the conjugate selectively binds to an antigen produced by or associated with tumor cells, and at least one second antibody or antibody fragment of the conjugate specifically binds to a hapten on the boron compound. The use of monoclonal, multispecific antibodies to pre-target tumor cells greatly enhances the efficacy of BNCT over previously used boronated compounds which accumulated in tumor cells preferentially, but not selectively. Moreover, the strong affinity between the second antibody or antibody fragment and the boron compound increases the efficiency of the delivery of boron to the tumor cells.

The specificity of BNCT also may be enhanced by administering a clearing agent following the localization of the targeting conjugate at the tumor cells. The use of a clearing agent ensures the rapid removal of unbound targeting conjugate. This enhancement may be further improved by the administration of an anti-idiotypic clearing agent, such as an anti-idiotypic monoclonal antibody specific for the determinant of the targeting conjugate

which binds to the tumor site. One advantage of using such a clearing agent is that it does not competitively remove bound targeting conjugate from the target site, as may occur with other clearing agents. As a result, a higher level of boron is ultimately delivered to the tumor cells. The clearance effect may be further enhanced by using a galactosylated clearing agent, because a galactosylated clearing agent is rapidly cleared through the liver.

The specificity of the therapy may be further enhanced by radiolabelling the boron compound with a detectable label. This enhancement allows the practitioner to determine the location of the boron before it is irradiated and permits the practitioner to delay irradiation until all non-localized boron has been removed from the body, thereby minimizing undesirable tissue damage. Radiolabelling the boron compound with a detectable label also facilitates focusing the thermal neutron beam used to irradiate the boron molecules, further maximizing the benefits of boron therapy and further reducing unintended tissue damage.

The specificity of BNCT may be further enhanced by performing a clearing step after the boron compound has been administered. This step may be performed in addition to or instead of the clearance step discussed above, and comprises administering a clearing agent with specificity for the boron compound. Any suitable clearing agent may be used. Advantageously, the clearing agent comprises an antibody, such as an antibody specific for the hapten on the boron compound recognized by the second antibody or antibody fragment of the targeting conjugate. This clearance may be further enhanced by galactosylating the clearing agent, as discussed above. This clearing step facilitates the rapid removal of non-localized boron compounds, which further reduces the likelihood of unintended tissue damage.

A method comprising one or more of the above described features will achieve BNCT with greater specificity and efficacy than has heretofore been possible.

As mentioned above, the present invention uses multivalent antibody conjugates to target boron to tumor cells. The targeting composition comprises a conjugate of at least one first antibody or antigen-binding antibody fragment which selectively binds to an antigen produced by or associated with the tumor cells and present at the tumor cells, and at least one second antibody or antibody fragment which specifically binds to a hapten on the boron compound.

Bispecific antibodies can be made by a variety of conventional methods, *e.g.*, disulfide cleavage and reformation of mixtures of whole IgG or, preferably $F(ab')_2$ fragments, fusions of more than one hybridoma to form polyomas that produce antibodies having more than one specificity, and by genetic engineering. Bispecific antibodies have been prepared by oxidative cleavage of Fab′ fragments resulting from reductive cleavage of different antibodies. This is advantageously carried out by mixing two different $F(ab')_2$ fragments produced by pepsin digestion of two different antibodies, reductive cleavage to form a mixture of Fab′ fragments, followed by oxidative reformation of the disulfide linkages to produce a mixture of $F(ab')_2$ fragments including bispecific antibodies containing a Fab′ portion specific to each of the original epitopes (*i.e.*, tumor-associated antigen and antigen or hapten present on the boron compound). General techniques for the preparation of multivalent antibodies may be found, for example, in Nisonnoff *et al.*, Arch Biochem. Biophys. 93: 470 (1961), Hammerling *et al.*, J. Exp. Med. 128: 1461 (1968), and U.S. Patent No. 4,331,647.

A more selective linkage can be achieved by using a heterobifunctional linker such as maleimide-hydroxysuccinimide ester. Reaction of the ester with an antibody or antibody fragment will derivatize amine groups on the antibody or antibody fragment, and the derivative can then be reacted with, *e.g.*, an antibody Fab fragment having free sulfhydryl groups (or, a larger fragment or intact antibody with sulfhydryl groups appended thereto by, *e.g.*, Traut's Reagent). Such a linker is less likely to crosslink groups in the same antibody and improves the selectivity of the linkage.

It is advantageous to link the antibodies or fragments at sites remote from the antigen binding sites. This can be accomplished by, *e.g.*, linkage to cleaved interchain sulfhydryl groups, as noted above. Another method involves reacting an antibody having an oxidized carbohydrate portion with another antibody which has at lease one free amine function. This results in an initial Schiff base (imine) linkage, which is preferably stabilized by reduction to a secondary amine, *e.g.*, by borohydride reduction, to form the final product. Such site-specific linkages are disclosed, for small molecules, in U.S. Patent No. 4,671,958, and for larger addends in U.S. Patent No. 4,699,784.

Alternatively, bispecific antibodies can be produced by fusing two hybridoma cell lines that produce anti-tumor-associated antigen Mab and anti-boron compound Mab. Techniques for producing tetradomas are described, for example, by Milstein *et al.*, Nature 305: 537 (1983) and Pohl *et al.*, Int. J. Cancer 54: 418 (1993).

Bispecific antibodies also can be produced by genetic engineering. For example, plasmids containing DNA coding for variable domains of an anti-tumor-associated antigen Mab can be introduced into hybridomas that secrete antibodies to the boron compound. The resulting "transfectomas" produce bispecific antibodies that bind tumor-associated antigen and the boron compound. Alternatively, chimeric genes can be designed that encode both anti-tumor-associated antigen and anti-boron compound binding domains. General techniques for producing bispecific antibodies by genetic engineering are described, for example, by Songsivilai *et al.*, Biochem. Biophys. Res. Commun. 164: 271 (1989); Traunecker *et al.*, EMBO J. 10: 3655 (1991); and Weiner *et al.*, J. Immunol. 147: 4035 (1991).

Functional bi-specific single-chain antibodies (bscAb), also called diabodies, also can be produced using recombinant methods. See, e.g., Mack *et al.*, *Proc. Natl. Acad. Sci.*, 92: 7021-7025, 1995. For example, bscAb are produced by joining two single-chain Fv fragments via a glycine-serine linker using recombinant methods. The V light-chain (V_L) and V heavy-chain (V_H)

domains of two antibodies of interest are isolated using standard PCR methods. The V_L and V_H cDNA's obtained from each hybridoma are then joined to form a single-chain fragment in a two-step fusion PCR. The first PCR step introduces the $(Gly_4\text{-}Ser_1)_3$ linker, and the second step joins the V_L and V_H amplicons. Each single chain molecule is then cloned into a bacterial expression vector.

Following amplification, one of the single-chain molecules is excised and sub-cloned into the other vector, containing the second single-chain molecule of interest. The resulting bscAb fragment is subcloned into an eukaryotic expression vector. Functional protein expression can be obtained by transfecting the vector into chinese hamster ovary cells. Bi-specific fusion proteins are prepared in a similar manner. Bi-specific single-chain antibodies and bi-specific fusion proteins are included within the scope of the present invention.

Bi-specific fusion proteins linking two or more different single-chain antibodies or antibody fragments are produced in similar manner. Recombinant methods can be used to produce a variety of fusion proteins. For example a fusion protein comprising a Fab fragment derived from a humanized monoclonal anti-CEA antibody and a scFv derived from a murine anti-diDTPA can be produced. A flexible linker, such as GGGS connects the scFv to the constant region of the heavy chain of the anti-CEA antibody. Alternatively, the scFv can be connected to the constant region of the light chain of hMN-14. Appropriate linker sequences necessary for the in-frame connection of the heavy chain Fd to the scFv are introduced into the VL and VK domains through PCR reactions. The DNA fragment encoding the scFv is then ligated into a staging vector containing a DNA sequence encoding the CH1 domain. The resulting scFv-CH1 construct is excised and ligated into a vector containing a DNA sequence encoding the VH region of an anti-CEA antibody. The resulting vector can be used to transfect mammalian cells for the expression of the bi-specific fusion protein.

A higher order multivalent, multispecific molecule can be obtained by adding various antibody components to a bispecific antibody, produced as above. For example, a bispecific antibody can be reacted with 2-iminothiolane to introduce one or more sulfhydryl groups for use in coupling the bispecific antibody to a further antibody derivative that binds at the same or a different epitope of the tumor-associated antigen or of the boron compound, using the bis-maleimide activation procedure described above. These techniques for producing multivalent antibodies are well known to those of skill in the art. See, for example, U.S. Patent No. 4,925,648, and Goldenberg, international publication No. WO 92/19273.

The targeting composition may comprise a conjugate comprising a plurality of antibodies or antibody fragments that specifically bind to the same or different epitopes of a tumor-associated antigen, or that bind to different tumor-associated antigens. Additionally or alternatively, the conjugate may comprise a plurality of antibodies or antibody fragments that specifically bind to the same or different epitopes of the boron compound.

In accordance with one aspect of this embodiment of the invention, monoclonal antibodies or fragments of monoclonal antibodies are used. Monoclonal antibodies are preferred because of their high specificities. They are readily prepared by what are now considered conventional procedures of immunization of mammals with immunogenic antigen preparation, fusion of immune lymph or spleen cells with an immortal myeloma cell line, and isolation of specific hybridoma clones. More unconventional methods of preparing monoclonal antibodies are not excluded, such as interspecies fusions and genetic engineering manipulations of hypervariable regions, since it is primarily the antigen specificity of the antibodies that affects their utility in the present invention.

Additionally or alternatively, humanized antibodies or fragments of humanized antibodies are used. Humanized monoclonal antibodies are produced by transferring mouse complementary determining regions from heavy and light

variable chains of the mouse immunoglobulin into a human variable domain, and then substituting human residues in the framework regions of the murine counterparts. The use of antibody components derived from humanized monoclonal antibodies obviates potential problems associated with the immunogenicity of murine constant regions. General techniques for cloning murine immunoglobulin variable domains are described, for example, in Orlandi *et al., Proc. Nat'l Acad. Sci. USA 86*:3833 (1989). Techniques for producing humanized Mabs are described, for example, in Jones *et al., Nature 321*:522 (1986); Carter *et al., Proc. Nat'l Acad. Sci. USA 89*:4285 (1992); Sandhu, *Crit. Rev. Biotech. 12*:437 (1992), and Singer *et al., J. Immun. 150*:2844 (1993). See also Shevitz *et al., J. Nucl. Med. 35*:112 (1994), describing hMN-14, a humanized an anti-CEA antibody having the human IgG_1/κ isotype. See also international publication No's: WO 97/11370, WO 97/34636 and WO 97/34632. It will be appreciated that newer techniques for production of monoclonals can also be used, e.g., interspecies monoclonals, chimeric (e.g., human/mouse) monoclonals, genetically engineered antibodies and the like.

Antibody fragments useful in the present invention include $F(ab')_2$, $F(ab)_2$, Fab', Fab, Fv and the like, including hybrid fragments. Preferred fragments are Fab', $F(ab')_2$, Fab, and $F(ab)_2$. Also useful are any subfragments retaining the hypervariable, antigen-binding region of an immunoglobulin and having a size similar to or smaller than a Fab' fragment. This includes genetically engineered and/or recombinant proteins, whether single-chain or multiple-chain, which incorporate an antigen-binding site and otherwise function *in vivo* as targeting vehicles in substantially the same way as natural immunoglobulin fragments. Such single-chain binding molecules are disclosed in U.S. Patent No. 4,946,778. Fab' antibody fragments may be conveniently made by reductive cleavage of $F(ab')_2$ fragments, which themselves may be made by pepsin digestion of intact immunoglobulin. Fab antibody fragments may be made by papain digestion of intact immunoglobulin, under reducing conditions, or by cleavage of $F(ab)_2$ fragments which result from careful papain

digestion of whole immunoglobulin. The fragments may also be produced by genetic engineering.

It should be noted that mixtures of antibodies, antibody fragments and immunoglobulin classes can be used, as can hybrid antibodies. Multispecific, including bispecific and hybrid, antibodies and antibody fragments are useful in the methods of the present invention, and are comprised of at least two different substantially monospecific antibodies or antibody fragments, wherein at least two of said antibodies or antibody fragments specifically bind to at least two different antigens produced by or associated with the cancer cells or at least two different epitopes or molecules of a marker substance produced by or associated with the cancer cells. Multispecific antibodies and antibody fragments with dual specificities can be prepared analogously to the anti-tumor marker hybrids disclosed in U.S. Patent. No. 4,361,544. Other techniques for preparing hybrid antibodies are disclosed in, e.g., U.S. Patent. No. 4,474,893 and 4,479,895, and in Milstein *et al.*, *Immunol. Today*, 5: 299 (1984).

Preferred are antibodies having a specific immunoreactivity to a marker substance produced by or associated with the cancer cells of at least 60% and a cross-reactivity to other antigens or non-targeted substances of less than 35%.

Antibodies against tumor antigens are known. For example, antibodies and antibody fragments which specifically bind markers produced by or associated with tumors have been disclosed, *inter alia*, in Hansen *et al.*, U.S. Patent No. 3,927,193, and Goldenberg, U.S. Patent Nos. 4,331,647, 4,348,376, 4,361,544, 4,468,457, 4,444,744, 4,818,709 and 4,624,846. In particular, antibodies against an antigen, e.g., a gastrointestinal, lung, breast, prostate, ovarian, testicular, brain or lymphatic tumor, a sarcoma or a melanoma, are advantageously used.

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An antibody preferred for use in the present invention is MN-14, a second generation CEA-antibody that has ten times more affinity for CEA than the first generation version, NP-4. Hansen *et al.*, *Cancer*, 71:3478-85 (1993). MN-14 internalizes slowly, making it suitable for a pre-targeting approach.

Another antibody for use in the present invention is an anti-carcinoembryonic antigen (CEA) antibody.

The antibody or antibody fragment that specifically binds to a hapten on the boron compound may bind to any hapten on the boron compound, including a hapten on a moiety appended to the boron-carrier compound or a hapten of the carrier itself. Examples of suitable antibodies include:

- an anti-carborane antibody.
- an anti-biotin antibody.
- an anti-DTPA Mab, such as the MAb designated 734. See LeDoussal et al., *Cancer Res.*, 50: 3445-52 (1990).
- an anti-DTPA Mab, such as the MAb designated DTIn1. See Kranenborg et al., *Cancer Res.*, 55: 5864-67 (1995).
- an antibody to a non-chelate hapten such as histamine-succinate. See Janevik-Ivanovska et al., *Bioconjugate Chem.*, 8: 526-33 (1997).
- antibodies to the dinitrophenyl (DNP) group such as those disclosed in Eshhar et al., *J. Immunol.*, 124: 775 (1980).
- antibodies to polymers such as polyhistidine. See Sigma Catalog (1997).
- antibodies to fluorescein isothiocyanate. See W. Hijmans et al., *Clin. Exp. Immunol.*, 4: 457 (1969). If the boron compound comprises other fluorochromes such as rhodamine, Hoecsht 33258, or Texas Red, antibodies to haptens on these compounds also could be used.
- antibodies to dextran. See Anastase et al., *J. Chromatogr. B. Biomed. Appl.* 1996 Nov 15; 686(2):141-50.

Antigen-binding fragments of the above antibodies also may be used in the present invention.

Cancer states that can be targeted and treated in accordance with the present invention include carcinomas, melanomas, sarcomas, neuroblastomas, leukemias, lymphomas, gliomas and myelomas.

The antibodies and antibody fragments useful in the methods of the present invention may conjugated by a variety of methods known in the art. Many of these methods are disclosed in the above-referenced U.S. Patents and Patent Applications. *See also* Childs *et al.*, *J. Nuc. Med.*, 26: 293 (1985).

The present invention also provides a boron compound comprising a carrier coupled to approximately 1,500 boron atoms. The carrier may be, for example, dextran. Other carrier molecules will be apparent to those skilled in the art and include aminodextrans, Shih *et al.*, U.S. Patent No. 5,057,313, other polysaccharides, natural and synthetic polypeptides, such as polylysines, polyglutamic acids and polycysteines, and synthetic polymers, such as polyethyleneimine, polyolefins, polyalcohols, polycarboxylic acids and starburst dendrimers. Another example of suitable carriers are copolymers, such as those with the formula $(Lys)_n$-$(aax)_q$-$(Glu)_m$-$(aay)_p$, where n, m, p and q are integers and aax and aay are non-specified amino acids which may be the same as or different from each other, and which are selected from the natural amino acids and their D-isomers.

In a preferred embodiment of the present invention, sulfhydryl-containing boron moieties, sodium borocaptate or BSH ($Na_2B_{12}H_{11}SH$), are used for boronating dextran. These have been documented to be non-toxic. Barth, *Cancer Res., supra*; Haselberger *et al.*, *Cancer Res.* 54: 6318-20 (1994). These are preferably boron-10 enriched, containing, for example up to 95-98% boron-10.

Preparation of a borocaptate-dextran conjugate is illustrated in Scheme I below. Other boron-10 enriched compounds also are known, such as boron-10-enriched carboranes. Examples of these are described in U.S. Patent No. 4,824,659. These compounds may be used in accordance with the present invention, for example, by conjugating the carboranes to the chosen carrier. In this embodiment, the second antibody or antibody fragment may specifically bind to the carborane moiety on the boron compound.

In one embodiment, the boron compound comprises biotin. For example, from about 1 to about 3 biotin moieties may be conjugated to a dextran carrier. The biotin is preferably a biotinidase-resistant biotin analog, described in more detail below. In this embodiment, the second antibody or antibody fragment may specifically bind to the biotin moiety on the boron compound.

While endogenous biotin is found at high levels in animals, it is believed to occur at lower levels in humans. Accordingly, binding between the targeting conjugate and endogenous biotin is not expected to present a problem in the treatment of humans. Moreover, those skilled in the art can address any problems posed by endogenous biotin by, for example, administering the boron compound shortly after the targeting conjugate is administered. For example, it is believed that administering the boron compound within two to three days of the targeting conjugate would minimize any problems associated with endogenous biotin.

The boron compound of the present invention amplifies the amount of boron that can be delivered per antibody molecule used in the first pre-targeting step, and is an important advantage of the present invention. The use of this compound in accordance with the present invention achieves or surpasses the high boron concentration in tumor required for effective BNCT. Accordingly, the compositions and methods of the present invention are useful for targeting boron atoms to tumor sites for therapy of common malignant tumors by BNCT.

In a preferred embodiment, the boron compound also is radiolabeled with a detectable label. This permits the determination of the location of the administered boron compound. Suitable radiolabels are known to those skilled in the art, and include, for example, gamma-emitting isotopes. The compound may be labelled by methods known in the art. For example, the boron compound may be conjugated to a chelating agent such as, for example, DTPA, which chelates the radiolabel, or a thiol ligand for direct labeling by Tc-99m using known methods, such as those described in U.S. Patent No. 5,514,363. If DTPA is used to chelate the label, the chelate can serve as a hapten for a bispecific antibody, as disclosed in, e.g., U.S. Patent No. 5,256,395. When a radiolabeled boron compound is used, the radiolabel can be detected before the boron is irradiated to ensure that the compound has localized at the tumor cells and that non-localized boron has cleared from circulation. This embodiment minimizes the risks of damaging healthy cells when the boron is irradiated,

because irradiation can be delayed until the boron compound has localized at tumor cells. The neutron beam advantageously is focused to sites of localized boron-10 moieties to further improve the precision of neutron capture.

Routes of administration of the compositions used in the present invention include intravenous, intraarterial, intrapleural, intraperitoneal, intrathecal, subcutaneous or by perfusion.

In one embodiment of the present invention, a two-step pre-targeting approach is used. As with the other embodiments of the invention, this approach separates the antibody localization step from the step of depositing boron at the tumor sites. For example tumor sites are pre-targeted with a composition comprising a conjugate of a first member a binding pair and an antibody, wherein the antibody binds to antigens produced by or associated with the tumor cells. After a time period for tumor targeting has passed a boron compound comprising a conjugate comprising a complementary member of the binding pair and boron atoms, is administered. This compound binds to the targeting composition localized at the tumor sites, thereby delivering boron atoms to the tumor sites. This method avoids concerns about the *in vivo* behavior of boronated antibodies because the boron compound does not comprise an antibody. After the boron compound has localized at the tumor site, the boron atoms may be irradiated according to conventional BNCT methods, thereby effecting therapy of the tumor cells.

Another embodiment of the present invention uses an intermediate step between the antibody conjugate delivery step and the boron delivery step. In this step, a clearing agent is used to effect the rapid clearance of circulating antibody and minimize the final boron concentration in circulation. In one embodiment, the clearing agent is an antibody that is anti-idiotypic to the first antibody or antibody fragment of the targeting composition, as described in international publication No: WO 96/40245. This antibody may be galactosylated to achieve rapid clearance by asialoglycoprotein receptors in the liver.

In one embodiment of the invention, an anti-idiotypic clearing agent is used, the boron compound is administered, and the patient is exposed to a thermal neutron beam shortly after the boron compound is administered, for example, immediately after the boron compound is administered, thereby irradiating the boron atoms at a time when the amount of boron localized at the targeted tumor cells is at a maximum. In a variation of this embodiment, the boron compound is labeled with a detectable label, and the detectable label is detected prior to the irradiation of the boron atoms. This latter variation allows the practitioner to ensure that the boron compound is localized before the boron is irradiated, thereby minimizing the risks of damage to healthy cells.

Alternatively, the boron compound can be administered parenterally within 24 hrs of the second (clearing) step, or up to 3 days later. The longer the delay after the first step, the lower the amount (and ratio) of clearing agent needed.

The multispecific antibody conjugate may be injected parenterally, usually at a dose of up to 1g of the first antibody or antibody fragment, for example within a dose range of from about 50 mg to about 500 mg. This can be administered as a single injection or in divided doses.

After 1-5 days, more preferably at less than 2 days and even at less than 1 day when the targeting composition comprises a small and rapidly targeting molecule, such as an antibody fragment or subfragment, a dose of unlabeled clearing agent may be administered parenterally. The dose may be, for example, 2.5 to 10 times the dose of the first step (which can be determined also by measuring the amount of antibody from the first step circulating in the blood at the time of the second step's injection). The clearing agent can be given as a single injection or in divided doses, wherein administering the clearing agent in 2 doses is preferred in certain circumstances. Then, the boron compound is administered with the appropriate time and dosage determined as described above.

Another embodiment of the present invention uses an intermediate step between the boron delivery step and the neutron irradiation step. In this step, a clearing agent is used to effect the rapid clearance of circulating boron compound and to minimize the amount of boron compound in circulation at the time of radiation. Any clearing agent may be used. Advantageously, the clearing agent comprises an antibody specific for the same hapten of the boron compound that is bound by the second antibody or antibody fragment of the targeting composition. An advantage of using such a clearing agent is that it does not competitively remove bound boron compound from the target site. The clearing agent may be galactosylated to achieve rapid clearance as described above.

As discussed above, galactosylated clearing agents remove unlocalized conjugates from circulation and direct them to the liver. When the target site is at or near the liver, further precautions may be taken to avoid unintended tissue damage to the liver during irradiation. For example, the second clearing step can be omitted, a low molecular weight clearing agent can be used to promote clearance through the kidneys, or the practitioner can wait an appropriate amount of time before irradiating the boron to allow the cleared boron compound to pass through the liver.

The timings of the two or three pre-targeting steps can be optimized to enhance the efficiency of boron delivery. The time of maximum tumor uptake of the targeting compositions can be determined by first determining the optimum dose, and also determining the time of maximum tumor uptake at this dose. This may be, for example, between 48 and 72 hours. Optimally, the clearing agent, such as an anti-idiotypic antibody, is administered at this time.

From initial studies, the present inventors have shown that the blood level of the targeting antibody conjugate drops dramatically at the 'zero' time upon administering an anti-idiotypic clearing agent. That is, circulating conjugate is cleared virtually instantaneously. The boron compound, therefore, may be administered within hours (2-4 hours) of the clearance step, and may be

administered immediately after the clearance step when an anti-idiotypic clearing agent is used.

The attainment of low levels of circulating boron, and, in particular, the attainment of near absolute clearance of circulating boron, has the advantage of reducing the systemic toxicity observed when boron atoms are irradiated by the neutron beam. That is, because little or no boron is in circulation, only targeted tumor cells are affected by the irradiation of boron atoms by the neutron beam.

In one embodiment of the invention, no clearing agent is used, and the boron compound is administered from about 5 to about 20 days after the administration of the targeting antibody conjugate. This embodiment is advantageous for patients who show rapid blood clearance and tumor accretion of the targeted compounds, for example, because they have a heavy tumor burden which is expressing large amounts of antigen.

In another embodiment, the boron-carrier compound is adapted for administration within about 48 to about 240 hours subsequent to administration of said conjugate.

The embodiments of the invention are further illustrated through examples which show aspects of the invention in detail. These examples illustrate specific elements of the invention and are not to be construed as limiting the scope thereof.

EXAMPLES

I. PREPARATION OF REAGENTS

1. Preparation of Bispecific Antibody

The interchain disulfide bridges of an $F(ab')_2$ fragment having specificity for a tumor-associated antigen (i.e., an antigen produced by or associated with the tumor cells and present at the tumor cells) is gently reduced with cysteine, taking care to avoid light-heavy chain linkage, to form Fab'-SH fragments. The SH group(s) are activated with an excess of bis-maleimide linker (1,6-bis-maleimidohexane). An antibody specific for the boron compound is converted to Fab'-SH and then reacted with the activated target-specific Fab'-SH fragment to obtain a bispecific antibody.

2. Preparation of a biotin-dextran-boron compound

A. Preparation of a sulfhydrylborane-dextran conjugate (compound 3 in Figure 1):

70,000 MW dextran is boronated with borocaptate using a published 2-step procedure. Holmberg *et al.*, *Bioconjugate Chem.*, 4: 570-73 (1993). This simple method involves allylation of the dextran's hydroxyl groups, followed by a free-radical type addition of borocaptate. This method has been found to incorporate 100-125 boron cages, or 1200-1500 boron atoms, per dextran chain. The product obtained by this method is water soluble. According to Holmberg *et al.*, *supra*, 70% of the hydroxyls were allylated and a 50% efficiency in the boronation of allyl dextran resulted in the conjugate having boron content of 150 μg boron/mg and a sulfur content of 1.5-4%. This corresponds to 100-120 boron cages, or 1200-1500 boron atoms, per dextran chain.

In particular, dextran (2g, 70 kD) was allylated in aqueous solution with 29 mmol of allyl bromide in the presence of 12.5 mmol of sodium hydroxide for 2 h at 60°C. After this time the reaction mixture was acidified, repeatedly precipitated from acetone, washed several times with ethanol and finally dialyzed. The intermediate product was then made basic with 40 mmol of sodium hydroxide and reacted with 2.8 g of 6-bromohexanoic acid at 70-80°C for 5 h. The solution was cooled, acidified and purified by dialysis.

The doubly derivatized dextran is boronated by reaction of dextran allyl groups with sodium borocaptate ('BSH' or $Na_2B_{12}H_{11}SH$; di-sodium undecahydro-mercapto-closo-dodecacarborate; Boron Biologicals, Raleigh, NC). Briefly, allyl dextran (20 mg) was reacted with 30 mg of sodium borocaptate and 20 mg of ammonium persulfate in 2 mL of water for 3 h at 50°C. The intermediate product is purified by PD-10 column chromatography and by repeated dialysis against water.

Boron content of the sulfhydrylborane-dextran conjugate is determined using ICP-atomic emission spectroscopy, as well as from sulfur content. Sulfur analysis indicated the presence of 124.3 boron cages while microanalysis for

boron content gives a figure of 137 boron cages (1644 boron atoms) per mole of dextran. Boron determinations of non-biological samples can be carried out by commercial outlets (Galbraith Laboratories).

The carboxylic acid derivatized boronated dextran-70 (12.5 mg) was treated with 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (12.5 mg) and a fifty-fold excess of ethylene diamine at pH 5.3 for 4 h at room temperature. Excess reactants were removed from the polymeric intermediate by repeated filtration through a Centricon*-30 membrane and the purified intermediate was buffer-exchanged into 0.1 M phosphate buffer at pH 7.7. This material was reacted with a fifteen-fold molar excess of sulfosuccinimido biotin for one hour and finally purified using a Centricon*-30 membrane. HABA assay indicated that the biotinylated-boronated-dextran-70 contained approximately two biotin moieties per polymeric unit.

While 70 KD molecular weight dextran is used in this example, *in vivo* pharmacokinetics of the boronated dextran may make the use of either higher or lower molecular weight dextran preferable.

B. Carboxyalkylation of sulfhydrylborane-dextran conjugate:

To react with an amine-containing biotin analog, the dextran conjugate is first derivatized with 6-bromohexanoic acid to introduce the necessary carboxylic acid groups. Carboxyalkylation and the allylation reaction described above are both chemically the same type of alkylation reaction. These two reactions can be combined in one operation by first reacting dextran, under basic conditions, with allyl bromide, and then reacting with bromohexanoic acid. A similar tandem operation has been described for the conjugation of mitomycin with an antibody using dextran as intermediate carrier. Noguchi *et al.*, *Bioconj. Chem.*, 3: 132-37 (1992). The order of alkylation is designed to limit the level of carboxylic acid groups introduced so as to achieve a biotin-dextran ratio of about one in the next step. The extent of carboxyalkylation is determined by titration with sodium methoxide. Any slight reduction in the number of boron

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atoms introduced as a result of this "double derivatization" should not be too worrisome due to the large number of boron atoms loadable by this method, and due to the 4-fold amplification of boron localization per mole of the SAv-IgG conjugate localized at tumor sites.

C. Biotinylation of sulfhydrylborane-dextran (Figure 1):

A preferred embodiment of the present invention uses a specially designed amine-containing biotin analog (compound 7 of Figure 1). This compound has a spacer arm between the biotin moiety and the amine terminus, and has a N-methyl substitution at the biotin peptide bond. Alternatively, a biotinidase-resistant biotin analog comprising a biotin peptide-bonded to an unnatural D-amino acid, and further terminating in an amino group for conjugation to carboxyl-substituted dextran borocaptate, may be used. These characteristics of the specific biotin-peptide bond prevent or minimize recognition by serum biotinidases, and the compounds are therefore more stable. Evangelatos *et al.*, *Analyt. Biochem.*, 196: 385-89 (1991).

The amine-containing biotinylation agent is condensed with carboxyalkylated sulfhydrylborane-dextran using water soluble carbodiimide ('EDC') and N-hydroxy sulfosuccinimide at a pH of about 6 at room temperature. The structure of the requisite final product is shown as compound 4 in Figure 1.

The extent of amide formation may be controlled by varying the molar excess of amine used. Noguchi *et al.*, *supra*. Such manipulation can be used to control the amount of biotin introduced, with the goal of introducing an average of about 1 biotin moiety per dextran chain. Final molar substitution ratios are derived from determinations of biotin-dextran and boron-dextran ratios and from binding to known concentrations of streptavidin.

3. Preparation of anti-CEA (IgG) x anti-indium-DTPA (Fab') bispecific antibody

Humanized MN-14 MAb (anti-CEA) IgG at 10 mg/mL in 0.2 M sodium borate buffer, pH 9.5, is treated with a ten-fold molar excess of the cross-linker sulfo-succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC, Pierce Chemical Co., Rockford IL) for 75 minutes, and the maleimide-activated antibody is purified from excess reactants by spin-column chromatography on G-50-80 Sephadex* buffer equilibrated in 0.2 M sodium phosphate, pH 7.0, containing 1 mM EDTA. Meanwhile, 10 mg of the anti-indium-DTPA antibody 734-F(ab')$_2$ in 1 mL of 20 mM HEPES/150 mM NaCl, pH 7.3, is treated with 100 uL of 100 mM freshly-prepared L-cysteine solution in the same buffer. The reaction is incubated for 30 minutes at 37 degrees Celsius and purified on the same spin-column system as the hMN-14. The two antibodies are mixed in a 3:1 molar ratio of Fab' to IgG and allowed to react for two hours at four degrees Celsius. The resulting bispecific antibody (hMN-14 x 734 [IgG x Fab'], containing 1-2 Fab' moieties per IgG) is purified by semi-preparative size-exclusion HPLC on a Tosohaas* TSK-3000 column developed in 0.2 M phosphate buffer and eluted at 8 mL per minute. The bispecific antibody is further purified from unreacted hMN-14 by loading the mixture onto a bed of Affigel*-DTPA equilibrated in 1 % HSA in 0.2 M phosphate, pH, 6.8. After washing unbound (non-anti-DTPA-MAb- containing) components from the column bed with 20 mL of phosphate buffer, the bispecific antibody is eluted using 10 mL of 100 mM EDTA, pH 3.8. The hMN-14 x 734 [IgG x Fab'] is purified from EDTA by repeated dialyses against 0.2 M phosphate buffered 0.9% sodium chloride, pH 6.8.

4. Preparation of anti-CEA (IgG) x anti-HSG (Fab') bispecific antibody

An anti-CEA x anti-HSG [(histamine-succinyl-glycyl)-polymer-carborane complex] bispecific antibody (IgG x Fab') is prepared analogously to the anti-CEA x anti-DTPA described above. Briefly, humanized MN-14 MAb (anti-

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CEA) IgG is first derivatized with sulfo-SMCC, and the product is purified by centrifuged size-exclusion chromatography ('spin-column'). Meanwhile, anti-HSG MAb, 679.1MC7 (as described by Morel, A. et al., *Molecular Immunology* 27:995-1000, 1990) is first converted to $F(ab')_2$ fragment by pepsin digestion, and then to Fab' by cysteine reduction as described above. The two antibodies are mixed in a 3:1 molar ratio of Fab' to IgG, and allowed to react for two hours at four degrees Celsius. The resulting bispecific antibody (hMN-14 x 679.1MC7 [IgG x Fab'], containing 1-2 Fab' moieties per IgG) is purified by semi-preparative size-exclusion HPLC on a Tosohaas TSK-3000 column developed in 0.2 M phosphate buffer and eluted at 8 mL per minute. The hMN-14 x 679.1MC7 [IgG x Fab'] is purified from EDTA by repeated dialyses against 0.2 M phosphate buffered 0.9% sodium chloride, pH 6.8.

5. <u>Preparation of DTPA-dextran-carborane</u>

A substantially mono-activated DTPA is prepared by using an in situ preparation comprising DTPA, sodium bicarbonate, N-hydroxysulfo-succinimide, and 1-ethyl-3,3-(dimethylamino)propyl carbodiimide in a molar ratio of 20:60:20:1. The activation reaction is allowed to proceed for 45 minutes at four degrees Celsius. Aminodextran (MW 40 kiloDalton) containing an average of four amino groups per mole of dextran (Carbomer Incorporated, Westborough, MA) in 0.2 M phosphate buffer, pH 8, is mixed with a fifty-fold molar excess of preactivated DTPA. The conjugation reaction is stirred for 18 hours at four degrees Celsius. Subsequently, the excess low molecular weight reactants are removed by repeated dialyses against water through a 10,000 MW cutoff membrane. The DTPA-dextran is converted to DTPA-dextran-carborane using the same methodology as described in Example 2; a two-step procedure using an allyl bromide reaction in the presence of NaOH, followed by reaction with sodium borocaptate. The final product is purified by repeated dialyses against water through a 10,000 MW cutoff membrane and lyophilized for long-term storage. The boron content is determined by ICP-atomic absorption

spectrometry. The number of chelates incorporated is determined by titration against an excess of indium-111/indium and radiomatic HPLC and ITLC analyses of the radiolabeled DTPA-dextran-carborane.

6. Preparation of HSG-dextran-carborane

Peptides containing one or two HSG units are prepared using solid phase peptide synthesis methods. Lysyl(HSG)-x-lysyl(HSG)-NH_2, where 'x' is one or more amino acids such as glycyl, tyrosyl and the like, is an example of such a peptide. The amine terminus of the peptide is reacted with activated carboxylic acid-derivatized-boronated dextran as described in Example 2. After conjugation, the excess low molecular weight reactants are removed by repeated dialyses against water through a 10,000 MW cutoff membrane, and the product is lyophilized for long-term storage. The boron content is determined by ICP-atomic absorption spectrometry. The number of HSG moieties incorporated is determined by amino acid analyses for histamine and glycyl units.

II. METHODS FOR TARGETING BORON ATOMS TO TUMOR CELLS

7. Methods using an anti-CEA x anti-carborane bispecific antibody

The following example illustrates a three-step pre-targeting method using a bispecific antibody and a clearing agent before the boron compound is administered. The three steps are as follows:

Step 1: A bispecific monoclonal antibody comprising an anti-CEA determinant and an anti-carborane determinant is administered to a cancer patient, and allowed to localize at tumor sites.

Step 2: The circulating conjugate is cleared using a galactosylated anti-idiotypic monoclonal antibody specific for the anti-CEA determinant of the bispecific antibody described above.

Step 3: A carborane-dextran-boron polymer comprising from about 1200 to about 1500 boron atoms is administered, and localizes at the tumor sites due to

the affinity between the carborane and the anti-carborane determinant of the bispecific monoclonal antibody localized at the tumor site in step 1.

Once the boron has localized at the tumor site, it is irradiated in accordance with conventional BNCT methods.

Another three-step pre-targeting method using a bispecific antibody and a clearing agent after the boron compound is administered is as follows:

Step 1: A bispecific monoclonal antibody comprising an anti-CEA determinant and an anti-carborane determinant is administered to a cancer patient, and allowed to localize at tumor sites.

Step 2: A carborane-dextran-boron polymer comprising from about 1200 to about 1500 boron atoms is administered, and localizes at the tumor sites due to the affinity between the carborane and the anti-carborane determinant of the bispecific monoclonal antibody localized at the tumor site in step 1.

Step 3: Circulating carborane-dextran-boron polymer is cleared using a monoclonal antibody specific for the same hapten of the boron compound that is bound by the anti-carborane determinant of the bispecific monoclonal antibody used in Step 1.

Once non-localized boron has been cleared, localized boron is irradiated in accordance with conventional BNCT methods.

8. <u>Methods using an anti-CEA x anti-biotin bispecific antibody</u>

The following example illustrates a three-step pre-targeting method using a bispecific antibody and a clearing agent before the boron compound is administered. The three steps are as follows:

Step 1: A bispecific monoclonal antibody comprising an anti-CEA determinant and an anti-biotin determinant is administered to a cancer patient, and allowed to localize at tumor sites.

Step 2: The circulating conjugate is cleared using a galactosylated anti-idiotypic monoclonal antibody specific for the anti-CEA determinant of the bispecific antibody described above.

Step 3: A biotin-dextran-boron compound comprising from about 1200 to about 1500 boron atoms is administered, and localizes at the tumor sites due to the affinity between the biotin and the anti-biotin determinant of the bispecific monoclonal antibody localized at the tumor site in step 1.

Once the boron has localized at the tumor site, it is irradiated in accordance with conventional BNCT methods.

A three-step pre-targeting method using a bispecific antibody and a clearing agent after the boron compound is administered is as follows:

Step 1: A bispecific monoclonal antibody comprising an anti-CEA determinant and an anti-biotin determinant is administered to a cancer patient, and allowed to localize at tumor sites.

Step 2: A biotin-dextran-boron compound comprising from about 1200 to about 1500 boron atoms is administered, and localizes at the tumor sites due to the affinity between the biotin and the anti-biotin determinant of the bispecific monoclonal antibody localized at the tumor site in step 1.

Step 3: Circulating biotin-dextran-boron polymer is cleared using a monoclonal antibody specific for the same hapten of the boron compound that is bound by the anti-biotin determinant of the bispecific monoclonal antibody used in Step 1.

Once non-localized boron has been cleared, localized boron is irradiated in accordance with conventional BNCT methods.

9. <u>Methods using a multivalent, multispecific targeting composition</u>

The following example illustrates a three-step pre-targeting method using a multivalent, multispecific targeting composition and a clearing agent before the boron compound is administered. The three steps are as follows:

Step 1: A multivalent, multispecific targeting composition, comprising a $F(ab')_2$ fragment comprising anti-CEA determinants and a Fab fragment comprising an anti-carborane determinant, is administered to a cancer patient, and allowed to localize at tumor sites.

Step 2: The circulating conjugate is cleared using an anti-idiotypic monoclonal antibody specific for the anti-CEA determinants of the multivalent, multispecific targeting composition described above. The clearing antibody may be galactosylated to achieve rapid clearance as described above.

Step 3: A carborane-dextran-boron polymer comprising about 1200 to about 1500 boron atoms is administered, and localizes at the tumor sites due to the affinity between the carborane and its determinant on the multivalent, multispecific targeting composition localized at the tumor site in step 1.

Once the boron has localized at the tumor site, it is irradiated in accordance with conventional BNCT methods.

Another three-step pre-targeting method using a multivalent, multispecific targeting composition and a clearing agent after the boron compound is administered is as follows:

Step 1: A multivalent, multispecific targeting composition, comprising a $F(ab')_2$ fragment comprising anti-CEA determinants and a Fab fragment comprising an anti-carborane determinant, is administered to a cancer patient, and allowed to localize at tumor sites.

Step 2: A boron compound comprising a particular antigen which is the hapten of the antibody described above and from about 1200 to about 1500 boron atoms is administered, and localizes at the tumor sites due to the affinity between the carborane and its determinant on the multivalent, multispecific targeting composition localized at the tumor site in step 1.

Step 3: Circulating carborane-dextran-boron polymer is cleared using a monoclonal antibody specific for the same hapten of the boron compound that is bound by the anti-carborane determinant of the multivalent, multispecific targeting composition used in Step 1.

Once non-localized boron has been cleared, localized boron is irradiated in accordance with conventional BNCT methods.

10. Methods using an hMN-14 x 734 bsMAb and borocaptate-dextran-DTPA

A patient presenting a CEA-expressing cancer is treated with 200 mg of hMN-14 x 734 (IgG x -Fab') bsMAb (1×10^{-6} moles) (as described in Example 3), infused over 30 minutes as a 5 mg/mL solution in 0.2 M phosphate buffered physiological saline, pH 6.8. The patient returns 7-14 days later, when the bsMAb localized to tumor sites has reached a maximum and excess bsMAb has substantially cleared from circulation. An infusion of an equimolar amount of borocaptate-dextran-DTPA (1×10^{-6} moles) is given to the patient. Non-localized borocaptate-dextran-DTPA is allowed to substantially clear from the circulation, and the patient is treated with BNCT in accordance with the methods described above.

11. Methods using an hMN-14 x 679.1MC7 bsMAb and borocaptate-dextran-HSG

A patient presenting a CEA-expressing cancer is treated with 200 mg of hMN-14 x 679.1MC7 (IgG x -Fab') bsMAb (1×10^{-6} moles) (as described in Example 4), infused over 30 minutes as a 5 mg/mL solution in 0.2 M phosphate buffered physiological saline, pH 6.8. The patient returns 7-14 days later, when the bsMAb localized to tumor sites has reached a maximum and excess bsMAb has substantially cleared from circulation. An infusion of an equimolar amount of borocaptate-dextran-HSG (1×10^{-6} moles) is given to the patient. Non-localized borocaptate-dextran-HSG is allowed to substantially clear from the circulation, and the patient is treated with BNCT as described above.

It will be apparent to those skilled in the art that various modifications and variations can be made to the processes and compositions of this invention. Thus, it is intended that the present invention cover the modifications and variations of this invention provided they come within the scope of the appended claims and their equivalents.

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CLAIMS:

1. Use, for targeting boron atoms to tumor cells in a patient, of:

(A) a conjugate of

(i) at least one first antibody or antigen-binding antibody fragment which selectively binds to an antigen produced by or associated with the tumor cells and present at the tumor cells, and

(ii) at least one second antibody or antibody fragment which specifically binds to a non-boron containing hapten on a carrier portion of a boron-carrier compound

wherein said conjugate is adapted to localize at said tumor cells upon administration to said patient of said conjugate and said boron-carrier compound.

2. The use of claim 1 further comprising a first clearing agent for clearing non-localized conjugate from circulation, wherein said first clearing agent is adapted for administration subsequent to administration of said conjugate and prior to administration of said boron-carrier compound.

3. The use of claim 1 or 2 further comprising a second clearing agent for clearing non-localized boron-carrier compound from circulation, wherein said second clearing agent is adapted for administration subsequent to administration of said boron-carrier compound.

4. Use, in the manufacture of a targeting composition for targeting boron atoms to tumor cells in a patient, of:

(A) a conjugate of

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(i) at least one first antibody or antigen-binding antibody fragment which selectively binds to an antigen produced by or associated with the tumor cells and present at the tumor cells, and

(ii) at least one second antibody or antibody fragment which specifically binds to a non-boron containing hapten on a carrier portion of a boron-carrier compound

wherein said conjugate is adapted to localize at said tumor cells upon administration to said patient of said conjugate and said boron-carrier compound.

5. The use of any one of claims 1 to 4, wherein said conjugate comprises a bispecific antibody.

6. The use of any one of claims 1 to 4, wherein said conjugate comprises a polyspecific antibody.

7. The use of claim 6, wherein said polyspecific antibody comprises a plurality of determinants that specifically bind to the same or different epitopes of an antigen produced by or associated with the tumor cells, or that bind to different antigens produced by or associated with the tumor cells.

8. The use of claim 6, wherein said polyspecific antibody comprises a plurality of determinants that specifically bind to the same or different epitopes of said boron-carrier compound.

9. The use of any one of claims 1 to 4, wherein said first antibody or antibody fragment is a monoclonal antibody or a fragment of a monoclonal antibody.

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10. The use of any one of claims 1 to 4, wherein said second antibody or antibody fragment is a monoclonal antibody or a fragment of a monoclonal antibody.

11. The use of any one of claims 1 to 4, wherein said first antibody or antibody fragment is a humanized antibody or a fragment of a humanized antibody.

12. The use of any one of claims 1 to 4, wherein said second antibody or antibody fragment is a humanized antibody or a fragment of a humanized antibody.

13. The use of any one of claims 1 to 12, wherein said first antibody or antibody fragment is an anti-carcinoembryonic antigen (CEA) antibody or a CEA-binding fragment of an anti-CEA antibody.

14. The use of any one of claims 1 to 13, wherein said carrier portion comprises biotin, diethylenetriaminepentaacetic acid (DTPA), histamine-succinate, fluorescein isothiocyanate, rhodamine, dinitrophenyl (DNP), dextran or polyhistidine, and said second antibody or antibody fragment comprises an antibody or antibody fragment that specifically binds to the non-boron containing hapten on said carrier portion.

15. The use of claim 3, wherein at least one of said first and second clearing agents is an antibody.

16. The use of claim 15, wherein said first or second clearing agent antibody is galactosylated.

17. The use of any one of claims 2 or 15, wherein said boron-carrier compound is adapted for administration within about 2 to about 24 hours subsequent to administration of said first clearing agent.

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18. The use of claim 2 or 15, wherein said first clearing agent comprises an antibody that is anti-idiotypic to said first antibody or antibody fragment.

19. The use of claim 3 or 15, wherein said second clearing agent comprises an antibody specific for the non-boron containing hapten on said boron-carrier compound that is bound by said second antibody or antibody fragment.

20. The use of claim 5, wherein said boron-carrier compound is carborane-dextran-boron polymer wherein the dextran is derivatized with from about 1200 to about 1500 boron atoms.

21. The use of any one of claims 1 to 20, wherein said boron-carrier compound is radiolabeled with a detectable label.

22. The use of claim 21, further comprising the use of said detectable label of said boron-carrier compound to detect the boron atoms of said boron-carrier compound.

23. The use of claim 22, further comprising the use of neutrons to irradiate the detected boron atoms of said boron-carrier compound localized at said tumor cells.

24. The use of claim 1, wherein said boron-carrier compound is adapted for administration within about 48 to about 240 hours subsequent to administration of said conjugate.

25. The use of claim 1, wherein said boron-carrier compound is adapted for administration within about 5 to about 20 days subsequent to administration of said conjugate.

26. The use of claim 1, further comprising the use of neutrons to irradiate the boron atoms of said boron-carrier compound localized at said tumor cells to effect boron neutron capture therapy (BNCT) of said tumor cells.

27. A sterile conjugate adapted for human injection for use in targeting boron atoms to tumor cells, comprising a conjugate of

(i) at least one first antibody or antigen-binding antibody fragment which selectively binds to an antigen produced by or associated with the tumor cells and present at the tumor cells, and

(ii) at least one second antibody or antibody fragment which specifically binds to a non-boron containing hapten on a carrier portion of a boron-carrier compound.

28. The conjugate of claim 27, wherein said first antibody or antibody fragment is an anti-carcinoembryonic antigen (CEA) antibody or a CEA-binding fragment of an anti-CEA antibody.

29. The conjugate of claim 28, wherein said carrier portion comprises biotin, diethylenetriaminepentaacetic acid (DTPA), histamine-succinate, fluorescein isothiocyanate, rhodamine, dinitrophenyl (DNP), dextran or polyhistidine, and said second antibody or antibody fragment comprises an antibody or antibody fragment that specifically binds to the non-boron containing hapten on said carrier portion.

30. A kit comprising in separate containers adapted for injection:

(A) a sterile conjugate of

(i) at least one first antibody or antigen-binding antibody fragment which selectively binds to an antigen

produced by or associated with the tumor cells and present at the tumor cells, and

(ii) at least one second antibody or antibody fragment which specifically binds to a non-boron containing hapten on a carrier portion of a boron-carrier compound;

(B) optionally, a first clearing composition;

(C) said boron-carrier compound;

(D) optionally, a second clearing composition; and

instructions for use in a method for targeting boron atoms to tumor cells in a patient.

31. The kit of claim 30, wherein said first antibody or antibody fragment is an anti-carcinoembryonic antigen (CEA) antibody or a CEA-binding fragment of an anti-CEA antibody.

32. The kit of claim 30 or 31, wherein said carrier portion comprises biotin, diethylenetriaminepentaacetic acid (DTPA), histamine-succinate, fluorescein isothiocyanate, rhodamine, dinitrophenyl (DNP), dextran or polyhistidine, and said second antibody or antibody fragment comprises an antibody or antibody fragment that specifically binds to the non-boron containing hapten on said carrier portion.

33. The kit of any one of claims 30 to 32, wherein said boron-carrier compound is carborane-dextran-boron polymer wherein the dextran is derivatized with from about 1200 to about 1500 boron atoms.

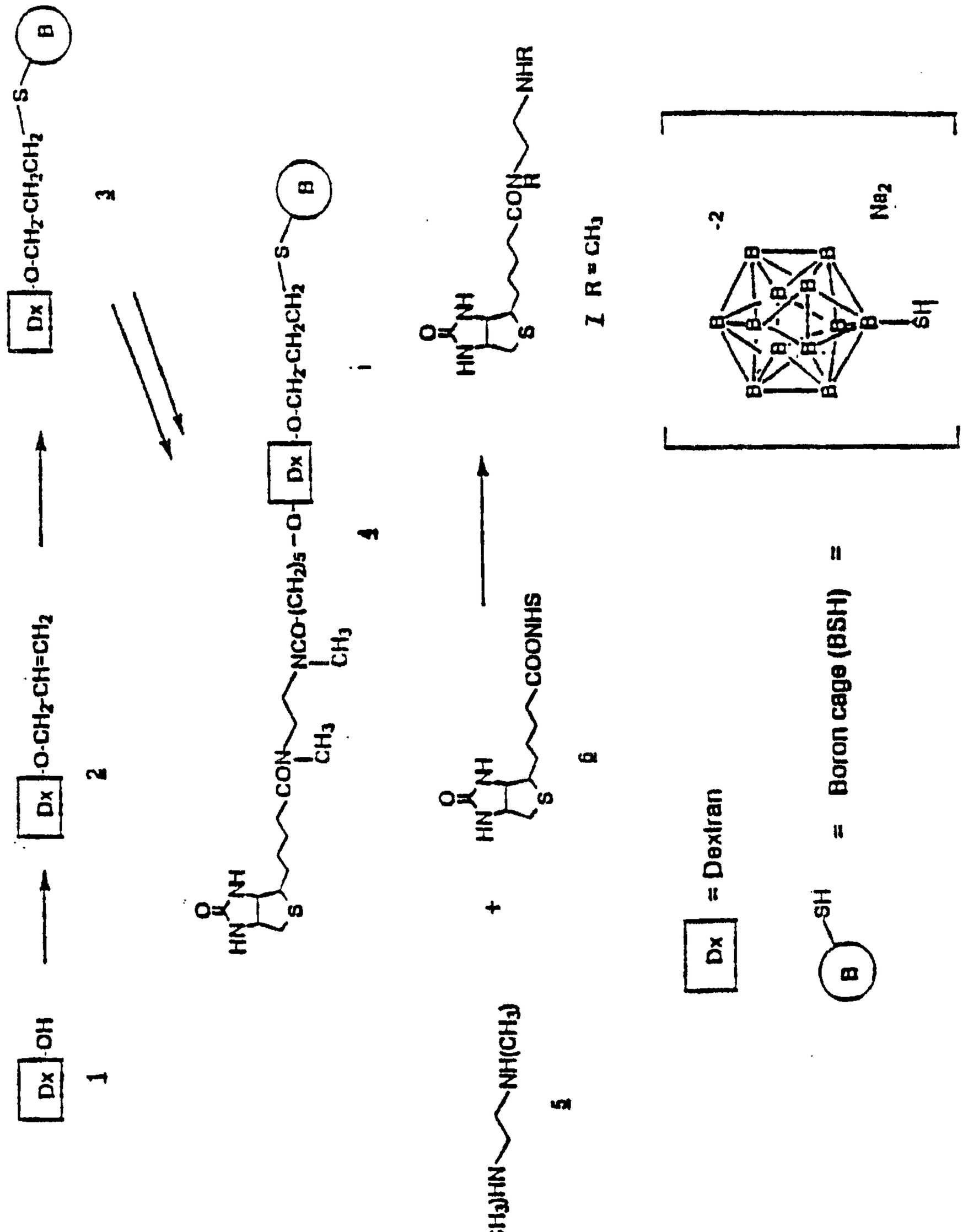
Dx-OH
1
Dx-O-CH2-CH=CH2
2
Dx-O-CH2-CH2-CH2-S-B
3
Dx-O-CH2-CH2-CH2-S-B
4
NH(CH3)
5
COONHS
6
NHR
Z R = CH3
-2
Na2
SH
Dx = Dextran
B = Boron cage (BSH)
(CH3)HN

Figure 1