Novel radioimmunoconjugates and uses thereof

Technical field of the invention

The present invention relates to radioimmunotherapy of hematologic cancer with a radiolabeled monoclonal antibody with an unexpectedly high cytotoxicity.

Background of the invention

Therapy with radiolabeled antibodies has been introduced against non-Hodgkin lymphoma (NHL) and is an approved method today. Two products are on the market, Zevalin™ and Bexxar™, and both targets the CD20 antigen (Jacene et al., 2007).

Also the immunotherapeutic agent rituximab (Rituxan™/Mabthera™) targets the CD20 antigen. One problem with treatment against the same target is the possibility of immunophenotypic drift during the disease course (Ngo et al., 2009) which could cause diminished effects of CD20 therapy when repeated over time as in rituximab therapy or if CD20-based radioimmunotherapy (RIT) is administered following prolonged rituximab therapy.

A large number of patients receiving CD20 directed therapy will eventually experience relapse (Buchegger et al., 2006; Gordon et al 2004). Thus, there is a significant need for RIT that targets another antigen than the CD20 in NHL patients.

In the early development of RIT, the two antigens CD37 and CD20 were evaluated as targets (Press et al., 2001). It was concluded that the CD20 targeting RIT was more appropriate and therefore the development of CD37 directed RIT was abandoned. Thus, it is known in the art that monoclonal antibodies are suitable for use in RIT against lymphoma, but that radioimmunoconjugate (RIC) targeting CD20 is superior to RIC targeting CD37 (Press et al., 2001).

In recent years CD37 has attracted some new interest (Heider et al., 2009; Grosmaire, 2007), mainly as target for immunotherapy using chimeric or

humanized antibody constructs. These works teaches away from using conventional murine IgG monoclonal antibodies, since murine antibodies may induce human anti-mouse antibody (HAMA) production in patients, which can cause discomfort and reduced efficacy of immunotherapies.

For RIT, conventional murine monoclonal antibodies are still considered interesting, since in general the protein doses used are lower and the treatment need not being repeated to the same extent as with immunotherapy. Also the clearance of murine IgG is generally slightly faster than humanized or chimeric versions of the same IgG, which may be more appropriate in terms of whole body radiation exposure from RIT, at least in some settings. It should be noted that both Bexxar and Zevalin are based on murine antibodies.

The present invention provides the anti-CD37 murine antibody HH1 as carrier for radioisotope. The original hybridoma clone that produces the murine anti-CD37 antibody HH1 was developed in the 1980's (Smeland et al., 1985) and the HH1 antibody has been in sale for in vitro use in immunohistochemistry for several years.

HH1 has not previously been evaluated for radioimmunotherapy in terms of biodistribution and cellular cytotoxicity. The current work was therefore undertaken to evaluate the suitability of HH1 in radioimmunotherapy. In contrast to the previous clinical and preclinical work with anti-CD37 RIC, which used ^{131}I directly radiolabeled to the tyrosine residues using the chloramineT/Iodogen methods, the HH1 was radiolabeled via a chelator using a metallic radionuclide instead of a halogen.

Using a metallic radionuclide labeled via a chelator-linker could be advantageous since the use of ^{131}I-labeled antibodies is associated with the exposure of the thyroid to various amounts of iodine released from the RIC's.

In a previous study to evaluate whether HH1 was suitable for producing a radioimmunoconjugate CHX-A-DTPA was conjugated to HH1 and the conjugate labeled with $^{205,206}Bi$ for in vitro modeling purposes (Henriksen et al., 1997).

The uptake in the cell line Raji was compared for bismuth conjugated to HH1 or streptavidin. In the latter case cells had been presaturated with biotinylated-HH1.

It was found that the number of chelators required to ensure functional RIC when labeled with ^{212}Bi or ^{213}Bi was a limiting factor. It was therefore suggested to use biotinylated HH1 instead of a HH1 based RIC. Once bound to the cells, the biotinylated HH1 could then be targeted with radiolabeled streptavidin.

Thus, the work suggests that HH1 labeled with an alpha-particle-emitting radionuclide was less useful due to insufficient specific activity at the chelator concentrations deemed tolerable for the HH1 to retain sufficient binding ability.

It was also indicated in the paper that a beta-emitter would be even less suitable for constructing a functional RIC compared with an alpha-emitter (Henriksen et al, 1997) as the authors stated that targeted radiotherapy with beta-emitter should be inferior in disseminated disease because cross-fire is essential for obtaining sufficient effect.

Thus, the above cited work teaches away from using a directly chelated HH1 in radioimmunotherapy and also away from using HH1 in a beta-emitter based RIC.

Summary

The present disclosure relates to a radioimmunoconjugate that binds human CD37 comprising murine monoclonal antibody HH1, a linker, and a radionuclide selected from the group consisting of ^{211}At, ^{213}Bi, ^{212}Bi, ^{212}Pb, ^{225}Ac, ^{227}Th, ^{90}Y, ^{186}Re, ^{188}Re, ^{199}Au, ^{194}Ir, ^{166}Ho, ^{159}Gd, ^{153}Sm, ^{149}Pm, ^{142}Pr, ^{111}Ag, ^{109}Pd, ^{77}As, ^{67}Cu, ^{47}Sc, and ^{177}Lu.

In one aspect, there is disclosed herein a radioimmunoconjugate that binds human CD37 comprising:

a) murine monoclonal antibody HH1,

b) a chelating linker, and

c) a radionuclide selected from the group consisting of ^{177}Lu, ^{212}Pb,^{225}Ac, ^{227}Th and ^{90}Y.

In an example of the present disclosure the linker is a chelating linker and the radionuclide is ^{177}Lu.

An example of the present disclosure relates to a pharmaceutical composition comprising a radioimmunoconjugate of the present disclosure, and a pharmaceutically acceptable carrier.

In another aspect, there is disclosed herein a pharmaceutical composition comprising a radioimmunoconjugate as defined above, and a pharmaceutically acceptable carrier.

In an example of the present disclosure the pharmaceutical composition of the present disclosure comprises one or more additional antibodies or radioimmunoconjugates.

In another example of the present disclosure one or more additional antibodies or radioimmunoconjugates target CD20.

In another aspect, there is disclosed herein a radioimmunoconjugate that binds human CD37 comprising:

a) murine monoclonal antibody HH1,

b) a chelating linker, and

c) a radionuclide selected from the group consisting of ^{177}Lu, ^{225}Ac, ^{212}Pb, ^{227}Th and ^{90}Y,

for use in the treatment of a B-cell malignancies selected from non-Hodgkin lymphoma and chronic lymphocytic leukemia.

In a further aspect, there is disclosed herein a radioimmunoconjugate that binds human CD37 comprising:

a) murine monoclonal antibody HH1,

b) a chelating linker, and

c) a radionuclide selected from the group consisting of ^{177}Lu, ^{212}Pb, ^{225}Ac, ^{227}Th and ^{90}Y,

for use in therapy.

A further example of the present disclosure relates to a pharmaceutical composition of the present disclosure for treating B-cell malignant cells expressing the CD37 antigen.

In a further aspect, there is disclosed herein the use of a pharmaceutical composition as defined above in the manufacture of a medicament for treatment of a B-cell malignancy selected from non-Hodgkin lymphoma and chronic lymphocytic leukemia.

In an example of the present disclosure the pharmaceutical composition is for treatment of non-Hodgkin lymphoma and chronic lymphocytic leukemia.

In a further aspect, there is disclosed herein the use of the radioimmunoconjugate as defined above, in the manufacture of a medicament for the treatment of B-cell malignancies.

An example of the present disclosure relates to the use of the radioimmunoconjugate of the present disclosure for the treatment of B-cell malignancies.

An example of the present disclosure relates to the use of the radioimmunoconjugate of the present disclosure administered in combination with or in addition to other therapy.

In an example of the present disclosure the therapy is selected from pretreatment, chemotherapy, monoclonal antibody therapy, surgery, radiotherapy, and/or photodynamic therapy.

In another example of the present disclosure the therapy comprises pre-treatment using anti-CD20 and/or anti-CD37 monoclonal antibody prior to the treatment with the radioimmunoconjugate of the present disclosure.

An example of the present disclosure relates to a method for treatment of a B-cell malignancy selected from non-Hodgkin lymphoma and chronic lymphocytic leukemia, comprising administration of an effective amount of a pharmaceutical composition of the present disclosure.

In one aspect, there is disclosed herein a kit for the production of the radioimmunoconjugate as defined above comprising two or more vials, wherein one vial contains a conjugate comprising a chelator linked to a murine monoclonal antibody HH1; and a second vial contains a radionuclide.

Another example of the present disclosure relates to a kit for the production of the radioimmunoconjugate of the present disclosure comprising two or more vials, wherein one vial contains a conjugate comprising a chelator linked to a murine monoclonal antibody HH1; and a second vial contains a radionuclide.

An example of the present disclosure relates to a kit of the present disclosure, wherein the content of one or several of the vials are either lyophilized or in a solution.

In another example of the present disclosure the radioimmunoconjugate is generated by mixing the content of the two vials.

Brief description of the figures

Figure 1

Cell-bound antibody immediately (A) and 96 hours (B) after washing for incubation of Raji, Rael and Daudi cells with ^{111}In-HH1, ^{111}In-rituximab, ^{125}I-HH1 and ^{125}I-rituximab.

Figure 2

Activity bound to Daudi cells after incubation with ^{177}Lu-HH1 or ^{177}Lu-rituximab for 2 h (A) and 18 h (B). Blocked cells were blocked with 100 µg/ml unlabeled antibody.

Figure 3

Growth of Daudi cells incubated with ^{177}Lu-HH1 (A) or ^{177}Lu-rituximab (B) for 2 h before washing.

Figure 4

Growth of Daudi cells incubated with ^{177}Lu-HH1 (A) or ^{177}Lu-rituximab (B) for 18 h before washing.

Figure 5

Biodistribution of ^{111}In-labeled via chelator to HH1 in mice with Daudi xenografts.

Figure 6

FITC-histograms of unlabeled Daudi cells, Daudi cells labeled with secondary antibody only, or labeled with HH1, ON.108, IPO.24 or 6D263.

Figure 7

Biodistribution of ^{177}Lu- in female nude mice with Daudi tumor.

Figure 8

Therapy of mice with iv injected Daudi cells. Survival of mice treated with 50 and 100 MBq/kg ^{177}Lu-HH1, cold HH1, cold rituximab and NaCl.

Detailed description of the invention

The present invention relates to the use of antibody HH1 in radioimmunotherapy.

The combination of a metal radionuclide, linker and anti-CD37 monoclonal antibody has surprisingly shown that radiolabeled HH1 has a relevant biodistribution and tumor uptake in a xenograft/nude mouse model.

This is important information that indicates suitability for use in radioimmunotherapy.

Radioimmunoconjugates

The present invention surprisingly shows that the radioimmunoconjugate ^{177}Lu-HH1 exhibited a significant cytotoxicity on disseminated tumor cells and that ^{177}Lu-HH1 was more cytotoxic than ^{177}Lu-rituximab against the tumor cells for a given dosage.

This finding was unexpected since more radioactivity was bound per cell and the retention of the bound radionuclide was similar or better for ^{177}Lu-rituximab.

This teaches against common knowledge in the field, which is that anti-CD20 antibody is better than anti-CD37 antibody for radioimmunotherapy. Furthermore, the present work differs from previous notion in that for a beta-emitter, cross-fire, which is not obtainable in disseminated cells, would be essential for obtaining sufficient effect (Henriksen et al., 1997).

The reason for the observed effect is not clear. Data from experiments with various dosages of unlabeled HH1 and rituximab did not indicate any effects from the unlabeled antibodies in the growth assay used.

One possible explanation could be that there are fewer cells with very low antigen density among CD37 vs. CD20 even though CD20 is on average more strongly expressed on the cell line used.

Retention data did not suggest better retention due to internalization of CD37, which would otherwise be a possible explanation, since some internalization has been reported with the CD37 antigen (Press et al, 2001).

Thus, the present invention relates to a radioimmunoconjugate that binds human CD37 comprising murine monoclonal antibody HH1, a linker, and a radionuclide selected from the group consisting of ^{211}At, ^{213}Bi, ^{212}Bi, ^{212}Pb, ^{225}Ac, ^{227}Th, ^{90}Y, ^{186}Re, ^{188}Re, ^{199}Au, ^{194}Ir, ^{166}Ho, ^{159}Gd, ^{153}Sm, ^{149}Pm, ^{142}Pr, ^{111}Ag, ^{109}Pd, ^{77}As, ^{67}Cu, ^{47}Sc, and ^{177}Lu.

In an embodiment of the present invention the linker is a chelating linker.

In another embodiment of the present invention the radionuclide is ^{177}Lu.

In yet another embodiment the radionuclide is another beta-emitter or an alpha-emitter.

The present invention suggests, with in vitro data, that radiolabeled HH1 binds more effectively to the CD37 antigen than radiolabeled rituximab do to the CD20

antigen, i.e., it reached maximum binding to the antigen with less circulating antibody required (Table 2, Figure 2).

It also required less time to reach maximum binding (Figure 2). These would be important features in vivo as well because this means that tumor cells can trap the RIC even at lower concentration of circulating antibodies, a situation that may occur in less available areas of solid tumors and for single tumor cells and micrometastases located in remote areas of normal tissues.

This is significantly different from previous data which indicated that higher antibody concentration was required with another anti-CD37 antibody than HH1 (Bernstein et al., 1990), also compared with an anti-CD20 antibody (Press et al., 1993), to saturate antigen and obtain favorable biodistribution.

In addition, the present invention shows that HH1 has some different antigen binding properties compared with a panel of three different anti-CD37 antibodies – despite that all of the antibodies substantially bind to the same epitope.

Blocking experiments, i.e., using cells pre-saturated with unlabeled antibody, showed that HH1 would block the CD37 on living cells from binding to radiolabeled HH1, substantially better than the three other anti-CD37 antibodies.

In a cell assay comparing radiolabeled antibodies, HH1 showed much better immunoreactive fraction compared with the three other antibodies. By immunoreactive fraction is meant the fraction of antibody that can bind antigen if there is an unlimited excess of antigens. Different antibodies can have different ability to preserve the immunoreactivity after going through a labeling procedure. The results in Example 6, Experiment IV, Table 5 shows that the immunoreactivity of HH1 was better preserved than the immunoreactivity of three commercially available antibodies.

On the other hand, immunohistochemistry analyzes showed that the three antibodies stained tissue sections from paraffin-embedded fixed tumor samples, while HH1 failed to do so. Differences in antibody antigen interactions were not detectable by flow cytometry.

Flow cytometry histograms were similar for HH1 and the three other anti-CD37 antibodies (Figure 6). All-in-all these data show that HH1 has a significant individual antigen interaction, which in several aspects cannot be predicted from studies with other anti-CD37 antibodies.

The novel anti-CD37 radioimmunoconjugate with strong cytotoxic properties described here consists of the murine monoclonal antibody HH1, a chelating linker, and the beta-emitter ^{177}Lu.

The radionuclide may be attached to the antibody by first reacting a bifunctional chelator, e.g., p-SCN-bn-DOTA (Macrocyclics, Tx, USA), with the antibody, followed by purification to remove unconjugated chelator, and then reaction of the chelator antibody conjugate with the radionuclide, followed by purification to remove any unconjugated radionuclide.

Alternatively, the chelator and the radionuclide can be combined firstly and subsequently conjugated to the antibody.

Chelating linkers like, e.g., p-SCN-bn-DOTA, can be used for conjugating other metal radionuclides to HH1 in similar fashion to that described for ^{177}Lu.

Any type of linker with sufficient complexing ability and a functional group allowing direct or indirect conjugation to a protein or a peptide could be used. Examples of such linkers are described in the literature (e.g. Brechbiel, 2008; Liu, 2008). Some useful examples are bifunctional cyclic chelators like p-SCN-bn-DOTA, DOTA-NHS-ester; bifunctional linear chelators like p-SCN-Bn-DTPA and CHX-A''-DTPA.

The radionuclides in the present invention will preferably be conjugated to a targeting molecule by using bifunctional chelators.

These could be cyclic, linear or branched chelators. Particular reference may be made to the polyaminopolyacid chelators which comprise a linear, cyclic or

branched polyazaalkane backbone with acidic (e.g. carboxyalkyl) groups attached at backbone nitrogens.

Examples of suitable chelators include DOTA derivatives such as p-isothiocyanatobenzyl-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (p-SCN-Bz-DOTA) and DTPA derivatives such as p-isothiocyanatobenzyl-diethylenetriaminepentaacetic acid (p-SCN-Bz-DTPA), the first being cyclic chelators, the latter linear chelators.

Metallation of the complexing moiety may be performed before or after conjugation of the complexing moiety to the targeting moiety.

The radiolabeling procedure will in general be more convenient in terms of time used etc if the chelator is conjugated to the antibody before the radiolabeling takes place.

The principles of preparing radiolabeled conjugates using chelators attached to antibodies is described broader in e.g. Liu, 2008.
Thus, HH1 can be used to prepare radioimmunoconjugates with differences in radiation properties and effective half-lives.

For example anti-CD37 radioimmunoconjugate consisting of the murine monoclonal antibody HH1, a chelating linker and a beta or alpha emitting radionuclide including, but not limited to, ^{177}Lu, ^{211}At, ^{213}Bi, ^{212}Bi, ^{212}Pb, ^{225}Ac, ^{227}Th, ^{90}Y, ^{186}Re, ^{188}Re, ^{199}Au, ^{194}Ir, ^{166}Ho, ^{159}Gd, ^{153}Sm, ^{149}Pm, ^{142}Pr, ^{111}Ag, ^{109}Pd, ^{77}As, ^{67}Cu, ^{47}Sc can be prepared and used for preparing pharmaceutical preparations and used in therapeutic applications.

Pharmaceutical compositions

A radioimmunotherapeutic product based on HH1 would typically be provided as a pharmaceutical composition consisting of a radionuclide, according to the description above, linked via a chelator to the murine monoclonal antibody HH1 dissolved in a buffer solution, which to a substantial degree maintain the chemical

integrity of the radioimmunoconjugate and is being physiologically acceptable for infusion into patients.

Thus, an aspect of the present invention relates to a pharmaceutical composition comprising a radioimmunoconjugate of the present invention, and a pharmaceutically acceptable carrier and/or excipient.

Acceptable pharmaceutical carriers include but are not limited to non-toxic buffers, fillers, isotonic solutions, etc. More specifically, the pharmaceutical carrier can be but are not limited to normal saline (0.9 %), half-normal saline, Ringer's lactate, 5 % Dextrose, 3.3 % Dextrose/0.3 % Saline. The physiologically acceptable carrier can contain a radiolytic stabilizer, e.g., ascorbic acid, which protect the integrity of the radiopharmaceutical during storage and shipment.

One embodiment of the present invention comprises the pharmaceutical composition of the present invention and one or more additional antibodies or radioimmunoconjugates. Antibodies include but are not limited to Rituximab, Epratuzumab, L19, F8, F16, Galiximab, Toralizumab, Alemtuzumab, Ofatumumab, Veltuzumab, Afutuzumab, Tositumomab, Reditux and Ibritumomab. Radioimmunoconjugates include but are not limited to Zevalin and Bexxar.

In another embodiment of the present invention one or more additional antibodies or radioimmunoconjugates target CD20. Antibodies include but are not limited to Rituximab, Veltuzumab, Ofatumumab, Afutuzumab, Tositumomab, Reditux and Ibritumomab. Radioimmunoconjugates include but are not limited to Zevalin and Bexxar.

A further embodiment of the present invention relates to a pharmaceutical composition of the present invention for treating B-cell malignant cells expressing the CD37 antigen.

In an embodiment of the present invention the pharmaceutical composition is for treatment of non-Hodgkin lymphoma and chronic lymphocytic leukemia.

Sequence identity

As commonly defined "identity" is here defined as sequence identity between genes or proteins at the nucleotide or amino acid level, respectively.

Thus, in the present context "sequence identity" is a measure of identity between proteins at the amino acid level and a measure of identity between nucleic acids at nucleotide level.

The protein sequence identity may be determined by comparing the amino acid sequence in a given position in each sequence when the sequences are aligned.

Similarly, the nucleic acid sequence identity may be determined by comparing the nucleotide sequence in a given position in each sequence when the sequences are aligned.

To determine the percent identity of two nucleic acid sequences or of two amino acids, the sequences are aligned for optimal comparison purposes (e.g., gaps may be introduced in the sequence of a first amino acid or nucleic acid sequence for optimal alignment with a second amino or nucleic acid sequence). The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared.

When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e., % identity = # of identical positions/total # of positions (e.g., overlapping positions) x 100). In one embodiment the two sequences are the same length.

One may manually align the sequences and count the number of identical nucleic acids or amino acids. Alternatively, alignment of two sequences for the determination of percent identity may be accomplished using a mathematical algorithm. Such an algorithm is incorporated into the NBLAST and XBLAST programs. BLAST nucleotide searches may be performed with the NBLAST

program, score = 100, wordlength = 12, to obtain nucleotide sequences homologous to a nucleic acid molecules of the invention. BLAST protein searches may be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to a protein molecule of the invention.

To obtain gapped alignments for comparison purposes, Gapped BLAST may be utilised. Alternatively, PSI-Blast may be used to perform an iterated search which detects distant relationships between molecules. When utilising the NBLAST, XBLAST, and Gapped BLAST programs, the default parameters of the respective programs may be used. See http://www.ncbi.nlm.nih.gov. Alternatively, sequence identity may be calculated after the sequences have been aligned e.g. by the BLAST program in the EMBL database (www.ncbi.nlm.gov/cgi-bin/BLAST).

Generally, the default settings with respect to e.g. "scoring matrix" and "gap penalty" may be used for alignment. In the context of the present invention, the BLASTN and PSI BLAST default settings may be advantageous.

The percent identity between two sequences may be determined using techniques similar to those described above, with or without allowing gaps. In calculating percent identity, only exact matches are counted.

An embodiment the invention relates to an isolated nucleic acid comprising a sequence sharing 80 % sequence identity with the HH1 antibody VH sequence (SEQ ID NO: 1) and/or VL sequence (SEQ ID NO: 3).

An embodiment the invention relates to an isolated nucleic acid comprising a sequence with the HH1 antibody VH sequence (SEQ ID NO: 1) and/or VL sequence (SEQ ID NO: 3).

In another embodiment of the invention the isolated nucleic acid comprises a sequence sharing at least 90 % sequence identity with the HH1 antibody VH sequence (SEQ ID NO: 1) and/or VL sequence (SEQ ID NO: 3), such as 90 % identity, 91 % identity, 92 % identity, 93 % identity, 94 % identity, 95 % identity, 96 % identity, 97 % identity, 98 % identity, or 99 % identity.

Another embodiment of the invention relates to an antibody comprising a polypeptide sequence sharing 80 % sequence identity with the HH1 antibody VH sequence (SEQ ID NO: 2) and/or VL sequence (SEQ ID NO: 4).

Another embodiment of the invention relates to an antibody comprising a polypeptide sequence with the HH1 antibody VH sequence (SEQ ID NO: 2) and/or VL sequence (SEQ ID NO: 4).

In another embodiment of the present invention, the antibody comprises a sequence sharing at least 90 % sequence identity with the HH1 antibody VH sequence (SEQ ID NO: 2) and/or VL sequence (SEQ ID NO: 4), such as 90 % identity, 91 % identity, 92 % identity, 93 % identity, 94 % identity, 95 % identity, 96 % identity, 97 % identity, 98 % identity, or 99 % identity.

Genetic vatiation

Genetic variation is caused by variation in the order of bases in the nucleotides in genes. This variation cause mutations in the genes and subsequently in the proteins that such genes encode.

These mutations can be either sense or missense mutations or substitutions.

An embodiment of the present invention relates to the isolated nucleic acid sequence of the HH1 monoclonal antibody VH chain (SEQ ID NO: 1) and/or VL chain (SEQ ID NO: 3) that comprises at least 50, such as 20, such as 10, such as 5, such as 4, such as 3, such as 2, such as 1 sense mutations.

Another embodiment of the present invention relates to the isolated nucleic acid sequence of the HH1 monoclonal antibody VH chain (SEQ ID NO: 1) and/or VL chain (SEQ ID NO: 3) that comprises 0-50, such as 1-50, such as 0-20, such as 1-20, such as 0-10, such as 1-10, such as 0-5, such as 1-5, such as 3, such as 1 sense mutations.

A missense mutation (a type of nonsynonymous mutation) is a point mutation in which a single nucleotide is changed, resulting in a codon that codes for a

different amino acid (mutations that change an amino acid to a stop codon are considered nonsense mutations, rather than missense mutations). A missense mutation can render the resulting protein non-functional.

However, not all missense mutations lead to appreciable protein changes. An amino acid may be replaced by an amino acid of very similar chemical properties, in which case, the protein may still function normally; this is termed a neutral, "quiet", or conservative mutation.

Alternatively, the amino acid substitution could occur in a region of the protein which does not significantly affect the protein secondary structure or function. When an amino acid may be encoded by more than one codon (so-called "degenerate coding") a mutation in a codon may not produce any change in translation; this would be a synonymous mutation (a form of silent mutation) and not a missense mutation.

An embodiment of the present invention relates to an antibody comprising a polypeptide sequence of the HH1 monoclonal antibody VH chain (SEQ ID NO: 1) and/or VL chain (SEQ ID NO: 3) that comprises at least 50, such as 20, such as 10, such as 5, such as 4, such as 3, such as 2, such as 1 missense mutations.

An embodiment of the present invention relates to an antibody comprising a polypeptide sequence of the HH1 monoclonal antibody VH chain (SEQ ID NO: 1) and/or VL chain (SEQ ID NO: 3) that comprises 0-50, such as 1-50, such as 0-20, such as 1-20, such as 0-10, such as 1-10, such as 0-5, such as 1-5, such as 3, such as 1 missense mutations.

A conservative substitution is a substitution of one amino acid with another with generally similar properties such that the overall functioning is likely not to be seriously affected.

In another embodiment of the present invention are the missense mutations conservative mutations or substitutions.

A further embodiment of the present invention relates to an isolated nucleic acid sequence or a polypeptide sequence with 80% sequence identity to the variable heavy chain (SEQ ID NO: 2) and/or variable light chain (SEQ ID NO: 4) sequences af HH1, wherein the sequence variation is conservative substitutions.

In another embodiment of the present invention is the sequence identity 80% identity, such as 90% identity, 91 % identity, 92 % identity, 93 % identity, 94 % identity, 95 % identity, 96 % identity, 97 % identity, 98 % identity, or 99 % identity and the sequence variation is conservative substitutions.

In order to improve the radiolabeling step it may be beneficial to introduce extra lysine into e.g., the Fc portion of HH1. This could reduce the probility of attaching lysine binding chelators into the antigen combining sites at the antibody, therebye reducing the risk of compromizing immunoreactivity during radiolabeling.

Methods for introducing lysine into e.g. the Fc portion of HH1 is known in the art e.g. from Hemminki et al., 1995.

An embodiment of the present invention relates to the radioimmunoconjugate of the present invention which has been modified by 10 Lys in the Fc portion of HH1, such as 8 Lys, such as 6 Lys, such as 5 Lys, such as 4 Lys, such as 3 Lys, such as 2 Lys, such as 1 Lys.

Treatment

Therapeutic use of a pharmaceutical solution according to the present invention may be for treatment against malignant cells expressing the CD37 antigen, including but not limited to non-Hodgkin lymphoma and chronic lymphocytic leukemia.

Other uses could be treatment of autoimmune diseases and treatment of transplantation related effects. The therapy could be based on, but are not limited to, beta-particle-radiation or alpha-particle-radiation or a combination of these.

The therapy could be administered either as a monotherapy or in combination with other therapies, preferentially standard treatments. Such other therapies may be pretreatment, surgery, chemotherapy, immunotherapy, photodynamic therapy, radioimmunotherapy or a combination of two or more of these. By administered is meant intravenous infusion or intravenous injection. More specifically, the radioimmunoconjugate of the present invention can be administered directly in a vein by a peripheral cannula connected to a drip chamber that prevents air embolism and allows an estimate of flow rate into the patient.

In one embodiment the radioimmunoconjugate can be administered in a repeated fashion.

In another embodiment of the present invention the radioimmunoconjugate could be administered in a repeated fashion but with different radionuclides, e.g., beta-radioimmunotherapy could be followed by alpha-radioimmunotherapy or vice versa.

An aspect of the present invention relates to the use of the radioimmunoconjugate of the present invention for the treatment of B-cell malignancies.

An embodiment of the present invention relates to the use of the radioimmunoconjugate of the present invention administered in combination with or in addition to other therapy.

In an embodiment of the present invention the other therapies is selected from pretreatment, chemotherapy, monoclonal antibody therapy, surgery, radiotherapy, and/or photodynamic therapy.

In another embodiment of the present invention the other therapies are bone marrow transplantation or stem cell transplantation and/or therapy.

Another embodiment of the present invention comprises therapeutic pre-treatment using anti-CD20 and/or anti-CD37 monoclonal antibody prior to the treatment with the radioimmunoconjugate of the present invention.

An aspect of the present invention relates to a method for treatment of a B-cell malignancy selected from non-Hodgkin lymphoma and chronic lymphocytic leukemia, comprising administration of an effective amount of the pharmaceutical composition of the present invention.

In an embodiment of the present invention the antibody dosing is 1-1000 mg per patient, more preferably 5-50 mg per patient, and ^{177}Lu amounting to 1 - 200 MBq/kg, more preferably 10-100 MBq/kg of bodyweight.

Kits

An aspect of the present invention relates to a kit for the production of the radioimmunoconjugate of the present invention comprising two or more vials, wherein one vial contains a conjugate comprising a chelator linked to a murine monoclonal antibody HH1; and a second vial contains a radionuclide.

A kit may require some procedures to be performed, e.g., radiolabeling and/or purification to take place before infusion.

An embodiment of the present invention relates to a kit of the present invention, wherein the content of one or several of the vials are either lyophilized or in a solution.

By mixing the contents of the two vials to generate the radioimmunoconjugate the final product will appear. Thus, in another embodiment of the present invention the radioimmunoconjugate is generated by mixing the content of the two vials. This product may need purification prior to use.

Examples

Example 1 - Radiolabeling of HH1

Iodination: Antibodies were labeled with ^{125}I through indirect iodination using IODOGEN pre-coated iodination tubes (Pierce, Rockford, IL) according to the manufacturer's description.

Labeling with ^{111}In and ^{177}Lu: Antibodies were first reacted with a chelator (p-SCN-Bn-DTPA or p-SCN-Bn-DOTA).

The DTPA or DOTA chelator was dissolved in 0.05 M HCl, and then added to the antibody, which was pH-adjusted to ca. 8 by washing with carbonate buffer, in a 5:1 ratio. pH was then checked again and if necessary adjusted. The solution was shaken in 60 min at room temperature, and then the reaction was terminated by adding 50 µl 200 mM glycine solution (per mg antibody). To remove free chelator the conjugated antibody was washed 4-5 times with PBS (PAA), and then adjusted to pH 5 by washing with ammonium acetate. ^{111}In or ^{177}Lu (Perkin Elmer, Boston, Ma, USA) was then added to 0.5 mg DOTA-Ab, and shaken for one hour at 42°C. Finally, the product was purified by elution on a gel filtration column, e.g., Sephadex G-25 PD10 (GE health) or similar. The overall labeling yield varied from 17 % to 63 %.

The quality of the radioimmunoconjugates was measured using lymphoma cells and a modified Lindmo method. Cell concentrations up to 108 cells pr ml were used to compensate for the modest specific activity of ^{111}In-conjugates. For ^{125}I-conjugates (which have a higher specific activity) it was enough to use cell concentrations up to $4*10^7$ cells pr ml.

The immunoreactivity and specific activity for the radioimmunoconjugates can be seen in Table 1.

Example 2 – Binding parameters

The association rate constant, k_a, the equilibrium dissociation constant, K_d, and the mean number of binding sites, B_{max}, was determined by a one-step curve fitting method (Dahle et al. 2007). The binding parameters were measured for HH1 and rituximab and for three different lymphoma cell lines; Raji, Rael and Daudi cells (Table 2). Specific binding was measured as a function of time and antibody concentration, and the solution of the differential equation describing the net rate of formation of the antigen-antibody complex was fitted to the experimental data points using the association rate constant, k_a, the equilibrium dissociation constant, K_d, and the mean number of binding sites, B_{max}, as parameters. Five million cells pr ml were used, four concentrations of ^{125}I-labeld antibody (100 ng/ml, 1000 ng/ml, 5000 ng/ml and 10000 ng/ml) and 7 incubation time points (5 min, 10 min, 20 min, 30 min, 1 h, 1.5 h and 2 h). After incubation, cells were washed twice with PBS, and then counted in a gamma counter.

Example 3 – Retention of cell-bound antibody

Retention of cell-bound antibody immediately and 96 hours after washing were measured after incubation of Raji, Rael and Daudi cells with ^{111}In-HH1, ^{111}In-rituximab, ^{125}I-HH1 and ^{125}I-rituximab (Figure 1).

One million cells in 1 ml RPMI 1640 medium with 10 % foetal calf serum, 1 % L-glutamine and 1 % penicillin/streptomycin were incubated with 1 µg/ml ^{125}I- or ^{111}In-labeled HH1 or rituximab for one hour, washed twice with medium and incubated further for four days. The cell bound activity was determined immediately after washing (Figure 1A) and after four days of incubation (Figure 1B) by measuring the number of cells (Vi Cell Viability Analyzer, Beckman Coulter, Fullerton, CA, USA) and the amount of radioactivity with a calibrated gamma detector (Cobra II auto-gamma detector, Packard Instrument Company, Meriden, CT, USA).

Example 4 – Treatment of lymphoma cells in vitro with ^{177}Lu-HH1 or ^{177}Lu-rituximab

Experiment I: Binding of ^{177}Lu-HH1 to Daudi cells

One ml of Daudi cell suspension (1 million cells/ml) was seeded in 24 tubes and half of the tubes were blocked with 100 µg/ml of either HH1 or rituximab and incubated for 30 minutes at 37 °C. Subsequently, each tube was added either ^{177}Lu-HH1 or ^{177}Lu-rituximab to a final concentration of 0, 1, 2.5, 5, 10 or 20 µg/ml and incubated further at 37 °C. The specific activity was 91.6 kBq/µg for ^{177}Lu-HH1 and 136.6 kBq/µg for ^{177}Lu-rituximab.

The amount of added activity was measured during the incubation period with a gamma detector (Cobra II auto-gamma detector, Packard Instrument Company). After 2 hours, half of the cells were washed and cell bound activity was measured (Figure 2A) while half of the cells were incubated over night (18 h) before washing and measurement of cell bound activity (Figure 2B).

There was no difference between cells incubated with HH1 and cells incubated with rituximab after 2 hour incubation, while the cell bound activity was twice as high for cells incubated with rituximab than for cells incubated with HH1 after 18 hour incubation (Figure 2).

Tables 3 and 4 indicate that radiolabeled HH1 saturates the antigen quicker and at lower antibody concentration than rituximab. The nonspecific binding seems to be similar for the two radioimmunoconjugates (RIC), and it increases with increasing concentration of RIC in the medium.

The maximum number of specific bound ^{177}Lu was about twice as high for rituximab as for HH1. However at the 1 µg/ml dosage, there were almost no differences in the number of specifically bound radioactive atoms.

Experiment II: Two hour incubation with ^{177}Lu-IgG: Cell growth data

Daudi cells were incubated with radioimmunoconjugates as in experiment I (Figure 2 A).

Growth of Daudi cells after 2 hour incubation with ^{177}Lu-HH1 or ^{177}Lu-rituximab was measured by seeding 50.000 cells from each tube in three wells in six 12-well plates. The amount of cells were measured for several time points the next 14 days using an automatic imaging system (Clone Select Imager, Gentix Ltd, Hampshire, UK).

There was no effect of unlabeled antibody alone on cell growth. However, the blocked cells treated with ^{177}Lu-antibody clearly did not grow as fast as the untreated control cells, indicating that there was an effect of unbound ^{177}Lu-antibody or unspecifically bound ^{177}Lu-antibody on the cells (Figure 3).

Treatment of unblocked cells with ^{177}Lu-antibody resulted in an increase in growth delay of 44 % for cells treated with 10 µg/ml ^{177}Lu-HH1 (Figure 3 A) and of 31 % for cells treated with 10 µg/ml ^{177}Lu-rituximab (Figure 3 B).

For treatment with 20 µg/ml the difference between the two antibodies was even larger since there was no regrowth of the cells treated with ^{177}Lu-HH1. This result was unexpected since the cells were labeled with the same amount of antibody (Figure 2 A).

Experiment III: Eighteen hour incubation with ^{177}Lu-IgG: Cell growth data.

Daudi cells were incubated with radioimmunoconjugates as in experiment I (Figure 2 B). Growth of Daudi cells after 18 hour incubation with ^{177}Lu-HH1 or ^{177}Lu-rituximab was measured by seeding 50.000 cells from each tube in three wells in six 12-well plates.

The amount of cells were measured for several time points the next 14 days using an automatic imaging system (Clone Select Imager, Gentix Ltd, Hampshire, UK). There was no effect of unlabeled antibody alone on cell growth.

The inhibition of cell growth on the blocked cells treated with ^{177}Lu-antibody was larger in this experiment (Figure 4) than in experiment II (Figure 3) because of 16 hour increased incubation time with radioimmunoconjugate in the medium.

Treatment of unblocked cells with ^{177}Lu-antibody resulted in an increase in growth delay of 107 % for cells treated with 2.5 µg/ml ^{177}Lu-HH1 (Figure 4 A) and of 52

% for cells treated with 2.5 µg/ml ^{177}Lu-rituximab (Figure 4 B). This result was unexpected since after 18 h of incubation the cells labeled with ^{177}Lu-rituximab had twice as much cell-bound activity attached than the cells labeled with ^{177}Lu-HH1 (Figure 2 B).

Example 5 – Biodistribution of HH1

Biodistribution of ^{111}In-labeled HH1 was studied in BALB/c-nude (nu/nu) mice with Daudi xenografts with size 32-256 mm^3 at the start of the study.

The radiolabeling was performed using pSCN-Bz-DOTA as a bifunctional chelating agent to complex the radionuclide and attach it to the antibody (see Example 1). The preparation was administered by tail vein injection of 100 µl solution to each animal.

An activity of 120 kBq was injected in each animal. Five animals were used per time point. Autopsies were performed after cervical dislocation at various time points after injection. The weight of each tissue sample was determined, and ^{111}In were measured by a calibrated gamma detector (Cobra II auto-gamma detector, Packard Instrument Company, Meriden, CT, USA). Samples of the injectates were used as references in the measurement procedures.

The uptake of ^{111}In-HH1 24 hours after injection of mice with Daudi xenografts and the biodistribution in normal tissues are presented in Figure 5. The radiolabeled antibody has a relevant tumor targeting and biodistribution. The chelator-conjugate ^{111}In-HH1 shows good stability *in vivo*.

Example 6 - Comparison of HH1 with three other anti-CD37 antibodies

Experiment I: Antigen blocking ability of anti-CD37 antibodies against radiolabeled HH1

To test whether the HH1 antigen interaction can be blocked by other anti-CD37 antibodies, Daudi cells were blocked by pre-incubation with either HH1, O.N.108, IPO-24 or 6D263 antibodies. Daudi cells (2 millions/ml) were incubated for 15

minutes with either HH1, O.N.108, IPO-24 or 6D263 antibodies (20 µg/ml) and added ^{125}I-labeled HH1 antibody and incubated for 1 hour.

Thereafter, the cells were centrifuged and washed 3 times and the activities in supernatant and cell pellets were counted using a X-ray/gamma counter. Compared with the HH1 blocked cells, the bound fraction of ^{125}I-labeled HH1 was 48 %, 44 % and 51 % higher for O.N.108, IPO-24 or 6D263 blocked cells, respectively.

Thus, the antigen binding of ^{125}I-HH1 was better blocked with HH1 than with the three other antibodies suggesting some differences in antigen interaction. In conclusion, HH1 has significant different antigen-binding properties compared with a panel of three other anti-CD37 monoclonal antibodies.

Experiment II: Antibody binding to paraffin embedded lymphoma tissue samples

To compare the ability of HH1 and three commercially available CD37 antibodies to bind to fixated lymphoma samples, biopsies from lymphoma patients were fixed in formalin, embedded in paraffin and cut in 10 µm slices that were mounted on object glasses.

The samples were labeled with the antibodies HH1, IPO.24, ON.108 and 6D263 and the grade of labeling was detected using rabbit anti mouse polyclonal antibody and peroxidase staining.

The antibodies IPO.24 and ON.108 resulted in the strongest labeling of the lymphoma samples. The 6D263 antibody labeled the sample a little weaker. The HH1 antibody labeling of the sections was insignificant.

Thus, it can be concluded, since HH1 did not bind while three other anti-CD37 antibodies were binding, that HH1 has a significantly different antigen interaction.

Experiment III: Flow cytometry of HH1, IPO.24, ON.108 and 6D263 antibodies

To investigate differences in antigen expression detected by the different CD37 antibodies vs. HH1 Daudi cells were washed twice with RPMI 1640 medium with 5

% foetal calf serum and labeled with 10 µg/ml of the primary antibodies HH1, IPO.24, ON.108 and 6D363 for 0.5 hour in 0.2 ml medium with 10 % FCS on ice.

Subsequently, the cells were washed twice with PBS with 0.25 % FCS and labeled with FITC-labeled polyclonal rabbit anti-mouse IgG Fab`2 (diluted 1:20) (Figure 6) for 0.5 hour on ice. Fluorescence from the FITC-label was detected by exciting with a 488 nm laser in a flow cytometer.

Dead cells and doublets were gated away using forward scatter, side scatter and propidium iodide signals. There was no significant variation among the different FITC histograms of the various CD37 antibodies (Figure 6).

In conclusion, HH1 and the tree other anti-CD37 antibodies IPO.24, ON.108 and 6D363, produce similar flow cytometry histograms.

Experiment IV: The binding fraction for HH1 and three commercially available anti-CD37 antibodies.

To compare the binding fraction of HH1 with the O.N.108, IPO-24 or 6D263 antibodies (Santa Cruz Biotechnology) using Daudi cells. Cell suspensions, representing a large antigen excess, consisting of 60 million Daudi cells in 0.2 ml RPMI 1640 medium with 5 % foetal calf serum were blocked for 15 minutes with HH1, O.N.108, IPO-24 or 6D263 antibodies (500 µg/ml) to account for non-specific binding of the antibody. Other parallels were unblocked.

Subsequently, ^{125}I-labeled HH1, O.N.108, IPO-24 or 6D263 antibody (5-10 ng/ml) was added and the cells were incubated for 2 hours at 4 °C with gentle shaking. Thereafter, the cells were centrifuged and washed 2 times with PBS with 1 % FCS. The cell pellets were transferred to clean tubes and counted using a gamma counter.

The binding fraction was determined as the difference in activity for the unblocked and the blocked vials vs. the added activity. The HH1 showed a much higher binding fraction compared with the other CD37 antibodies (Table 5). In conclusion, HH1 showed a much higher immunoreactivity against living cells compared with IPO.24, ON.108 and 6D363 when the antibodies were radiolabeled

in similar fashion. This result indicates that HH1 has different antigen interaction than the three other antibodies.

Example 7 - DNA and amino acid sequence of low and heavy chain variable regions

The gene and protein sequence of the variable regions of the HH1 anti-CD37 antibody is as follows:

The VH αCD37 gene sequence corresponds to SEQ ID NO: 1 and the VH αCD37 protein sequence corresponds to SEQ ID NO: 2.

VH αCD37

```
gagatccagctgcagcagtctggacctgagctggtgaagcctggggcttcagtgaaggta
 E  I  Q  L  Q  Q  S  G  P  E  L  V  K  P  G  A  S  V  K  V

tcctgcaaggcttctggttactcattcactgactacaacatgtactgggtgaagcagagc
 S  C  K  A  S  G  Y  S  F  T  D  Y  N  M  Y  W  V  K  Q  S

catggaaagagccttgagtggattggatatattgatccttacaatggtgatactacctac
 H  G  K  S  L  E  W  I  G  Y  I  D  P  Y  N  G  D  T  T  Y

aaccagaagttcaagggcaaggccacattgactgttgacaagtcctccagcacagccttc
 N  Q  K  F  K  G  K  A  T  L  T  V  D  K  S  S  S  T  A  F

atccatctcaacagcctgacatctgaggactctgcagtctattactgtgcaagatcccct
 I  H  L  N  S  L  T  S  E  D  S  A  V  Y  Y  C  A  R  S  P

tatggtcactatgctatggactactggggtcaaggaacctcagtcaccgtctcctca
 Y  G  H  Y  A  M  D  Y  W  G  Q  G  T  S  V  T  V  S  S
```

The VL αCD37 gene sequence corresponds to SEQ ID NO:3 and the VL αCD37 protein sequence corresponds to SEQ ID NO: 4.

VL aCD37

```
gacattgtgatgacccagtctcacaaactcttgtccacatcagtaggagacagggtcagc
 D  I  V  M  T  Q  S  H  K  L  L  S  T  S  V  G  D  R  V  S

atcacctgcaaggccagtcaggatgtgagtactgctgtagactggtatcaacagaaacca
 I  T  C  K  A  S  Q  D  V  S  T  A  V  D  W  Y  Q  Q  K  P

ggacaatctcctaaactactgattaactgggcatccacccggcacactggagtccctgat
 G  Q  S  P  K  L  L  I  N  W  A  S  T  R  H  T  G  V  P  D

cgcttcacaggcagtggatctgggacagattatactctcaccatcagcagtatgcaggct
 R  F  T  G  S  G  S  G  T  D  Y  T  L  T  I  S  S  M  Q  A

gaagacctggcactttattactgtcgacaacattatagcactccattcacgttcggctcg
 E  D  L  A  L  Y  Y  C  R  Q  H  Y  S  T  P  F  T  F  G  S

gggacaaagttggaaataaaa
 G  T  K  L  E  I  K
```

The amino acid sequence is significantly different from the amino acid sequence of the CD37 binding antibody A0 (Heider et al., 2009). The overlap between the variable light chain of HH1 and A0 is 56 %, while the overlap between the variable heavy chain is 82 %.

Example 8 - Binding of radiolabeled HH1 on cells saturated with CD20 antibody rituximab.

Background

Non-Hodgkin lymphoma patients often receives rituximab as a standard therapy. It would be advantageous if radiolabeled HH1 could be used in patients even if they are undergoing rituximab therapy. Daudi lymphoma cells, which express both the CD20 and the CD37 antigens, were used as a model.

Methods

Daudi lymphoma cells (3, 3 millions in 0.5 ml) were either pre- and co-treated with excessive amounts (100 µg/ml of rituximab) for five minutes and thereafter added 1 µg of ^{125}I-labeled HH1 or given ^{125}I-labeled HH1 without rituximab pretreatment. To determine non-specific binding of ^{125}I-HH1 the same configuration as above but with pretreatment with unlabeled HH1 (10 µg/ml was used). The cells were incubated for two hours in PBS at room temperature and counted in a gamma counter, washed three times in 1 ml PBS followed by centrifugation, and finally recounted for cell bound radioactivity.

Results

With rituximab pre-treatment/co-treatment, 26.0% (total bound, 27.4% – nonspecific bound, 1.4%) of the added ^{125}I-HH1 bound specifically to the cells.

Without rituximab pre-treatment/co-treatment, 25.5% (26.9 -1.4) bound specifically (all numbers represents mean of three parallelles). I.e, there were no significant difference in the binding of ^{125}I-HH1 due to the presence of rituximab.

Conclusion

Pre- and co- treatment with excessive amounts of rituximab did not alter the cell binding ability of radiolabeled HH1 and, thus, did not block the access to the CD37 antigen.

This indicates that radioimmunotherapy with radiolabeled HH1 may be suitable in patients subsequently to or during immunotherapy with anti-CD20 antibody as well as in patients not treated with rituximab.

Example 9 - Treatment of SCID mice inoculated intravenously with Daudi lymphoma cells using ^{177}Lu-HH1.

Background

Treatment of lymphoma patients with current CD20 directed radioimmunotherapy can be problematic in patients previously treated with rituximab because of antigenic drift and possible blockage of CD20 antigen. Therefore, radioimmunotherapy targeting other antigens might be more effective. By intravenous injection of human lymphoma cells in severe combined immune deficient (SCID) mice we made an intravenous tumor model. When SCID mice are inoculated intravenously with Daudi lymphoma cells they will develop hind leg paralysis due to the growth of the Daudi tumor cells.

Experimental

SCID mice were injected intravenously with 10 million Daudi cells one week before administration of 50 or 100 MBq/kg ^{177}Lu-HH1, 50 µg HH1, 50 µg rituximab or NaCl. The mice were monitored for hind leg paralysis and bodyweight loss as well as WBC count every other week. Discontinuation of symptom-free survival was used as an end point. To prepare the radiolabeled antibody, HH1 was first labeled with p-SCN-Bn-DOTA and purified. After buffer exchange ^{177}Lu (Perkin Elmer, Boston, Ma, USA) was added to the DOTA-HH1, and shaken for 40 minutes at 40°C. Specific activity was approximately 3200 MBq/mkg for the final product. Each preparation was dissolved in isotonic saline to a total injection volume of 100 µl per animal.

Results

Median symptom free survival was 26 days (range 21 to 33) for saline, 40 days (range 23 to 44) for HH1 and 40 days (range 33 to 44) for rituximab (Figure 8). For 50 kBq/kg ^{177}Lu-HH1, 80% of the animals were alive after 79 days. Two of the mice in the 100 kBq/g group died before any of the animals in the saline groups, and the blood cell counts indicated radiotoxicity. A third animal in the 100 kBq/g group died at day 49. The other animals (70%) were alive at day 79. The survival of the mice treated with ^{177}Lu-HH1 were significantly higher than the survival of

mice treated with NaCl, HH1 or Rituximab ($p<0.005$, Mann Whitney Log Rank Test)

Conclusion

The data shows that the groups receiving ^{177}Lu-HH1 in dosages of 50 or 100 kBq/g of b.w. groups had considerablye better survival than the groups receiving saline or immunotherapy with either HH1 or rituximab. The toxicity data indicate that the activity should be kept below 100 kBq/g b.w. These data indicate that ^{177}Lu-HH1 has relevant properties for in vivo radioimmunotherapy.

Example 10 - Biodistribution of ^{177}Lu-HH1 in nude mice with CD37 expressing tumor xenografts.

Background

Lutetium-177 labeled HH1 antibody was evaluated for in vivo tissue distribution and tumor targeting.

Experimental procedure

Labeling of antibodies with radionuclides

The antibody was first labeled with the chelator p-SCN-Bn-DOTA. DOTA was dissolved in 0.05M HCl, added to the antibody in a 5:1 ratio and pH-adjusted to 8-9 by washing with carbonate buffer using Amicon centrifuge filters (Millipore, USA) with a molecular weight cut-of of 30 kDa.

The pH was then checked again and if necessary adjusted. The solution was shaken during 60 min at room temperature, and then the reaction was terminated by adding 50 µl 200 mM glycine solution (per mg antibody). To remove free chelator the conjugated antibody was washed 4-5 times with PBS (PAA)(using Amicon and centrifugation), and then adjusted to pH 5 by washing with ammonium acetate. ^{177}Lu (Perkin Elmer, Boston, Ma, USA) was then combined

with 0.5 mg DOTA-HH1 in a 2 ml polypropylene tube (Eppendorf, Germany), and shaked for one hour at 40°C. Specific activities were typically 25-120 MBq/kg for ^{177}Lu-conjugates.

Immunoreactivity

The quality of the radioimmunoconjugates were measured using lymphoma cells and a modified Lindmo method. Cell concentrations up to 108 cells pr ml were used. The conjugates used in the experiments had immunoreactivity above 50 %.

Biodistribution of radioimmunoconjugates

Biodistributions of ^{177}Lu-HH1 was determined in male BALB/c nude (nu/nu) mice implanted with 1·1·1 mm Daudi tumor xenografts three weeks before. The preparations were administered by tail vein injection of 100 µl solution in each animal. A mean acitivity of 500 kBq per mice for ^{177}Lu-HH1. Four to five animals were used per time point. Autopsies were performed after cervical dislocation at various time points after injection. The weight of each tissue sample was determined, and ^{177}Lu were measured by a calibrated gamma detector (Cobra II auto-gamma detector, Packard Instrument Company, Meriden, CT, USA). Samples of the injectates were used as references in the measurement procedures.

Results and discussion

Uptake and retention of ^{177}Lu-labeled HH1 and in normal tissues of BALB/c-nude (nu/nu) female mice with Daudi xenografts are presented in Figure 7. There were no signs of redistribution of nuclide from/to any organs after the initial uptake of radioimmunoconjugates, which indicate that the radioimmunoconjugates were stable.

Injection of ^{177}Lu-HH1 in nude mice with tumor showed a low uptake in bone. Free ^{177}Lu is known to accumulate in bone so this result indicates that the radioimmunoconjugate was stable in vivo. The uptake in the tumors was significantly higher than in other organs at later time points.

This indicates that ^{177}Lu has relevant half life for radioimmunotherapy using the HH1 antibody.

The biodistribution data of ^{177}Lu-HH1 show a relevant normal tissue uptake and clearance and significant retention in CD37 expressing tumor xenographs.

The HH1 antibody seems to be well suited for radioimmunotherapy. The ^{177}Lu-HH1 conjugate seems particularly suitable, as favorable tumor to normal tissue ratios were obtained before a larger fraction of the radionuclide was decayed.

Tables

Table 1

Immunoreactivity and specific activity for the radioimmunoconjugates.

Radioimmunoconjugate	IRF[1] (%)	Specific activity[1](MBq/mg)	# exp.
^{125}I-HH1	66 ± 17 (39-92)	75 ± 15 (51-104)	17
^{111}In-HH1	66 ± 14 (51-78)	22 ± 12 (9-32)	3
^{177}Lu-HH1	56	92	1
^{125}I-rituximab	62 ± 6 (54-68)	69 ± 30 (34-118)	6
^{111}In-rituximab	45	16	1
^{177}Lu-rituximab	60	137	1

[1]Mean ± SD (range)

Table 2

The mean number of antigens (B_{max}) on Raji, Rael and Daudi cells, the equilibrium dissociation constant (K_d) and the association rate constant (k_a) for the antibodies rituximab and HH1.

Antibody	Cell line	B_{max}(Ag/cell)	K_d (nM)	k_a (nM/h)
HH1	Raji	146 000 ± 7 600	6.3 ± 1.7	0.36 ± 0.14
HH1	Rael	263 000 ± 27 000	12.7 ± 5.5	0.07 ± 0.01
HH1	Daudi	340 000 ± 5 000	2.7 ± 0.3	0.72 ± 0.07
rituximab	Raji	272 000 ± 69 000	4.8 ± 0.9	0.08 ± 0.006
rituximab	Rael	626 000 ± 36 000	12.0 ± 2.0	0.08 ± 0.007

Table 3

Number of ^{177}Lu atoms bound per Daudi cell after 2 hours of incubation.

Antibody dosage (µg/ml)	^{177}Lu-HH1			^{177}Lu-rituximab		
	Unblocked	Blocked	Specific	Unblocked	Blocked	Specific
0[a]	12	7	5	8	53	-45
1	8318	449	7869	8554	372	8182
2.5	9105	720	8385	11629	885	10744
5	10025	1837	8188	13658	2019	11639
10	13646	3521	10125	17344	2769	14575
20	16290	8473	7812	30095	9709	20386

([a]The different counts for the control samples is indicative of the variation in background radiation to the counter).

Table 4

Number of ^{177}Lu atoms bound per Daudi cell after 18 hours of incubation.

Antibody dosage (µg/ml)	^{177}Lu-HH1			^{177}Lu-rituximab		
	Unblocked	Blocked	Net	Unblocked	Blocked	Net
0	12	5	7	10	53	-43
1	10327	301	10026	12831	356	12475
2.5	11757	787	10970	18836	1385	17451
5	12123	1857	10266	24097	1871	22226
10	11548	3205	8343	24249	2860	21389
20	15233	5445	9788	26639	5824	20815

Table 5

Binding fraction of four anti-CD37 antibodies.

Antibody	IRF
HH1	50 %
O.N.108	24 %
IPO-24	16%
6D263	21 %

References

Bernstein ID, Eary JF, Badger CC, Press OW, Appelbaum FR, Martin PJ, Krohn KA, Nelp WB, Porter B, Fisher D, Miller R, Brown S, Levy R, Donnall Thomas E. High dose radiolabelled antibody therapy of lymphoma. Cancer Res. 1990, 50(3 Suppl), 1017s-1021s.

Brechbiel MW. Bifunctional chelates for metal nuclides. Q J Nucl Med Mol Imaging 2008, 52 (2), 166-173.

Buchegger F, Antonescu C, Delaloye AB, Helg C, Kovacsovics T, Kosinski M, Mach JP, Ketterer N. Long-term complete responses after ^{131}I-tositumomab therapy for relapsed or refractory indolent non-Hodgkin's lymphoma. Br J Cancer. 2006, 94(12), 1770-6.

Dahle, J., Krogh, C., Melhus, K. B., Kaalhus, O., Larsen, R. H. and Stokke, T. A one-step method for determining the maximum number of bound antibodies, and the affinity and association rate constant of antibody binding. *Nuclear Medicine Communications*. 2007, 28, 742-747.

Gordon LI, Molina A, Witzig T, Emmanouilides C, Raubtischek A, Darif M, Schilder RJ, Wiseman G, White CA. Durable responses after ibritumomab tiuxetan radioimmunotherapy for CD20+ B-cell lymphoma: long-term follow-up of a phase 1/2 study. Blood. 2004, 103(12), 4429-31.

Grosmaire LS, Hayden-Ledbetter MS, Ledbetter JA, Thompson PA, Simon SA, Brady W. B-cell reduction using CD37-specific and CD20-specific binding molecules. US 2007/0059306 A1

Heather A. Jacene, Ross Filice, Wayne Kasecamp, and Richard L. Wahl. Comparison of ^{90}Y-Ibritumomab Tiuxetan and ^{131}I-Tositumomab in Clinical Practice. J Nucl Med. 2007, 48, 1767–1776.

Heider KH, Borges E, Ostermann E. Anti CD37 antibodies. WO 2009/019312.

Henriksen G, Funderud S, Hoff P. Bi-labelled antibody and Bi-labelled streptavidin. Comparison of targeting efficiacy of a lymphoma cell line in vitro. J Labelled Compds Radiopharm. 1997, 34(12), 1039-1046.

Hemminki A., Hoffrén A-M., Takkinen K., Vehniäinen M., Mäkinen M-L., Pettersson K., Teleman O., Söderlund H., Teeri T.T.. Introduction of lysine residues on the light chain constant domain improves the labeling properties of a recombinant Fab fragment. Protein Engineering. Vol. 8, No. 2, pp. 185-191, 1995.

Liu S. Bifunctional coupling agents for radiolabeling of biomolecules and target-specific delivery of metallic radionuclides. Adv Drug Deliv Rev. 2008, 60 (12), 1347-1370.

Ngo NT, Brodie C, Giles C, Horncastle D, Klammer M, Lampert IA, Rahemtulla A, Naresh KN. The significance of tumour cell immunophenotype in myeloma and its impact on clinical outcome. J Clin Pathol. 2009, 62(11), 1009-15.

Press OW, Eary JF, Appelbaum FR, Martin PJ, Badger CC, Nelp WB, Glenn S, Butchko G, Fisher D, Porter B, Matthews DC, Fisher LD, and Bernstein ID Radiolabeled-antibody therapy of B-cell lymphoma with autologous bone marrow support. N Engl J Med. 1993, 329(17), 1219-24.

Press OW, Leonard JP, Coiffier B, Levy R, Timmerman J. Immunotherapy of Non-Hodgkin's lymphomas. Hematology Am Soc Hematol Educ Program. 2001, 221-40.

Smeland E, Funderud S, Ruud E, Kiil Blomhoff H, Godal T. Characterization of two murine monoclonal antibodies reactive with human B cells. Their use in a high-yield, high-purity method for isolation of B cells and utilization of such cells in an assay for B-cell stimulating factor. Scand J Immunol. 1985, 21(3), 205-14.

Claims

1. A radioimmunoconjugate that binds human CD37 comprising:
   a) murine monoclonal antibody HH1,
   b) a chelating linker, and
   c) a radionuclide selected from the group consisting of ^{177}Lu, ^{212}Pb, ^{225}Ac, ^{227}Th and ^{90}Y.

2. The CD37 binding radioimmunoconjugate according to claim 1, wherein the radionuclide is ^{177}Lu.

3. A pharmaceutical composition comprising a radioimmunoconjugate according to any one of claims 1-2, and a pharmaceutically acceptable carrier.

4. The pharmaceutical composition according to claim 3, further comprising one or more additional antibodies or radioimmunoconjugates.

5. The pharmaceutical composition according to claim 4, wherein the one or more additional antibodies or radioimmunoconjugates target CD20.

6. The pharmaceutical composition according to any one of claims 3-5, for treating B-cell malignant cells expressing the CD37 antigen.

7. The pharmaceutical composition according to claim 6, for treatment of non-Hodgkin lymphoma and chronic lymphocytic leukemia.

8. The radioimmunoconjugate according to any one of claims 1-2, for use in the treatment of B-cell malignancies.

9. Use of the radioimmunoconjugate according to any one of claims 1-2, in the manufacture of a medicament for the treatment of B-cell malignancies.

10. The use according to claim 9, wherein the radioimmunoconjugate is to be administered in combination with or in addition to other therapy.

11. The use according to claim 10, wherein the therapy is selected from pretreatment, chemotherapy, monoclonal antibody therapy, surgery, radiotherapy, and/or photodynamic therapy.

12. The use according to claims 10 or 11, wherein the therapy comprises pretreatment using anti-CD20 and/or anti-CD37 monoclonal antibody prior to the treatment with the radioimmunoconjugate according to any one of claims 1-2.

13. Use of a pharmaceutical composition according to any one of claims 3-7 in the manufacture of a medicament for treatment of a B-cell malignancy selected from non-Hodgkin lymphoma and chronic lymphocytic leukemia..

14. A radioimmunoconjugate that binds human CD37 comprising:

a) murine monoclonal antibody HH1,

b) a chelating linker, and

c) a radionuclide selected from the group consisting of ^{177}Lu, ^{225}Ac, ^{212}Pb, ^{227}Th and ^{90}Y,

for use in the treatment of a B-cell malignancies selected from non-Hodgkin lymphoma and chronic lymphocytic leukemia.

15. A kit for the production of the radioimmunoconjugate according to any one of claims 1-2 comprising two or more vials, wherein one vial contains a conjugate comprising a chelator linked to a murine monoclonal antibody HH1; and a second vial contains a radionuclide.

16. The kit according to claim 15, wherein the content of one or several of the vials are either lyophilized or in a solution.

17. A radioimmunoconjugate that binds human CD37 comprising:

a) murine monoclonal antibody HH1,

b) a chelating linker, and

c) a radionuclide selected from the group consisting of ^{177}Lu, ^{212}Pb, ^{225}Ac, ^{227}Th and ^{90}Y,

for use in therapy.