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**(54) Title:** METHOD OF DECREASING CYTOTOXIC SIDE-EFFECTS AND IMPROVING EFFICACY OF IMMUNOCONJUGATES

**(57) Abstract:** Disclosed are methods, compositions and kits for improving targeting, in particular tumor targeting, of immunoconjugates. The method and composition relies on the sequestration of non-target cells that also express the antigen the immunoconjugate targets. Sequestration of those non-target cells in a variety of ways is disclosed. The methods, compositions and kits allow appropriate sequestration of non-target cells while maintaining a high degree of effectiveness of the immunoconjugates against target cells.

## METHOD OF DECREASING CYTOTOXIC SIDE-EFFECTS AND IMPROVING EFFICACY OF IMMUNOCONJUGATES

### CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. provisional application 61/016,613, filed

5 December 26, 2007, and U.S. provisional application 61/016,630, filed December 26, 2007, both of which are incorporated herein by reference in their entirety.

### FIELD OF THE INVENTION

The present invention relates to methods and compositions that improve the targeting of immunoconjugates, in particular immunoconjugates directed at antigens that 10 are expressed on target as well as non-target cells.

### BACKGROUND

Immunoconjugates are promising candidates for the treatment of different medical indications, in particular for the treatment of a wide variety of cancers. For example, Tassone et al. (2004) reports excellent cytotoxic activity of the maytansinoid 15 immunoconjugate B-B4-DM1 against CD138 positive multiple myeloma cells (see also US Patent Publ. 20070183971).

The publications and other materials, including patents, used herein to illustrate the invention and, in particular, to provide additional details respecting the practice are incorporated by reference. For convenience, the publications are referenced in the 20 following text by author and date and are listed alphabetically by author in the appended bibliography.

A substantial body of research has concentrated on the development of systems in which an effector agent can be selectively delivered to a desired location or cell population, i.e., systems for a more targeted treatment of ailments with fewer toxic side 25 effects. In spite of considerable progress that has been made, many of those delivery systems for the treatment of various diseases, for example, the treatment of cancer, are still ineffective and/or subject the patient to considerable risk.

Immunoconjugates comprise at least one targeting agent attached to at least one effector molecule. Such immunoconjugates can be categorized according to their 30 effector molecules into, for example, drug immunoconjugates, immunotoxin conjugate and radioimmunoconjugates (Payne, 2003).

Efficiency in killing target cells is one key factor in the usefulness of an immunoconjugate. Efficiency can be influenced by the potency of the effector molecule

(Blättler and Chari, 2001), by the ability of the effector to retain its potency (Chari et al., 1995; Liu et al., 1996; Ojima et al., 2002; Senter et al., 2002 and Sievers and Linenberger, 2001), by the tumor accessibility (Charter, 2001), by the level of expression of the target antigen on the target cell, by targeting agent affinity, and by the ability of the target cell to internalize the immunoconjugate (Wargalla, 1989).

One approach towards better efficiency involves the radioimmunoconjugate Zevalin. Y-90 Zevalin (Yttrium-90-labeled Ibritumomab-Tiuxetan) and In-111 Zevalin (Indium-111-labeled Ibritumomab-Tiuxetan) are radioimmunconjugates (Biogen-IDEK) based on Ibritumomab, the murine counterpart of Rituximab, conjugated with the chelator Tiuxetan, that has affinity for In-111 and Y-90. Both Ibritumomab (murine) and Rituximab (human), bind to CD20 which is widely expressed on B cells. CD20 does not internalize, modulate or shed and most likely plays a role in the  $Ca^{2+}$  in- and efflux of cells. Rituximab when administered by itself (without Zevalin) is highly cytotoxic and results in the elimination of B cells (including cancerous B cells) and thus has become part of the standard treatment of aggressive lymphomas. The eliminated B cells are replaced by healthy B cells from lymphoid stem cells.

The Zevalin therapeutic regime involves both the unconjugated antibody Rituximab and the immunoconjugate Zevalin. Generally, a single infusion of Rituximab precedes a fixed dose of In-111 Zevalin administered as a 10 minutes IV push. This step is, after seven to nine days, followed by a second step that involves a second infusion of Rituximab prior to administration of Y-90 Zevalin as a 10 minutes IV push. The pre-treatment with Rituximab removes the bulk of B cells, while the remaining cells, which may include tumor cells resistant to treatment with Rituximab, can then be targeted with Zevalin. In-111/Y-90 Zevalin is generally not employed by itself due to side effects that would result from the B cell mediated distribution of the radioactivity throughout the patient's body.

This Zevalin treatment, which is indicated for patients with relapsed or refractory low-grade, follicular, or transformed B-cell non-Hodgkin's lymphoma, including patients with Rituximab refractory follicular non-Hodgkin's lymphoma, showed higher response rates in clinical trials compared to only Rituximab. It also showed promising results for patients who no longer responded to Rituximab.

Ideally an immunoconjugate specifically binds to the target cell population, such as a particular class of tumor cells, and hardly or not at all to any other cell, in particular not to cells that perform vital functions in the host organism. While some antigens well correlate to certain disease profiles, in the majority of cases the antigens are also expressed on cells that are not associated with the disease, that is, non-target cells.

5 Depending on the importance of these healthy cells for the organism, the success of an immunoconjugate will to a large extent depend on how far these cells are affected by the treatment.

10 Thus, there remains a need for reducing the potential of side effects of immunoconjugates resulting from their interaction with non-targets that express the relevant antigen of the target cells. These include, in particular, cytotoxicity towards non-target cells as well as the overall reduction in efficiency of the immunoconjugate due to association with target molecules that have dissociated from the target cells. The 15 reduction of cytotoxicity towards non-target cells is particularly important when the non-target cells play a vital role in the host system.

## **SUMMARY OF THE INVENTION**

The present invention is directed at a method for improving targeting of immunoconjugates targeting cell bound antigen expressed on target cells comprising:  
(a) providing a medium comprising said antigen, wherein said antigen is cell bound on target cells, non-target cells and/or is present in soluble form;  
(b) sequestering cell bound antigen, soluble antigen and/or antigen expressing cells,  
(c) administering an immunoconjugate targeting cell bound antigen expressed on target 25 cells comprising a targeting agent for said antigen that is functionally attached to an effector molecule, wherein said sequestering in (b) improves the target cell targeting of said immunoconjugate. Preferably, the antigen internalizes into cells to which it is bound and/or is subject to shedding. The non-target cells may comprise cells such as epithelial cells, liver cells or any other cells that are not readily regenerated.

30 In particular, the present invention is directed at a method for improving tumor targeting of immunoconjugates targeting CD138 comprising:  
(a) providing a medium comprising cell bound CD138 and/or soluble CD138;  
(b) sequestering cell bound CD138, soluble CD138 and/or CD138 expressing cells,

(c) administering an immunoconjugate targeting cell bound CD138 comprising a targeting agent for CD138 that is functionally attached to an effector molecule, wherein said sequestering in (b) improves the tumor targeting of said immunoconjugate.

The present invention is also directed at pharmaceutical compositions and kits comprising an immunoconjugate targeting cell bound antigen (e.g., CD138) expressed on target cells (e.g., tumor cells) comprising a targeting agent for said antigen that is functionally attached to an effector molecule, and an unconjugated targeting agent.

The present invention provides a use of an immunoconjugate targeting cell bound CD138 comprising a targeting agent for CD138 that is functionally attached to an effector molecule for the manufacture of a medicament for the treatment of a tumor, wherein the medicament is to be administered to a patient in which cell bound CD138, soluble CD138 and/or CD138 expressing cells have been sequestered.

The present invention further provides a use of an immunoconjugate targeting cell bound CD138 comprising a targeting agent for CD138 that is functionally attached to an effector molecule for the manufacture of a medicament for the treatment of a tumor, wherein the medicament is to be administered as part of a treatment regime comprising the steps of:

- (a) providing a medium comprising cell bound CD138 and/or soluble CD138;
- (b) sequestering cell bound CD138, soluble CD138 and/or CD138 expressing cells, and
- (c) administering the immunoconjugate.

In addition, the present invention provides an immunoconjugate targeting cell bound CD138 comprising a targeting agent for CD138 that is functionally attached to an effector molecule for use in the treatment of a tumor, wherein the immunoconjugate is to be administered to a patient in which cell bound CD138, soluble CD138 and/or CD138 expressing cells have been sequestered.

Further the present invention provides a use of an immunoconjugate targeting cell bound CD138 and an unconjugated CD138 targeting agent for the manufacture of a combined preparation for simultaneous, separate or sequential use in the treatment of a tumor, wherein the immunoconjugate comprises a targeting agent for CD138 that is functionally attached to an effector molecule and wherein the unconjugated CD138

targeting agent is capable of sequestering cell bound CD138, soluble CD138 and/or CD138 expressing cells.

Also, the present invention provides a medicament comprising an immunoconjugate targeting cell bound CD138 and an unconjugated CD138 targeting agent as a combined preparation for simultaneous, separate or sequential use in the treatment of a tumor, wherein the immunoconjugate comprises a targeting agent for CD138 that is functionally attached to an effector molecule and wherein the unconjugated CD138 targeting agent is capable of sequestering cell bound CD138, soluble CD138 and/or CD138 expressing cells.

In particular, in the above uses the sequestering of cell bound CD138, soluble CD138 and/or CD138 expressing cells improves the tumor targeting of the immunoconjugate.

The present invention also provides a use of an immunoconjugate targeting cell bound antigen expressed on target cells comprising a targeting agent for said antigen that is functionally attached to an effector molecule for the manufacture of a medicament for treating a patient, wherein the medicament is to be administered to a patient in which cell bound antigen, soluble antigen and/or antigen expressing cells have been sequestered.

The present invention further provides a use of an immunoconjugate targeting cell bound antigen expressed on target cells comprising a targeting agent for said antigen that is functionally attached to an effector molecule for the manufacture of a medicament for treating an individual, wherein the medicament is to be administered as part of a treatment regime comprising the steps of:

- (a) providing a medium comprising said antigen, wherein said antigen is cell bound on target cells, non-target cells and/or is present in soluble form;
- (b) sequestering cell bound antigen, soluble antigen and/or antigen expressing cells; and
- (c) administering the immunoconjugate.

The present invention also provides an immunoconjugate targeting cell bound antigen expressed on target cells comprising a targeting agent for said antigen that is

functionally attached to an effector molecule for use in the treatment of an individual, wherein the immunoconjugate is to be administered to a patient in which cell bound antigen, soluble antigen and/or antigen expressing cells have been sequestered.

5        Further, the present invention provides a use of an immunoconjugate targeting cell bound antigen expressed on target cells and an unconjugated targeting agent for the manufacture of a combined preparation for simultaneous, separate or sequential use in the treatment of an individual, wherein the immunoconjugate comprises a targeting agent for the antigen that is functionally attached to an effector molecule and wherein  
10      the unconjugated targeting agent is capable of sequestering cell bound antigen, soluble antigen and/or antigen expressing cells.

Also, the present invention provides a medicament comprising an immunoconjugate targeting cell bound antigen expressed on target cells and an unconjugated targeting agent as a combined preparation for simultaneous, separate or sequential use in the treatment of an individual, wherein the immunoconjugate comprises a targeting agent for said antigen that is functionally attached to an effector molecule and wherein the unconjugated targeting agent is capable of sequestering cell bound antigen, soluble antigen and/or antigen expressing cells.

20        In one embodiment of the invention, the sequestering of cell bound antigen, soluble antigen and/or antigen expressing cells in the above uses improves the target cell targeting of said immunoconjugate.

25        In particular, the present invention can be applied to the treatment or prevention of a disease in an individual wherein the disease is one in which the target antigen expressed on the target cells is also present in soluble form and/or on non-target healthy cells and/or tissue. In one embodiment of the invention the target cell is a pre-cancerous or cancer cell.

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**BRIEF DESCRIPTION OF THE DRAWINGS**

**Figure 1** shows in (A) CD138 expression on the surface of NCI-H929 and MOLP-2 (right) determined by FACS analysis. Bound nBT062 antibody as detected using a FITC- (5) conjugated secondary antibody is shown in black. In the representative histogram plots, cell counts are plotted against fluorescence intensity measured in the FITC channel (FL1-H). Cells treated with the secondary antibody only served as controls (white). (B)

shows the sensitivity of NCI-H929 and MOLP-2 cells against nBT062-SPDB-DM4 (conjugate) and against free toxin lacking the antibody moiety as measured in cell (10) viability assays. Corresponding  $IC_{50}$  values are given. The relative expression of CD138 on the cell surface was determined by FACS analysis and relative fluorescence intensities (RFI) are given. In addition, absolute receptor numbers per cell are shown as quantified by using the QIFIKIT. In (C) the Figure shows the sensitivity of MOLP-2 and NCI-H929 cells towards nBT062.

**Figures 2 (A) and (B)** depict the cytotoxicity of nBT062-SPDB-DM4 against NCI-H929 cells in the presence of increasing concentrations of nBT062. Each plot also shows the dose/response curve obtained in the absence of nBT062. Sensitivity of NCI-H929 cells against the free toxin is shown in (A) (second plot).

**Figure 3 (A) and (B)** depict the cytotoxicity of nBT062-SPDB-DM4 against MOLP-2 cells in the presence of increasing concentrations of nBT062. Each plot also shows the dose/response curve obtained in the absence of nBT062. Sensitivity of MOLP-2 cells against the free toxin is shown in (A) (second plot).

**Figure 4** depict the influence of increasing concentrations of nBT062 on the  $IC_{50}$  values against NCI-H929 and MOLP-2 cells. The  $IC_{50}$  values were calculated based on the dose-response curves shown in Figure 2 and 3 and plotted against the concentration of nBT062 used for preincubation.

**Figure 5** shows a functional attachment of the effector to the antibody in the immunoconjugate BT062.

## DETAILED DESCRIPTION OF VARIOUS AND PREFERRED EMBODIMENTS OF THE INVENTION

The present invention relates to a method of improving targeting, in particular tumor targeting, of immunoconjugates, in particular immunoconjugates having cell bound antigens as targets that are also expressed on non-target cells. The method comprises sequestering the antigen (e.g., CD138) which may be cell bound or soluble, via unconjugated targeting agent(s). In certain embodiments, the method also comprises sequestering cells expressing cell bound antigen. The sequestration preferably "shields" non-target cells from destruction by the immunoconjugate and/or reduces immunoconjugate binding to soluble antigen and/or antigen expressed on non-target cells. Thus, side effects resulting from immunoconjugate treatment are reduced and/or the efficiency of binding is improved allowing administration of a lower dosage of immunoconjugate and/or a higher dosage of immunoconjugate with a respectively lower occurrence of side effects.

In one embodiment, the unconjugated targeting agent according to the present invention is administered to cells of a subject in need of therapeutic treatment. The unconjugated targeting agent binds an antigen (e.g., CD138) which may or may not be cell bound. An immunoconjugate is administered subsequently or concurrently (e.g., in a single dosage or as part of a kit). Alternatively or additionally, e.g., the blood of the patient is first treated (pretreated) to sequester soluble, cell bound antigen and/or antigen expressing cells. Compared to the direct administration of the immunoconjugate (i.e., without the pretreatment with the unconjugated targeting agent) the viability of accessible non-target tumor cells upon treatment with the immunoconjugate is improved by about 5%, about 10%, about 20%, about 30%, about 40%, about 50%, about 60% or even more.

In one example, the unconjugated targeting agent is nBT062 (DSM ACC287), which is administered to a patient with cancer as a single dose. In this example, an effective amount of nBT062 is administered intravenously so that the unconjugated targeting agent reaches soluble CD138 (CD138) and readily accessible cell bound CD138 promptly. Within four hours, the immunoconjugate BT062, which is described in more detail herein, is administered in an effective amount intravenously to the same

patient. The immunoconjugate is internalized and the effector molecule(s) is/are released from the antibody target by natural means. The administration procedure may be repeated on the same patient after 5 to 10 days. The effective ratio of nBT062: BT062 employed is 10:1 and 3:1, respectively.

5        In a second example, nBT062 and BT062 are administered simultaneously as part of a pharmaceutical composition to a patient in need of tumor treatment. The target cells are CD138 expressing tumor cells. Both, nBT062 and BT062, are each administered in an effective amount. The dosage of the individual components of the composition and the effective ratio of nBT062 to BT062 is determined in cell culture prior 10 to administration to allow an improvement of tumor targeting of the immunoconjugate. In particular, the viability of tumor target cells is, compared to the direct administration of the immunoconjugate, only slightly reduced by 1%, while the viability of the accessible non-target cells, also compared to the direct administration of the immunoconjugate, is doubled from 40% to 80%. When an effective amount of the immunoconjugate has 15 bound the target cell, the immunoconjugate is internalized and the effector molecule or molecules are released from the antibody target by natural means.

20        In a third example, the alpha blocker prozosin is administered orally to a patient in need of tumor treatment prior to or concurrent with nBT062. Prozosin stimulates the peripheral circulation in the patient thus allowing nBT062 to more efficiently reach its destination, which, in the case of the unconjugated targeting agent, is a non-target cell, e.g., cells of the epithelium. The next day, BT062 is administered to said patient in an effective amount. An improvement of tumor targeting of the immunoconjugate is obtained. An effective ratio of 1:1 nBT062/BT062 is employed. When an effective 25 amount of the immunoconjugate has bound the target cell, the immunoconjugate is internalized and the effector molecule or molecules are released from the antibody target by natural means.

30        In a fourth example, blood of a patient is run through a column in which nBT062 has been immobilized on sepharose beads that are packed into the column. sCD138 ("soluble"/ "shielded" CD138) binds to the immobilized nBT062. The treatment is performed continuously for 2 hours. The so depleted blood is reintroduced into the patient, and sCD138 is washed off the column with a saline solution to prepare the

column for the next procedure. Within 24 hours, the patient is treated with an effective amount of BT062.

In a fifth example, the patient's blood is pretreated as in the third example, but the patient is treated with a combination of antitrombin-3 and BT062, which are 5 administered, each in an effective amount and at an effective ratio, intravenously.

In a sixth example, the patient's blood is subjected to plasmapheresis for two hours. Blood cells obtained are resuspended in replacement donor plasma or saline with added proteins and are reintroduced into the patient. The immunoconjugate is then administered to the patient as in examples 3 or 4.

10 CD138 or sydecan-1 (also described as SYND1; SYNDECAN; SDC; SCD1; CD138 ANTIGEN, SwissProt accession number: P18827 human) is an integral membrane glycoprotein that was originally described to be present on cells of epithelial origin, and subsequently found on hematopoietic cells (Sanderson, 1989). In malignant hematopoiesis, CD138 is highly expressed on the majority of multiple myeloma (MM) 15 cells, ovarian carcinoma, kidney carcinoma, gall bladder carcinoma, breast carcinoma, prostate cancer, lung cancer, colon carcinoma cells and cells of Hodgkin's and non-Hodgkin's lymphomas, chronic lymphocytic leukemia (CLL) (Horvathova, 1995), acute lymphoblastic leukemia (ALL), acute myeloblastic leukemia (AML) (Seftalioglu, 2003 (a); Seftalioglu, 2003 (b)), gallbladder (GB) carcinoma (Roh et al, 2008), solid tissue 20 sarcomas, colon carcinomas, as well as other hematologic malignancies and solid tumors that express CD138 (Carbone et al., 1999; Sebestyen et al., 1999; Han et al., 2004; Charnaux et al., 2004; O'Connell et al., 2004; Orosz and Kopper, 2001).

Other cancers that have been shown to be positive for CD138 expression are many ovarian adenocarcinomas, transitional cell bladder carcinomas, kidney clear cell 25 carcinomas, squamous cell lung carcinomas; breast carcinomas and uterine cancers (see, for example, Davies et al., 2004; Barbareschi et al., 2003; Mennerich et al., 2004; Anttonen et al., 2001; Wijdenes, 2002).

In the normal human hematopoietic compartment, CD138 expression is restricted 30 to plasma cells (Wijdenes, 1996; Chilos, 1999) and is not expressed on peripheral blood

lymphocytes, monocytes, granulocytes, and red blood cells. In particular, CD34<sup>+</sup> stem and progenitor cells do not express CD138 and anti-CD138 mAbs do not affect the number of colony forming units in hematopoietic stem cell cultures (Wijdenes, 1996). In non-hematopoietic compartments, CD138 is mainly expressed on simple and stratified epithelia within the lung, liver, skin, kidney and gut. Only a weak staining was seen on endothelial cells (Bernfield, 1992; Vooijs, 1996). It has been reported that CD138 exists 5 in polymorphic forms in human lymphoma cells (Gattei, 1999).

Monoclonal antibodies B-B4, BC/B-B4, B-B2, DL-101, 1 D4, MI15, 1.BB.210, 10 2Q1484, 5F7, 104-9, 281-2 in particular B-B4 have been reported to be specific to CD138. Of those B-B4, 1D4 and MI15 recognized both the intact molecule and the core protein of CD138 and were shown to recognize either the same or closely related epitopes (Gattei, 1999). Previous studies reported that B-B4 did not recognize soluble CD138, but only CD138 in membrane bound form (Wijdenes, 2002).

15 B-B4, a murine IgG1 mAb, binds to a linear epitope between residues 90-95 of the core protein on human syndecan-1 (CD138) (Wijdenes, 1996; Dore, 1998). Consistent with the expression pattern of CD138, B-B4 was shown to strongly react with plasma cell line RPMI8226, but not to react with endothelial cells. Also consistent with 20 the expression pattern of CD138, B-B4 also reacted with epithelial cell lines A431 (keratinocyte derived) and HepG2 (hepatocyte derived). An immunotoxin B-B4-saporin was also highly toxic towards the plasma cell line RPMI8226, in fact considerably more toxic than free saporin. However, from the two epithelial cell lines tested, B-B4-saporin showed only toxicity towards cell line A431, although in a clonogenic assay B-B4 saporin 25 showed no inhibitory effect on the outgrowth of A431 cells (Vooijs, 1996). Other researchers reported lack of specificity of MM-associated antigens against tumors (Couturier, 1999).

nBT062 is a CD138 specific chimeric human/murine antibody based on B-B4, 30 which has the same binding specificity for CD138 as its parental antibody, B-B4 (B-B4 chimeric antibodies are generally also referred to herein as c-B-B4). Chinese hamster ovary cells expressing nBT062 have been deposited with the DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1, D-38124 Braunschweig on December 11, 2007. The identification number is DSM ACC2875. BT062 is an immunoconjugate comprising the CD138-specific chimeric antibody nBT062

that is conjugated via a linker with a cytostatic maytansinoid derivative. Immunoconjugates comprising nBT062 and a maytansinoid effector molecule are often characterized in terms of their linker and maytansinoid effector, e.g., BT062-SMCC-DM1, is an immunoconjugate comprising nBT062, SMCC (a noncleavable linker containing a thioester bond) and DM1 as an effector. More generically, an immunoconjugate containing nBT062 and an effector molecule may also be described as nBT062-linker-effector or just as nBT062-effector (nBT062N, wherein N is any effector described herein).

A “targeting agent” according to the present invention is able to associate with a molecule expressed by a target cell and includes peptides and non-peptides. In particular, targeting agents according to the present invention include targeting antibodies and non-immunoglobulin targeting molecules, which may be based on non-immunoglobulin proteins, including, but not limited to, AFFILIN® molecules, ANTICALINS® and AFFIBODIES®. Non-immunoglobulin targeting molecules also include non-peptidic targeting molecules such as targeting DNA and RNA oligonucleotides (aptamers), but also physiological ligands, in particular ligands of the antigen in question, such as CD138.

A “targeting antibody” according to the present invention is or is based on a natural antibody or is produced synthetically or by genetic engineering and binds to an antigen on a cell or cells (target cell(s)) of interest. A targeting antibody according to the present invention includes a monoclonal antibody, a polyclonal antibody, a multispecific antibody (for example, a bispecific antibody), or an antibody fragment. The targeting antibody may be engineered to, for example, improve its affinity to the target cells (Ross, 2003) or diminish its immunogenicity. The targeting antibody may be attached to a liposomal formulation including effector molecules (Carter, 2003). An antibody fragment comprises a portion of an intact antibody, preferably the antigen binding or variable region of the intact antibody. Examples of antibody fragments according to the present invention include Fab, Fab', F(ab')<sub>2</sub>, and Fv fragments, but also diabodies; domain antibodies (dAb) (Ward, 1989; United States Patent 6,005,079); linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragments. In a single chain variable fragment antibody (scFv) the heavy and light chains (VH and VL) can be linked by a short amino acid linker having, for example, the sequence (glycine<sub>4</sub>serine)<sub>n</sub>, which has sufficient flexibility to allow the two domains to assemble a

functional antigen binding pocket. Addition of various signal sequences may allow for more precise targeting of the targeting antibody. Addition of the light chain constant region (CL) may allow dimerization via disulphide bonds, giving increased stability and avidity. Variable regions for constructing the scFv can, if a mAb against a target of interest is available, be obtained by RT-PCR which clones out the variable regions from mRNA extracted from the parent hybridoma. Alternatively, the scFv can be generated *de novo* by phage display technology (Smith, 2001). A bispecific antibody according to the present invention may, for example, have at least one arm that is reactive against a target tissue and one arm that is reactive against a linker moiety (United States Patent Publication 20020006379). A bispecific antibody according to the present invention may also bind to more than one antigen on a target cell (Carter, 2003). An antibody according to the present invention may be modified by, for example, introducing cystein residues to introduce thiol groups (Olafsen, 2004).

In accordance with the present invention, the targeting antibody may be derived from any source and may be, but is not limited to, a camel antibody, a murine antibody, a chimeric human/mouse antibody such as nBT062 or a chimeric human/monkey antibody, in particular, a chimeric human/monkey antibody with the monkey portion stemming from a cynomolgus monkey.

Humanized antibodies are antibodies that contain sequences derived from a human antibody and from a non-human antibody and are also within the scope of the present invention. Suitable methods for humanizing antibodies include CDR-grafting (complementarity determining region grafting) (EP 0 239 400; WO 91/09967; United States Patents 5,530,101; and 5,585,089), veneering or resurfacing (EP 0 592 106; EP 0 519 596; Padlan, 199; Studnicka et al., 1994; Roguska et al., 1994), chain shuffling (United States Patent 5,565,332) and Delimmunosation<sup>TM</sup> (Biovation, LTD). In CDR-grafting, the mouse complementarity-determining regions (CDRs) from, for example, mAb B-B4 are grafted into human variable frameworks, which are then joined to human constant regions, to create a human B-B4 antibody. Several antibodies humanized by CDR-grafting are now in clinical use, including MYLOTARG (Sievers et al., 2001) and HECEPTIN (Pegram et al, 1998).

The resurfacing technology uses a combination of molecular modeling, statistical analysis and mutagenesis to alter the non-CDR surfaces of antibody variable regions to resemble the surfaces of known antibodies of the target host. Strategies and methods for the resurfacing of antibodies, and other methods for reducing immunogenicity of

antibodies within a different host, are disclosed, for example, in United States Patent 5,639,641. Human antibodies can be made by a variety of methods known in the art including phage display methods. See also United States Patents 4,444,887, 4,716,111, 5,545,806, and 5,814,318; and international patent application publications WO 5 98/46645, WO 98/50433, WO 98/24893, WO 98/16654, WO 96/34096, WO 96/33735, and WO 91/10741.

Fully human antibodies may also been used. Those antibodies can be selected by the phage display approach, where CD138 or an antigenic determinant thereof is used to selectively bind phage expressing, for example, B-B4 variable regions (see, 10 Krebs, 2001). This approach is advantageously coupled with an affinity maturation technique to improve the affinity of the antibody.

In one embodiment, the targeting antibody is, in its unconjugated form, moderately or poorly internalized. Moderate internalization constitutes about 30% to about 75% internalization of antibody, poor internalization constitutes about 0.01% to up 15 to about 30% internalization after 3 hours incubation at 37°C. In another preferred embodiment the targeting antibody binds to CD138, for example, antibodies B-B4, BC/B-B4, B-B2, DL-101, 1 D4, MI15, 1.BB.210, 2Q1484, 5F7, 104-9, 281-2 in particular B-B4 or nBT062. nBT062 is a chimerized B-B4 antibody. Hybridoma cells, which were generated by hybridizing SP02/0 myeloma cells with spleen cells of Balb/c mice have 20 been deposited with the DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1, D-38124 Braunschweig on December 11, 2007. The identification number is DSM ACC2874. When, in the context of the present invention, the name of a specific antibody is combined with the term "targeting antibody" such as "nBT062 targeting antibody," this means that this targeting antibody 25 has the binding specificity of the antibody nBT062. If a targeting antibody is said to be "derived from" a specified antibody, this means that this targeting antibody has the binding specificity of this antibody, but might take any form consistent with the above description of a targeting antibody.

30 "Non-immunoglobulin targeting molecules" according to the present invention include targeting molecules derived from non-immunoglobulin proteins as well as non-peptidic targeting molecules. Small non-immunoglobulin proteins which are included in this definition are designed to have specific affinities towards, in particular surface expressed CD138. These small non-immunoglobulin proteins include scaffold based

engineered molecules such as Affilin® molecules that have a relatively low molecular weight such as between 10 kDa and 20 kDa. Appropriate scaffolds include, for example, gamma crystalline. Those molecules have, in their natural state, no specific binding activity towards the target molecules. By engineering the protein surfaces through

5 locally defined randomization of solvent exposed amino acids, completely new binding sites are created. Former non-binding proteins are thereby transformed into specific binding proteins. Such molecules can be specifically designed to bind a target, such as CD138, and allow for specific delivery of one or more effector molecules (see, scil Proteins GmbH at [www.scilprotein.com](http://www.scilprotein.com), 2004). Another kind of non-immunoglobulin  
10 targeting molecules are derived from lipocalins, and include, for example ANTICALINS®, which resemble in structure somewhat immunoglobulins. However, lipocalins are composed of a single polypeptide chain with 160 to 180 amino acid residues. The binding pocket of lipocalins can be reshaped to recognize a molecule of interest with high affinity and specificity (see, for example, Beste et al., 1999). Artificial bacterial  
15 receptors such as those marketed under the trademark Affibody® (Affibody AB) are also within the scope of the present invention. These artificial bacterial receptor molecules are small, simple proteins and may be composed of a three-helix bundle based on the scaffold of one of the IgG-binding domains of Protein A (*Stylococcus aureus*). These molecules have binding properties similar to many immunoglobulins, but are  
20 substantially smaller, having a molecular weight often not exceeding 10kDa and are also comparatively stable. Suitable artificial bacterial receptor molecules are, for example, described in United States Patents 5,831,012; 6,534,628 and 6,740,734.

Other “non-immunoglobulin targeting molecules” are physiological ligands of the antigen in question. Physiological ligands of CD138 include for example, but not limited  
25 to, ADAMTS4 (aggrecanase-1), antithrombin-3, bFGF, cathepsin G, CCL5 (RANTES), CCL7, CCL11, CCL17, CD44, collagens (collagen type 1, collagen type 2, collagen type 3, collagen type 4, collagen type 5, collagen type 6), CXCL1, elastase, gp120 , HGF [hepatocyte growth factor], laminin-1, laminin-2, laminin-5, midkine, MMP-7, neutrophil elastase, and pleiotrophin (HBNF, HBGF-8).

30 Non-peptidic targeting molecules include, but are not limited to, to DNA and RNA oligonucleotides that bind to CD138 (aptamers).

An “unconjugated targeting agent” as used herein, is in certain embodiments of the invention, a targeting agent that is not part of an immunoconjugate as defined herein,

i.e. a targeting agent that is not attached to an effector molecule. However, in other embodiments and as will be detailed below, the “unconjugated targeting agent” is attached to a non-effector molecule, for example, to a matrix. In yet other embodiments, the unconjugated targeting agent may be attached to a dye molecule for monitoring.

5 Unconjugated targeting agents according to the present invention preferably are not systemically cytotoxic for the cells they bind to that is, over time the unconjugated targeting agents do not induce cell death of populations of those cells.

The targeting agents of the present invention bind antigens expressed on target 10 cells, in particular tumor cells, but also antigens expressed on non-target cells, though generally to different degrees. Non-target cells according to the present invention include cells expressing the target specific antigen, i.e., the antigen targeted by the immunoconjugate, such as CD138, but generally do so in considerably lower 15 concentrations (such as a third, a fourth, a fifth or a sixth of the expression on the tumor cells) and do not have tumor cell/disease properties. Such non-target cells vary from antigen to antigen, but may include epithelial cells, liver cells or any other cells that are 20 not readily regenerated.

An “effector molecule” according to the present invention is a molecule or a 25 derivative, or an analogue thereof that is attached to a targeting agent and exerts a desired effect, for example apoptosis, or another type of cell death, or a continuous cell cycle arrest on the target cell or cells. Effector molecules according to the present invention include molecules that can exert desired effects in a target cell and include, but are not limited to, toxins, drugs, in particular low molecular weight cytotoxic drugs, 30 radionuclides, biological response modifiers, pore-forming agents, ribonucleases, proteins of apoptotic signaling cascades with apoptosis-inducing activities, cytotoxic enzymes, prodrug activating enzymes, antisense oligonucleotides, antibodies or cytokines as well as functional derivatives or analogues/fragments thereof. Toxins may include bacterial toxins, such as, but not limited to, Diphtheria toxin or Exotoxin A, plant toxins, such as but not limited to, Ricin. Proteins of apoptotic signaling cascades with apoptosis-inducing activities, include, but are not limited to, Granzyme B, Granzyme A, Caspase-3, Caspase-7, Caspase-8, Caspase-9, truncated Bid (tBid), Bax and Bak.

In certain embodiments the effector has high non-selective toxicity, including systemic toxicity, when in its native form (“unconjugated effector”), that is, is non

selective with regard to one or more target cells or types of target cells, in particular disease targets. The "native form" of an effector molecule of the present invention is an effector molecule before being attached to the targeting agent to form an immunoconjugate, that is, as it exists when not attached to the targeting agent. It can 5 also be referred to as just the effector molecule or the unconjugated effector. In another preferred embodiment, the non-selective toxicity of the effector molecule is substantially eliminated upon conjugation to the targeting agent, that is, to the degree that any remaining non-selective toxicity is clinically acceptable. In another preferred embodiment, the effector molecule causes, upon reaching the target cell, death or 10 continuous cell cycle arrest in the target cell. A drug-effector molecule according to the present invention includes, but is not limited to, a drug including, for example, small highly cytotoxic drugs that act as inhibitors of tubulin polymerization such as maytansinoids, dolastatins, auristatin and crytophycin; DNA alkylating agents like CC-1065 analogues or derivatives (United States Patents 5,475,092; 5,585,499; 6,716,821) 15 and duocarmycin; enediyne antibiotics such as calicheamicin and esperamicin; and potent taxoid (taxane) drugs (Payne, 2003). Maytansinoids and calicheamicins are particularly preferred. An effector maytansinoid includes maytansinoids of any origin, including, but not limited to synthetic maytansinol and maytansinol analogue and derivative. Doxorubicin, daunomycin, methotrexate, vinblastine, neocarzinostatin, 20 macromycin, treimon and  $\alpha$ -amanitin are some other effector molecules within the scope of the present invention. Also within the scope of the present invention are antisense DNA molecules as effector molecules. When the name of, for example, a specific drug or class of drugs is combined herein with the term "effector" or "effector molecule," reference is made to an effector of an immunoconjugate according to the 25 present invention that is based on the specified drug or class of drugs.

Maytansine is a natural product originally derived from the Ethiopian shrub *Maytenus serrata* (Remillard, 1975; United States Patent 3,896,111). This drug inhibits tubulin polymerization, resulting in mitotic block and cell death (Remillard, 1975; Bhattacharyya, 1977; Kupchan, 1978). The cytotoxicity of maytansine is 200-1000-fold 30 higher than that of anti-cancer drugs in clinical use that affect tubulin polymerization, such as *Vinca* alkaloids or taxol. However, clinical trials of maytansine indicated that it lacked a therapeutic window due to its high systemic toxicity. Maytansine and maytansinoids are highly cytotoxic but their clinical use in cancer therapy has been greatly limited by their severe systemic side-effects primarily attributed to their poor

selectivity for tumors. Clinical trials with maytansine showed serious adverse effects on the central nervous system and gastrointestinal system.

Maytansinoids have also been isolated from other plants including seed tissue of *Trewia nudiflora* (United States Patent 4,418,064)

5 Certain microbes also produce maytansinoids, such as maytansinol and C-3 maytansinol esters (United States Patent 4,151,042).

The present invention is directed to maytansinoids of any origin, including synthetic maytansinol and maytansinol analogues which are disclosed, for example, in United States Patents 4,137,230; 4,248,870; 4,256,746; 4,260,608; 4,265,814; 10 4,294,757; 4,307,016; 4,308,268; 4,308,269; 4,309,428; 4,313,946; 4,315,929; 4,317,821; 4,322,348; 4,331,598; 4,361,650; 4,362,663; 4,364,866; 4,371,533; 4,424,219 and 4,151,042.

In a preferred embodiment, the maytansinoid is a thiol-containing maytansinoid and is more preferably produced according to the processes disclosed in United States 15 Patent 6,333,410 to Chari et al or in Chari et al.(Chari, 1992).

DM-1 (N<sup>2</sup>-deacetyl-N<sup>2</sup>-(3-mercaptopropyl)-maytansine) is a preferred effector molecule in the context of the present invention. DM1 is 3- to 10-fold more cytotoxic than maytansine, and has been converted into a pro-drug by linking it *via* disulfide bond(s) to a monoclonal antibody directed towards a tumor-associated antigen.

20 Certain of these conjugates (sometimes called "tumor activated prodrugs" (TAPs)) are not cytotoxic in the blood compartment, since they are activated upon associating with a target cells and internalized, thereby releasing the drug (Blättler, 2001). Several antibody-DM1 conjugates have been developed (Payne, 2003), and been evaluated in clinical trials. For example, huC242-DM1 treatment in colorectal cancer patients was 25 well tolerated, did not induce any detectable immune response, and had a long circulation time (Tolcher, 2003).

Other particularly preferred maytansinoids comprise a side chain that contains a sterically hindered thiol bond such as, but not limited to, maytansinoids N<sup>2</sup>-deacetyl- N<sup>2</sup>-(4-mercaptopropyl)-maytansine, also referred to as "DM3," and N<sup>2</sup>-deacetyl- N<sup>2</sup>-(4-methyl-4-mercaptopropyl)-maytansine, also referred to as "DM4."

30 DNA alkylating agents are also particularly preferred as effector molecules and include, but are not limited to, CC-1065 analogues or derivatives. CC-1065 is a potent antitumor-antibiotic isolated from cultures of *Streptomyces zelensis* and has been shown to be exceptionally cytotoxic *in vitro* (United States Patent 4,169,888). Within the scope

of the present invention are, for examples the CC-1065 analogues or derivatives described in United States Patents 5,475,092, 5,585,499 and 5,739,350. As the person skilled in the art will readily appreciate, modified CC-1065 analogues or derivatives as described in United States Patent 5,846,545 and prodrugs of CC-1065 analogues or derivatives as described, for example, in United States Patent 6,756,397 are also within the scope of the present invention. In certain embodiments of the invention, CC-1065 analogues or derivatives may, for example, be synthesized as described in United States Patent 6,534,660.

Another group of compounds that make preferred effector molecules are taxanes, especially highly potent ones and those that contain thiol or disulfide groups. Taxanes are mitotic spindle poisons that inhibit the depolymerization of tubulin, resulting in an increase in the rate of microtubule assembly and cell death. Taxanes that are within the scope of the present invention are, for example, disclosed in United States Patents 6,436,931; 6,340,701; 6,706,708 and United States Patent Publications 20040087649; 20040024049 and 20030004210. Other taxanes are disclosed, for example, in United States Patent 6,002,023, United States Patent 5,998,656, United States Patent 5,892,063, United States Patent 5,763,477, United States Patent 5,705,508, United States Patent 5,703,247 and United States Patent 5,367,086. As the person skilled in the art will appreciate, PEGylated taxanes such as the ones described in United States Patent 6,596,757 are also within the scope of the present invention.

Calicheamicin effector molecules according to the present invention include gamma 1I, N-acetyl calicheamicin and other derivatives of calicheamicin. Calicheamicin binds in a sequence-specific manner to the minor groove of DNA, undergoes rearrangement and exposes free radicals, leading to breakage of double-stranded DNA, resulting in cell apoptosis and death. One example of a calicheamicin effector molecule that can be used in the context of the present invention is described in United States Patent 5,053,394.

An "immunoconjugate" according to the present invention comprises at least one targeting agent, in particular a targeting antibody, and one or more effector molecules. The immunoconjugate might comprise further molecules for example for stabilization. For immunoconjugates, the term "conjugate" is generally used to define the operative association (i.e., functional attachment) of the targeting agent with one or more effector molecules and is not intended to refer solely to any type of operative association, and is

particularly not limited to chemical "conjugation". So long as the targeting agent is able to bind to the target site and the attached effector functions sufficiently as intended, particularly when delivered to the target site, any mode of attachment will be suitable. The conjugation methods according to the present invention include, but are not limited to, direct attachment of the effector molecule to the targeting antibody, with or without prior modification of the effector molecule and/or the targeting antibody or attachment via linkers. Linkers can be categorized functionally into, for example, acid labile, photosensitive, enzyme cleavable linkers etc. Other suitable linkers may include disulfide bonds and non-cleavable bonds, such as, but not limited to Sulfosuccinimidyl maleimidomethyl cyclohexane carboxylate (SMCC), which is a heterobifunctional linker capable of linking compounds with SH-containing compounds. Bifunctional and heterobifunctional linker molecules, such as carbohydrate-directed heterobifunctional linker molecules, such as S-(2-thiopyridyl)-L-cysteine hydrazide (TPCH), are also within the scope of the present invention (Vogel, 2004). The effector molecule, such as a maytansinoid, may be conjugated to the targeting antibody via a two reaction step process, including as a first step modification of the targeting antibody with a cross-linking reagent such as N-succinimidyl pyridylthiopropionate (SPDP) to introduce dithiopyridyl groups into the targeting antibody. In a second step, a reactive maytansinoid having a thiol group, such as DM1 or DM4, may be added to the modified antibody, resulting in the displacement of the thiopyridyl groups in the modified antibody, and the production of disulfide-linked cytotoxic maytansinoid/antibody conjugate (United States Patent 5,208,020). However, one-step conjugation processes such as the one disclosed in United States Patent Publication 20030055226 to Chari et al are also within the scope of the present invention. In one embodiment of the present invention multiple effector molecules of the same or different kind are functionally attached to a targeting antibody. Fig. 5 shows an exemplatory functional attachment as present in the immunoconjugate BT062 (nBT062-SPDP-DM4). As the Figure indicates on average, 3.5 DM4 molecules are attached to the antibody.

CC-1065 analogues or derivatives may be conjugated to the targeting agent via for example PEG linking groups as described in United States Patent 6,716,821.

Calicheamicins may be conjugated to the targeting antibodies via linkers (United States Patent 5,877,296 and United States Patent 5,773,001) or according to the conjugation methods disclosed in United States Patent 5,712,374 and United States Patent 5,714,586. Another preferred method for preparing calicheamicin conjugates is

disclosed in United States Patent Publication 20040082764. The immunoconjugates of the present invention also include recombinant fusion proteins.

The unconjugated targeting agents and/or immunoconjugates of the present invention can be provided *in vivo* and *ex vivo*. In many embodiments the treatment 5 regime will include an *in vivo* as well as an *ex vivo* portion. For example, the blood of the patient may be first subjected to plasmapheresis and after reintroduction of at least the blood cells (e.g., in plasma expander) into the patient, the patient may be subjected to treatment with the immunoconjugate. Other *ex vivo* methods include treatment of cell supernatant. Depending on the particular treatment regime, different media may be 10 contacted with the unconjugated targeting agent and/or immunoconjugate. These media include, but are not limited to, blood, plasma, tissue and bone marrow.

The unconjugated targeting agent preferably binds to antigen having different configurations, including antigen present in soluble form such as soluble (shed) CD138 15 (sCD138). Such sCD138 is produced, e.g., during a process referred to as shedding. Shedding can occur naturally, but may also be induced by certain drugs that are administered to, e.g., a MM patient.

The unconjugated targeting agent may also bind to cell bound antigen such as 20 CD138. This cell bound antigen may be expressed on the surface of a non-target cell, which are also referred to herein as non-tumor cells when the target cells are tumor cells. The unconjugated targeting agent may bind to the antigen expressed on such a non-tumor cell/non-target cell to an extent that it shields the non-tumor cell/ non-target cell to different degrees from destruction by the immunoconjugate that is administered 25 concomitantly or subsequently, or in certain embodiments, prior to the administration of the unconjugated targeting agent.

The time intervals between a "pretreatment" with the unconjugated targeting agent and the administration of the immunoconjugate may differ and may be as short as about 20 mins., about 30 mins, but may also be about 1 hour, about 2 hours, about 3 30 hours, about 4hrs, about 5hrs, but generally do not exceed 24 hours.

When the unconjugated targeting agent is said to sequester cells expressing CD138 reference is made to sequestering whole cells expressing CD138. For example, an unconjugated targeting agent that is attached to a matrix may sequester such a cell from blood contacted with this matrix.

A vasodilatory agent, as used herein, is any agent with vasodilatory activity. Included are in particular, alpha 1 receptor antagonists, i.e., alpha blockers such as, but not limited to, prozosin, terazosin or doxazosin. However, any other agent with vasodilatory activity is included in this definition such as, but not limited to, sodium nitroprussid, calcium antagonists, nitrates or ACE inhibitors (inhibitors of Angiotensin-Converting Enzyme (ACE)).

These agents may be administered using different routes. However, oral administration is preferred. In the context of the present invention such vasodilatory agents may, preferably, be administered either before or with the unconjugated targeting agent. This administration is generally followed by an administration of a suitable immunoconjugate. In a preferred embodiment, the administration of the immunoconjugate is timed at least ten to twelve hours subsequent to the administration of the vasodilatory agent. However, the most appropriate timing will depend on the particular vasodilatory agent used and is well within the skill of the person of ordinary skill in the art. It is preferred that the administration of the immunoconjugate will be timed so that the vasodilatory effects of the agent have substantially subsided.

A method according to the present invention is said to "improve tumor targeting" of an immunoconjugate when the immunoconjugate employed according to this method binds, and preferably destroys, a higher percentile of tumor cells than when the immunoconjugate, under otherwise equivalent conditions (route of administration, concentration etc.) is used as the sole effective ingredient. Tumor cells according to the present invention include cancer cells as well as pre-cancerous cells which may or may not form solid tumors. Thus, any abnormally growing cell is part for this definition.

A method according to the present invention is said to improve targeting of target cells of an immunoconjugate if the target cells are associated with a disease or disease state, but are not necessarily tumor cells.

The improvement in targeting, in particular tumor targeting may be expressed as a percentile. For example, about 20% improved tumor targeting means that a given amount of immunoconjugate, when administered to a patient, binds, e.g., 20% more of the target tumor cells than when administered as the sole effective agent.

A method according to the present invention is also said to "improve targeting of target cells" or to "improve tumor targeting" of an immunoconjugate when non-target cells, e.g., CD138 expressing non-tumor cells are shielded from binding by the immunoconjugate, preferably to an extent that prevents or diminishes the destruction of

5 said non-tumor (non-target) cells, preferably to a degree that is clinically acceptable, which may, depending on the non-tumor cell in question and the locus and route of administration, vary. Acceptable levels of destruction of non-target/non-tumor cells may vary widely and might be, in certain embodiments be as low as about 1% of the total population of a certain type of accessible non-tumor cells of a patient's body or non-tumor cell in a particular organ or up to about 2, 3, 4, 5, 6, 7, 8, 9, 10, 15 or 20%. In certain cases, in particular when the administration can be confined, e.g., to a particular organ, this percentile may be even higher. In this context, an effective amount of an immunoconjugate means an amount that results in destruction, preferably by apoptosis, 10 of tumor cells that the immunoconjugate binds to. An effective amount of unconjugated targeting agent means an amount that "shields" or "masks", e.g., non-tumor cells expressing CD138, that is, an amount of unconjugated targeting agent that is sufficient to impede or block access of an effective amount of immunoconjugate to non-tumor cells so that destruction of the cells is prevented. By masking non-tumor cells toxicity of the 15 immunoconjugate towards the total accessible non-tumor cells/non-target cells is limited to a degree acceptable, e.g. to a clinically acceptable degree. Certain concentrations of unconjugated targeting agent may result in decreasing the sensitivity of total accessible non-tumor cell/non-target cell population to an immunoconjugate. The decrease in sensitivity of the total accessible non-tumor cells/non-target cells may reflected by an 20 increase in  $IC_{50}$  values, and can be quantified by sequestration values:

$$\text{Sequestration [%]} = 100 \times (\text{IC}_{50} \text{ treat} / \text{IC}_{50} \text{ direct}) - 100$$

25  $IC_{50}$  treat [nM] . . .  $IC_{50}$  value for the immunoconjugate when cells are treated (pre- or concomitant) with the unconjugated targeting agent

$IC_{50}$  direct [nM] . . .  $IC_{50}$  value for the immunoconjugate when cells are treated directly with the unconjugated targeting agent

30 The  $IC_{50}$  treat value might be above 2, above 3, above 4, above 5, above 6, above 7, above 8, above 9, above 10 times the  $IC_{50}$  direct value, resulting in sequestration values of above 100%, above 200%, above 300%, above 400%, above 500%, above 600%, above 700%, above 800%, above 900% and above 1000%. However, in certain embodiments, lower sequestration values such as above 20% or 35 above 50% might already be desirable.

$IC_{50}$  values and sequestration values for both accessible non-target cells (e.g., CD138<sup>low</sup>) and accessible target cells (e.g., CD138<sup>high</sup>) can be calculated and compared and relative sequestration values can be calculated:

5 **Relative Sequestration** =sequestration (accessible non-target cells)/sequestration (accessible target cells)

Values higher than 1 indicate that the shielding of ,e.g., CD138<sup>low</sup> cells is higher than that of, e.g., CD138<sup>high</sup> cells for a given concentration of unconjugated targeting  
10 agent, e.g., nBT062.

In the context of the present invention relative sequestration values of equal to or more than 10, equal to or more than 20, equal to or more than 30, equal to or more than 40 or equal to or more than 50 are preferred.

15

When an unconjugated targeting agent is administered to shield the accessible non-target cells, in particular non-tumor cells, as explained elsewhere herein it will shield also target cells. In a preferred embodiment the effective amount of unconjugated targeting agent does not or only slightly decrease the sensitivity of tumor target cells, resulting in no or a relative slight increase in viable tumor cells. The effective amount of immunoconjugate administered preferably still allows the desired degree of destruction tumor cells.

25

In a preferred embodiment a ratio of unconjugated targeting agent to immunoconjugate is chosen so that the sensitivity of the total accessible non-target cells is decreased to clinically acceptable levels so that the percentile of viable accessible non-target cells compared to the same cell not treated with an unconjugated targeting agent is increased, e.g., more than 10%, 20%, 30%, 40%, 50%, 60%, up to 100%, while the sensitivity of the target, e.g., tumor cells is only decreased by preferably few percentile points, such as 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9% or 10%, 15%, 20%, preferably 0 to 10%, even more preferably, 0 to 5%. The **effective ratio** of unconjugated targeting agent to immunoconjugate is thus a ratio that decreases the sensitivity of accessible total non-tumor/non-target cells (and thus increase the % of viable non-tumor cells) to a clinically acceptable level, while retaining the sensitivity of

the tumor cells (and thus maintain or only slightly increase the % of viable tumor cells) at a clinically desirable level.

While the acceptability of non-target cell destruction and/or target cell survival 5 and thus the ratios of unconjugated targeting agent to immunoconjugate might vary from patient to patient (depending, e.g., on the overall condition or disease state of the patient) and the form and locus of administration (intravenous vs. contained injection into, e.g., a particular organ), the following ratio of unconjugated targeting agent to immunoconjugate are in one embodiment of the present invention preferred: about 1:2 to 10 about 10:1, more preferably about 1:1 to about 8:1, even more preferably about 1:1 to about 5:1 and in particular about 2:1, about 3:1, about 4:1. In one embodiment of the present invention, administration is started at high ratios of unconjugated targeting agent/immunoconjugate, which is successively reduced. For example, in a first 15 treatment a ratio of about 20:1 is used allowing full protection of non-target cells and is subsequently lowered to about 10:1. If no adverse side effects are observed, the ratios are lowered to about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1, about 2:1 and/or about 1:1 and, in certain embodiments, about 1:2. In certain 20 embodiments of the present invention, pretreatment with, e.g., a vasodilatory agent allows the reduction of the ratio to about 1:1, about 1:2, about 1:3, about 1:4 or about 1:5. The person skilled in the art, however, will be readily able to adjust the ratios as required for a particular situation and patient.

The improvement in tumor targeting of an immunoconjugate may alternatively or 25 additionally result from sequestration by physical separation of, e.g., sCD138 or entire cells expressing CD138. The later of these methods is in particular of interest in the treatment of late stage cancers, such as multiple myelomas. As a result, the same amount of immunoconjugate administered will bind more tumor cells, since sCD138 or entire cells expressing CD138 are removed from, e.g. blood. This may allow a reduction 30 of the dose of immunoconjugate to be administered to achieve the desired target cell destruction and thus reduce potential or actual side effects of the immunoconjugate.

Plasmapheresis is one way, even though not the only way, of achieving physical separation of, e.g., sCD138. It involves the removal, treatment, and return of (components of) blood plasma from the blood circulation. Plasma is then removed from

the blood by a cell separator via discontinuous or continuous flow centrifugation or plasma filtration. As will be described in more detail below, the method may or may not be employed in conjunction with an unconjugated targeting agent. Preferably, during plasmapheresis a percentile of soluble antigen is removed, e.g., at least about 5%, at 5 least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50% or more are removed from the blood of a patient.

A targeting agent may be attached to a matrix. If a CD138 specific targeting agent is attached to a matrix, this targeting agent is referred to herein as a "CD138-specific adsorber." For example, to deplete sCD138 from a biological fluid, a targeting 10 agent, such as nBT062, may be immobilized on a matrix. Such an immobilization could be based on covalent bounds such as a coupling to aldehyde activated beads or other surfaces by primary amines via "reductive amination", but also on non-covalent bounds such as by binding of nBT062 to protein A or protein G or by binding of, e.g., a biotinylated antibody to a streptavidin or avidin coated matrix. As the person skilled in 15 the art will appreciate, typical matrixes include, but are not limited to, agarose or sepharose beads, polyesters or activated polyester surfaces.

The improved targeting, in particular tumor targeting of the method of the present invention can in certain embodiments at least in part be attributed to the relatively high 20 level of expression of antigen, such as CD138, on target cells, in particular tumor cells as opposed to non-target/non-tumor cells and/or a faster processing/internalization of, e.g., CD138 by tumor cells as opposed to non-tumor cells. Tassone et al. (2004) reports a CD138 expression on the mRNA level of primary positive multiple myeloma (MM) cells that is 50 to 200 times higher than on plasma cells. A FACS analysis revealed an about 25 six times higher CD138 amount on the surface of MM cells relative to plasma cells. However, as the person skilled in the art will appreciate, a number of methods may be employed to determine the relative expression of an antigen such as CD 138 on tumor 30 cells relative to non-tumor cells, which include the methods described above, but also, e.g., a direct comparison of the protein levels or the QIFIKIT method further described below. Any increased level of expression (e.g., 2, 3, 4, 5, 6, 7, 8 or 9 fold when measured by FACS or the QIFIKIT method) that the person skilled in the art would understand to constitute a basis for differentiating between tumor and non-tumor cells is within the scope of the present invention. Thus, a specific dosage of, e.g., unconjugated targeting agent may bind to a relative higher percentile of CD138 expressed at non-

tumor cells than CD138 expressed at tumor cells. By providing a certain dosage of unconjugated targeting agent, for example, on average, about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90% or even more of CD138 expressed on non-tumor cells is bound by the unconjugated targeting agent, while a lower percentile, such as about 5%, about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80% or more of CD138 expressed on tumor cells is bound. As the person skilled in the art will appreciate, different percentile combinations are within the scope of the present invention. For example, at a certain dosage of unconjugated targeting agent, on average, about 50% of CD138 expressed on non-tumor cells is bound by the unconjugated targeting agent, while due to higher expression levels and/or reduced accessibility (see following discussion), on average, only about 30% of CD138 expressed on tumor cells is bound by said unconjugated targeting agent. The relative higher level of binding will effectively shield a significant amount of non-tumor cells (e.g., about 80%, about 70%, about 60%, about 50%, about 40%, about 30%, about 20% about 10% or about 5%) from destruction by an immunoconjugate, while a substantial portion, but preferably substantially all, tumor cells can still be, e.g., subject to apoptosis by the immunoconjugate.

In Fig. 1B expression of CD138 on NCI-H929 (CD138<sup>high</sup>) and MOLP-2 (CD138<sup>low</sup>) model cells is shown. As can be seen the amount of CD138 receptors expressed on the surfaces of NCI-H929 cells relative to that expressed on MOLP-2 cells as measured by QIFIKIT generally correlates to the relative amount found on multiple myeloma cells relative to plasma cells, that is, tumor cells express significantly higher levels of CD138 than non-transformed (non-tumor) cells.

Fig. 1B also details the results of the cell viability assays described in the experimental section. The sensitivity of CD138<sup>high</sup> cells towards the CD138 specific immunoconjugate nBT062-SPDP-DM4 (expressed in terms of the IC<sub>50</sub> (nM)) is higher than the sensitivity of CD138<sup>low</sup> cells towards this immunoconjugate. On the other hand, the sensitivity of CD138<sup>low</sup> (MOLP-2) cells towards free DM4 is higher than that of the CD138<sup>high</sup> (NCI-H929) cells, with the sensitivity of the CD138<sup>high</sup> cells towards the free DM4 toxin (also expressed in terms of the IC<sub>50</sub> (nM)) almost equalling their sensitivity towards the immunoconjugate.

This forces the conclusion that the differences in the sensitivities CD138<sup>high</sup> and CD138<sup>low</sup> cells against the immunoconjugate are a specific property of the

immunoconjugate and not just a reflection of reduced sensitivity of MOLP-2 cells against the effector molecule (here: DM4) of the immunoconjugate. The  $IC_{50}$  values for both cell lines,  $CD138^{high}$  and  $CD138^{low}$ , as provided in Figure 1B, were calculated based on the dose response curves shown in Figure 2A and 3A.

5

The impact of preincubation of  $CD138^{high}$  and  $CD138^{low}$  cells with an unconjugated targeting agent, here nBT062, was shown by the cell viability assays described in the experimental section. The fraction of viable cells was calculated in reference to untreated cells (set to 100% viable cells). Figure 1C show the impact of incubation of 10 unconjugated targeting agent (nBT062) by itself on MOLP-2 ( $CD138^{low}$ ) and NCI-H929 ( $CD138^{high}$ ) cells. The Figure makes clear that the unconjugated targeting agent used does not have an cytotoxic effect on either cell line.

Figure 2A+B (NCI-H929 cells) and Figure 3A+B (MOLP-2 cells) show the 15 results of the different assays. Incubation, here preincubation for 30 min., of the cells with nBT062 at increasing concentrations reveals a protective effect for both cell lines. This shows that the unconjugated targeting agent, here nBT062, shields cell bound CD138, resulting in the decreased sensitivity of both cell lines against treatment with BT062. Increased concentrations of nBT062 thus led to an increase of  $IC_{50}$  values for 20 subsequently administered conjugate nBT062-SPDB-DM4 for both cell lines, that is, to a decrease of sensitivity towards this immunoconjugate. However and importantly, at equivalent concentrations of nBT062, the sensitivity of  $CD138^{low}$  cells against subsequent nBT062-SPDB-DM4 treatment at a give concentration was significantly 25 more reduced than the sensitivity of the  $CD138^{high}$  cells. In Figure 4, the  $IC_{50}$  values for BT062 for both cell lines are plotted against the nBT062 concentration that was preadministered. As can be seen, at certain concentration ranges of nBT062 preadministered, for the MOLP-2 ( $CD138^{low}$ ) cells, the nBT062 provides an effective shield against treatment with BT062, i.e., the  $IC_{50}$  increases considerably, while in the 30 same concentration ranges of nBT062, the NCI-H929 ( $CD138^{high}$ ) remain highly sensitive to BT062. This concentration range of unconjugated targeting agent is of interest in the context of the present invention, as it represents the concentration range at which non-target cells are well protected, while target cells can still be effectively diminished.

As discussed above, to quantify the nBT062 mediated sensitivity reduction against the immunoconjugate, the degree of shielding (or more generically "sequestration") of both cell lines at a given concentration of nBT062 can be calculated as described above.

5 IC<sub>50</sub> values as well as the sequestration values for both cell lines are provided in Table 1. The relative sequestration was calculated as described above. Values higher than 1 indicate that the shielding of CD138<sup>low</sup> cells is higher than that of CD138<sup>high</sup> cells for a given concentration of nBT062 (Table 1).

10 Table 1: IC<sub>50</sub> values of MOLP-2 and NCI-H929 cells in the absence or presence of different nBT062 concentrations. The reduction of sensitivity (increase of IC<sub>50</sub> value) calculated based on the formula below is given as % sequestration. The relative sequestration is calculated based on the protection values obtained on the individual cell lines for each nBT062 concentration (formula given below).

nBT062 conc. (nM)	Sensitivity				Relative sequestration <sup>(2)</sup>	
	MOLP-2; CD138 <sup>low</sup>		NCI-H929; CD138 <sup>high</sup>			
	IC50 (nM)	sequestration <sup>(1)</sup>	IC50 (nM)	sequestration <sup>(1)</sup>		
--	0.398	--	0.146	--	--	
0.39	0.739	86%	0.158	8%	10.8	
0.78	2.522	534%	0.166	14%	38.1	
1.56	2.879	623%	0.232	59%	10.6	
3.125	3.283	725%	0.325	123%	5.9	

15 <sup>(1)</sup>sequestration=100\*(IC50 value with nBT062/IC50 value without nBT062)-100

<sup>(2)</sup>relative sequestration=sequestration CD138<sup>low</sup>/sequestration CD138<sup>high</sup>

20 The results show that at certain concentrations of an unconjugated targeting agent, the relative sequestration is in fact significantly higher than 1. In the context of the present invention relative sequestration values of equal to or more than 10, equal to or more than 20, equal to or more than 30, equal to or more than 40 or equal to or more than 50 are preferred.

25 In Table 2, the viability of CD138<sup>low</sup> and CD138<sup>high</sup> cells after pre-treatment with an unconjugated targeting agent is shown relative to control cells that grew in the same concentration of unconjugated targeting agent. Both cell lines were treated with 0.4 or 0.8 nM of nBT062-SPDB-DM4 and either not pretreated (first row) or pretreated with increasing concentrations of nBT062. For example, at a concentration of 0.4 nM 30 immunoconjugate, pre-treatment with 0.78nM unconjugated targeting agent raised the viability of the model non-target cells from 41 to 99%, while the viability of the model target cells was only slightly increased from 6 to 10%. Thus, at a ratio of unconjugated

targeting agent to immunoconjugate of 2:1, the goal of finding a ratio of unconjugated targeting agent to immunoconjugate that allows for a survival of the high percentile of CD138<sup>low</sup> (non target cells) and a low survival of CD138<sup>high</sup> cells (target cells) was almost optimally achieved. Thus, the data of Table 2 shows that by incubating the cells with 5 certain concentrations of unconjugated targeting agent, CD138<sup>low</sup> (non-target cells) can be almost completely shielded from any effect of the immunoconjugate, that is, become close to or completely insensitive to subsequent immunoconjugate treatment, while the efficacy of BT062 against target cells is only slightly reduced.

10 **Table 2:** The percentages of viable cells (% of untreated control) for the individual cell lines are given as examples for given concentrations of BT062 and nBT062.

BT062 conc. (nM)	nBT062 conc. (nM)	MOLP-2	NCI-H929	nBT062/BT062 ratio
		Viable cells (% control)	Viable cells (% control)	
0.4	--	41	6	--
0.4	3.125	90	23	7.8
0.4	1.56	71	9	3.9
0.4	0.78	99	10	2.0
0.4	0.39	71	7	1.0
0.8	--	37	5	--
0.8	6.25	100	27	7.8
0.8	3.125	87	9	3.9
0.8	1.56	76	8	2.0
0.8	0.78	64	7	1.0
0.8	0.39	46	6	0.5

15 The acceptability of non-target cell destruction and/or target cell survival and thus the ratios of unconjugated targeting agent to immunoconjugate might vary from condition to condition, patient to patient (depending, e.g., on the overall condition or disease state of the patient) and the form and locus of administration (intravenous vs. contained injection into, e.g., a particular organ).

20 Accessibility of cells differs in place (locus of administration) and time (status of target cell treatment). Thus, differing accessibility of CD138 expressing non-tumor cells and CD138 expressing tumor cells, will, in certain embodiments, also contribute to results achieved with the method and composition of the present invention. For example, nBT062 may bind soluble CD138 and/or CD138 expressed on, at least certain 25 types of, non-tumor cells, e.g., on cells floating in blood, more readily than CD138

expressed on tumor cells. At least a part of the later may, in certain embodiments of the invention, be relatively inaccessible to, e.g., nBT062. In certain embodiments, such relatively inaccessible CD138 will become more accessible during the course of the treatment as immunoconjugates cause apoptosis in cells. Subsequent to apoptosis the 5 dead cells are removed, e.g. by phagocytosis, and access to previously inaccessible or hard to access tumor cells is provided.

“Sequestering” a substance, such as soluble or cell bound CD138, or a cell in the context of the present invention refers (i) to a binding of a substance or cell which 10 diminishes or prevents further binding, in particular by immunoconjugates or (ii) to a physical separation of the substance or cell. A binding by, e.g., an unconjugated targeting agent to an individual CD138 antigen may reduce the affinity of the immunoconjugate to the individual CD138 antigen and may even render the individual CD138 unavailable for binding by an immunoconjugate targeting CD138. A reduction in 15 affinity may, e.g., be the result of employing an unconjugated targeting agent that differs from the targeting agent of the immunoconjugate. For example, the unconjugated targeting agent may be a physiological CD138 ligand such as ADAMTS4 and the targeting agent of the immunoconjugate may be nBT062.

Thus, binding by, e.g., an unconjugated targeting agent may decrease non-tumor 20 cell binding of the immunoconjugate. This will improve tumor targeting by protecting non-tumor cells from binding of an effective amount of immunoconjugate (also referred to herein “shielding” or “masking” of non-tumor cells) and thus decrease undesirable side effects associated with the administration of the immunoconjugates. The improved tumor targeting may be reflected by an increase in the effectiveness of a certain dosage 25 of the immunoconjugate or alternatively, by the fact that a lower dosage can be employed to obtain an equivalent effect.

As discussed above, sequestering can also be solely based on a physical separation. For example, during plasmapheresis, plasma is separated from blood cells. Soluble antigen (e.g., sCD138) will be part of the plasma. At least part of the plasma 30 may be replaced by a plasma expander, thus reducing the overall amount of soluble CD138 present. This will reduce the amount of non-tumor bound CD138 antigen available for binding by the immunoconjugate. As a result, e.g., the same dosage of immunoconjugate will result in a higher degree of binding to cell bound CD138 including

a higher degree of binding to tumor cell bound CD138, than obtained without the sequestration by physical separation.

It is understood that a combination of sequestering based on binding and physical separation is also possible. For example, the sCD138 contained in plasma which is separated from blood cells during plasmapheresis may be bound, e.g., by the targeting agent, such as a CD138 specific adsorber and the plasma may be reintroduced into the patient. As outlined above, the present invention distinguishes between targeting agents that are functionally attached to an effector molecule (as part of an immunoconjugate) and unconjugated targeting agent. In the case of, e.g., a CD138 specific adsorber, the unconjugated targeting agent is attached to or associated with non-effector molecule(s) such as a matrix.

The unconjugated targeting agent and immunoconjugates according to the present invention can be administered by any route, including intravenously, parenterally, orally, intramuscularly, intrathecally or as an aerosol. The mode of delivery will depend on the desired effect. A skilled artisan will readily know the best route of administration for a particular treatment in accordance with the present invention. The appropriate dosage will depend on the route of administration and the treatment indicated, and can readily be determined by a skilled artisan in view of current treatment protocols.

Pharmaceutical compositions containing an unconjugated targeting agent and the immunoconjugate of the present invention as active ingredients can be prepared according to conventional pharmaceutical compounding techniques. See, for example, Remington's Pharmaceutical Sciences, 17th Ed. (1985, Mack Publishing Co., Easton, Pa.). Typically, effective amounts of active ingredients will be admixed with a pharmaceutically acceptable carrier. The carrier may take a wide variety of forms depending on the form of preparation desired for administration, for example, intravenous, oral, parenteral, intrathecal, transdermal, or by aerosol.

For oral administration, the unconjugated targeting agent and/or immunoconjugate can be formulated into solid or liquid preparations such as capsules, pills, tablets, lozenges, melts, powders, suspensions or emulsions. In preparing the compositions in oral dosage form, any of the usual pharmaceutical media may be employed, such as, for example, water, glycals, oils, alcohols, flavoring agents,

preservatives, coloring agents, suspending agents, and the like in the case of oral liquid preparations (such as, for example, suspensions, elixirs and solutions); or carriers such as starches, sugars, diluents, granulating agents, lubricants, binders, disintegrating agents and the like in the case of oral solid preparations (such as, for example, powders, 5 capsules and tablets). Because of their ease in administration, tablets and capsules represent the most advantageous oral dosage unit form, in which case solid pharmaceutical carriers are obviously employed. If desired, tablets may be sugar-coated or enteric-coated by standard techniques. The active agent must be stable to passage through the gastrointestinal tract. If necessary, suitable agents for stable 10 passage can be used, and may include phospholipids or lecithin derivatives described in the literature, as well as liposomes, microparticles (including microspheres and macrospheres).

For parenteral administration, the unconjugated targeting agent and/or the immunoconjugate may be dissolved in a pharmaceutical carrier and administered as 15 either a solution or a suspension. Illustrative of suitable carriers are water, saline, phosphate buffer solution (PBS), dextrose solutions, fructose solutions, ethanol, or oils of animal, vegetative or synthetic origin. The carrier may also contain other ingredients, for example, preservatives, suspending agents, solubilizing agents, buffers and the like. When the unconjugated targeting agent and/or immunoconjugate are being administered 20 intracerebroventricularly or intrathecally, they may also be dissolved in cerebrospinal fluid.

In accordance with the present invention, MM is treated as follows, with the use of nBT062 and BT062 as an example. This example is not intended to limit the present invention in any manner, and a skilled artisan could readily determine other 25 unconjugated targeting agent and/or immunoconjugates that are within the scope of the present invention and other treatment regimes which could be utilized for the treatment of diseases such as MM. The two components are administered to a patient in need thereof at a ratio of 3:1 (nBT062/BT062) concomitantly. Due to the binding of nBT062 to tumor and non-tumor cells, the toxicity of BT062 towards non-tumor cells is reduced. 30 In particular, the viability of the non-tumor cells is, at the specified amount of BT062 employed, increased by 40%, namely from 40% to 80%. Due to different factors such as a high expression of CD138 on patients' MM cells and/or the relative high turnover of CD138 on those cells, BT062 can bind, even after administration of nBT062, to tumor cells in an effective amount that will cause destruction of the tumor cells. In particular,

the viability of the tumor cells is only increased by a couple of percentage points, namely from 6% to 9%. Thus, the unconjugated targeting agent and the immunoconjugate of embodiments of this invention provide a means for the effective administration of the effector molecules to tumor cell sites where the effector molecules 5 can be released from the immunoconjugate with clinically acceptable side effects. This targeted delivery and release provides a significant advance in the treatment of multiple myeloma.

The present invention is further described by reference to the following Examples, which are offered by way of illustration and are not intended to limit the 10 invention in any manner. Standard techniques well known in the art or the techniques specifically described below are utilized.

## EXPERIMENTAL SECTION: MATERIAL AND METHODS

### *Cell Lines*

15 *MOLP-2* is a human multiple myeloma cell line (DSMZ No.: ACC 607), which expresses CD138 in relatively low amounts (see details below and Fig. 1b). This cell line is also highly sensitive towards, e.g., the toxin DM4, indicating that its internal mechanisms to resist drug treatment are, if at all, underdeveloped. These facts underline that this cell line is an excellent model for non-target cells, in particular non-target CD138<sup>low</sup> cells.

20 *NCI-H929* is a human multiple myeloma cell line (DSMZ No.: ACC 163), which expresses CD138 in, compared to *MOLP-2* cells, relatively high amounts. (see details below and Fig. 1b). This cell line has also a high degree of resistance against free effector molecule, e.g., the toxin DM4, indicating that its internal mechanisms to resist 25 drug treatment are, as in other tumor cells, well developed. These facts make this cell line an excellent model for tumor cells, in particular target CD138<sup>high</sup> cells as, e.g. found in multiple myeloma.

### *FACS analysis for the relative determination of CD138 expression*

30  $1 \times 10^5$  *MOLP-2* and *NCI-H929* multiple myeloma cells were each separately incubated with nBT062 to detect CD138 molecules on their cell surface. The cells were washed twice after addition of nBT062 antibody diluted in 100  $\mu$ l PBS for 30 min at room temperature. Antibody concentrations used in this assay were 125-0.98 ng/ml. Bound antibody was detected upon subsequent incubation of the cells with 1/50 diluted FITC-

conjugated goat anti-human IgG (Immunotech, Inc.) in PBS using a FACS Calibre flow cytometer (Beckton Dickinson). The FITC fluorescence was measured in the FL-1 channel. The mean fluorescence of untreated cells, isotype control treated cells and cells treated with the secondary antibody only were subtracted from the obtained values (mean value of controls). All determinations were performed in duplicate.

5 The data for the determination of CD138 expression on the surface of NCI-H929 and MOLP-2 multiple myeloma cell lines by FACS analysis via unconjugated antibody nBT062 binding is shown in the histogram plots of Fig. 1A.

10 *Determination of absolute CD138 receptor numbers via QIFIKIT*

The QIFIKIT assay (DAKO USA) allows for the quantification of cell surface antigens by flow cytometry using an indirect immunofluorescence assay. This method was used to determine absolute receptor numbers of CD138 on the cell surface of MOLP-2 and NCI-H929 multiple myeloma cells. QIFIKIT consists of a series of 6 bead populations,

15 approximately 10 µm in diameter and coated with different, but well-defined quantities of a mouse monoclonal antibody. The number of monoclonal antibody molecules on the 6 bead populations ranges from 0 to 400 000-800 000 and are provided with the kit. The beads mimic cells labeled with a specific primary mouse monoclonal antibody.

Specimen cells were labeled with primary mouse Mab at saturating concentration. Under 20 this condition the primary Mab is expected to bind to the respective cell surface antigen monovalently. Therefore, the number of bound antibody molecules corresponds to the number of antigenic sites. The cells are incubated at saturating concentration, in parallel with the QIFIKIT beads, with Polyclonal Goat Anti-Mouse Immunoglobulins/FITC, Goat F(ab')<sub>2</sub>.

25 A calibration curve was constructed by plotting the fluorescence intensity of the individual bead populations against the number of Mab molecules on the beads. The number of antigenic sites on the specimen cells were then determined by interpolation.

Cells (1x10<sup>6</sup> cells/mL) in 50 µl/well were incubated for 45 min at 4°C with different 30 concentrations of nBT062 (125-0.98 ng/mL). Set up and calibration beads were prepared according to the manufacturer's recommendation. The wells were washed twice with 100 µl PBS. Bound nBT062 was detected using 100 µl FITC-conjugated secondary antibody (diluted 1:50 in PBS, incubation time 45 min, 4°C in the dark).

Samples were analyzed by flow cytometry according to the manufacturer's recommendation.

The absolute receptor numbers of CD138 were quantified using the Qifikit is shown in Fig. 1B.

5

#### *Cell viability assay*

To assess the impact of preincubation of CD138<sup>high</sup> and CD138<sup>low</sup> cells with an unconjugated targeting agent, cell viability assays were performed.

10 NCI-H929 and MOLP-2 multiple myeloma cell lines were cultured in microtiter plates at a density of about 5000 cells/well. The cells were preincubated with unconjugated nBT062 at different concentrations for 30 min. Subsequently, cultures are treated with increasing concentrations of BT062. The cultures were incubated for 5 days before 10 µl of the tetrazolium salt containing WST-1 reagent (Roche) was added, followed by further incubation for 1-3 h to measure the cell viability. Color development due to formation of 15 the formazan metabolite was quantified by determining the absorbance at 450 nm versus 690 nm (reference wavelength) in a microplate reader. Wells containing medium and WST-1 reagent served as blank control. The percentage of viable cells was calculated from the data obtained relative to data of cells grown in normal growth medium (set to 100%). In control experiments, cells were treated under the same 20 conditions with equimolar concentrations of free toxin or with unconjugated nBT062 and cell viability was measured as described above.

The above results are confirmed by the following co-culture experiments

#### *Cell viability in co-cultures*

25 Cytotoxicity of BT062 towards CD138 expressing cells is measured by flow cytometry. About 2x10<sup>6</sup> cells expressing low levels of CD138 (non-target cells, CD138<sup>low</sup>) are incubated for 5 min at room temperature (RT) with the fluorescent dye PKH67-GL (Sigma, Deisenhofen, Germany) according to the manufacturer's instructions. Incorporation of the dye is stopped by addition of heat-inactivated FBS. Then cells are 30 washed twice with 15 ml of PBS.

Labelled cells are seeded together with unlabelled cells expressing high levels of CD138 ("target cells", CD138<sup>high</sup>) in 96 well microtiter plates and incubated overnight at 37°C.

The cells are preincubated with different concentrations of unconjugated nBT062 in order to block a fraction of binding sites. In control samples, nBT062 is omitted.

Subsequently, the cocultures are treated with increasing concentrations of BT062. After

an additional incubation for 24-120 h, the cultures are transferred to polypropylene

5 tubes. Cells are centrifuged for 5 min at 500·g, supernatant is removed and 200 µl/tube

of a propidium iodide (PI) solution (1 µg/ml in PBS) is added. After incubation for 5-10

minutes at room temperature, fluorescence is determined by FACS analysis. The

fraction of viable cells in reference to untreated cells is calculated using the

CellQuestPro software (BD Biosciences). Viable CD138<sup>low</sup> cells are determined as

10 PKH67-GL positive and PI negative. Viable CD138<sup>high</sup> cells (target cells) are determined

as PKH67-GL negative and PI negative. Number of spontaneously lysed cells,

measured in cultures that are treated identically but in the absence of BT062, are added.

Alternatively, in another set of experiments, CD138<sup>high</sup> are labelled with PKH67-GL and

coculturing is performed with unlabelled CD138<sup>low</sup> cells.

15

*Decreasing sensitivity of CD138<sup>low</sup> non-target cells towards BT062 via nBT062 preincubation*

The effect of preincubating the cells with unconjugated nBT062 on the viability of

CD138<sup>high</sup> and CD138<sup>low</sup> cells is analyzed in coculture assays. CD138<sup>low</sup> cells are

20 labelled with the fluorescent dye PKH67-GL before they are cocultured with unlabeled

CD138<sup>high</sup> cells in order to allow differentiation of target and non-target cells in

subsequent cell viability assays. Alternatively, CD138<sup>high</sup> cells are labelled with the same

dye. Cocultures are treated with different concentrations of unconjugated nBT062 in

order to partially block binding sites. Subsequently, BT062 is added to the cells and cell

25 viability is analyzed by PI-staining and subsequent FACS analysis.

The viability of non-target cells in cocultures after treatment with BT062 is increased by

preincubation with the unconjugated antibody nBT062. Importantly, the efficacy of

BT062 against target cells is only slightly reduced. This result further demonstrates that

the cytotoxicity of BT062 towards CD138<sup>low</sup> non-target cells can be reduced by

30 preincubation of the cells with nBT062, whereby the cytotoxicity of BT062 towards

CD138<sup>high</sup> target cells is maintained.

### SEQUESTRATION OF SOLUBLE CD138

#### *Measurement of soluble CD138 in cell culture supernatants*

5 Levels of soluble CD138 (sCD138) in cell culture supernatants of CD138 expressing cells are measured using a solid phase sandwich ELISA (Human CD138 ELISA kit, Diaclone, Besançon, France) according to the manufacturer's instructions.

#### *Cell viability assay in cultures supplemented with sCD138*

10 Soluble CD138 is purified from cell culture supernatants by antibody affinity chromatography using columns loaded with the anti-CD138 antibody B-B4 following standard procedures.

In order to obtain cell cultures of CD138 positive cells with known concentrations of sCD138 in their culture supernatant, media of freshly seeded cells are supplemented

15 with different concentrations of purified sCD138. Cell culturing is performed in microtiter plates at a density of 5000 cells/well (for suspension cells) or at 900 cells/well (for adherent cells). The cells are preincubated with unconjugated nBT062 in order to block binding sites of sCD138. Subsequently, cultures are treated with increasing concentrations of BT062. The cultures are incubated for 1 to 5 days before 10 µl of the

20 tetrazolium salt containing WST-1 reagent (Roche) is added, followed by further incubation for 1-3 h. Color development due to formation of the formazan metabolite is quantified by determining the absorbance at 450 nm versus 690 nm as reference wavelength in a microplate reader. Wells containing medium and WST-1 reagent serve as blank. The percentage of viable cells is calculated from these data relative to cells

25 grown in normal growth medium (set to 100%).

#### *Increasing cytotoxic activity of BT062 towards CD138<sup>high</sup> target cells by masking soluble CD138 with nBT062*

The cytotoxic activity of BT062 against CD138 expressing multiple myeloma cells is decreased by the presence of soluble CD138 in cell culture supernatants *in vitro*, by the presence of sCD138 in blood or tissues of multiple myeloma patients or in experimental animals *in vivo*. In order to prevent sCD138 to function as a competitor, nBT062 is used in an *in vitro* model system to block binding sites on sCD138. Therefore, CD138 expressing cells are cultured in microtiter plates with conditioned medium that contains

sCD138 or in fresh cell culture medium supplemented with known concentrations of purified sCD138. Unconjugated nBT062 is added to the cultures prior or simultaneously to the addition of BT062. Cell viability is determined in WST-1 cell viability assays. The results show that the activity of BT062 can be enhanced by preincubation of the 5 cells with nBT062, a result of masking of sCD138 binding sites with the unconjugated antibody nBT062.

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**What is claimed is:**

1. A method for improving targeting, in particular tumor targeting, of immunoconjugates targeting cell bound antigen expressed on target cells comprising:

5 (a) providing a medium comprising said antigen, wherein said antigen is cell bound on target cells, non-target cells and/or is present in soluble form;  
(b) sequestering cell bound antigen, soluble antigen and/or antigen expressing cells,  
(c) administering an immunoconjugate targeting cell bound antigen expressed on target cells comprising a targeting agent for said antigen that is functionally attached to an  
10 effector molecule, wherein said sequestering in (b) improves target cell targeting of said immunoconjugate.

2. The method of claim 1, wherein an unconjugated targeting agent sequesters said cell bound antigen, soluble antigen and or said antigen expressing cells.

15 3. The method of claim 1, wherein said antigen internalizes into the cell to which it is bound and/or is subject to shedding.

20 4. The method of claim 1, wherein said non-target cells comprise ill regenerating cells such as epithelial cells, brain, heart, kidney and liver cells.

5. The method of claim 2, wherein said unconjugated targeting agent is a targeting antibody or a physiological ligand.

25 6. The method of claim 2, wherein a relative sequestration of non-target cells to target cells is higher than 5 or higher than 10, preferably higher than 20 most preferably higher than 30.

30 7. The method of claim 2, wherein a ratio of unconjugated targeting agent to immunoconjugate provided is about 10:1 to about 1:2, preferably 5:1 to 2:1.

8. The method of claim 2, wherein a vasodilatory agent, such as prazosin, terazosin or doxazosin, is provided prior to or concomitant with the unconjugated targeting agent.

9. The method of claim 2, wherein an antigen specific adsorber comprises said unconjugated targeting agent and wherein soluble antigen and/or antigen expressing cells are sequestered ex vivo via said antigen specific adsorber.

5 10. The method of claim 1, wherein said medium is blood and said sequestering comprises separating soluble antigen from blood cells during plasmapheresis, wherein said soluble antigen is part of plasma.

11. The method of claim 1, wherein the antigen is CD138, and wherein:

10 - the medium in (a) comprises cell bound CD138 and/or soluble CD138;  
- cell bound CD138, soluble CD138 and/or CD138 expressing cells is sequestered (b);  
and  
- an immunoconjugate targeting cell bound CD138 comprising a targeting agent for  
CD138 that is functionally attached to an effector molecule is administered in (c).

15 12. The method of claim 11, wherein an unconjugated CD138 targeting agent  
sequesters said cell bound CD138, soluble CD138 and/or said CD138 expressing cells.

20 13. The method of claim 12, wherein said unconjugated CD138 targeting agent is an  
unconjugated targeting antibody or a physiological ligand.

14. The method of claim 11, wherein a relative sequestration of CD138 expressing non-target cells to CD138 expressing target cells obtained is higher than 5 or higher than 10, preferably higher than 20 most preferably higher than 30.

25 15. The method of claim 12, wherein a ratio of unconjugated CD138 targeting agent to  
immunoconjugate provided is about 10:1 to about 1:2, preferably 5:1 to 2:1.

30 16. The method of claim 12, wherein a vasodilatory agent, such as prazosin, terazosin or  
doxazosin, is provided prior to or concomitant with the unconjugated CD138 targeting  
agent.

17. The method of claim 12, wherein a CD138 specific adsorber comprises said unconjugated CD138 targeting agent and wherein soluble CD138 and/or CD138 expressing cells are sequestered *ex vivo* via said CD138 specific adsorber.

5 18. The method of claim 11, wherein said medium is blood and said sequestering comprises separating soluble CD138 from blood cells during plasmapheresis, wherein said soluble CD138 is part of plasma.

10 19. The method of claim 11, wherein said medium is blood, plasma, tissue or bone marrow.

20. The method of claim 11 or 15, wherein said immunoconjugate targeting cell bound CD138 is administered concurrently with or subsequent to said unconjugated CD138 targeting agent.

15 21. The method of claim 20, wherein said sequestering in (b) and administering in (c) is separated by a time interval.

22. The method of claim 21, wherein the time interval is at least 20 minutes.

20 23. The method of claim 18, wherein at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80% of soluble CD138 and/or CD138 expressing cells in the blood of a patient are removed during said plasmapheresis.

25 24. The method of claim 11, wherein said targeting agent in (b) and/ or (c) is a targeting antibody.

30 25. The method of claim 24, wherein said targeting antibody is or is derived from antibody B-B4.

26. The method of claim 24, wherein said antibody is nBT062.

27. A pharmaceutical composition comprising:

an immunoconjugate targeting cell bound antigen expressed on target cells comprising a targeting agent for said antigen that is functionally attached to an effector molecule,

an unconjugated targeting agent, wherein said unconjugated targeting agent is not systemically cytotoxic, and

a pharmaceutically acceptable carrier.

28. The pharmaceutical composition of claim 27, wherein said antigen internalizes into the cell to which it is bound.

10

29. The method of claim 27, wherein said unconjugated targeting agent is an unconjugated targeting antibody or a physiological ligand.

15

30. The pharmaceutical composition of claim 27, wherein said unconjugated targeting agent and said immunoconjugate are present at a ratio of about 10:1 to about 1:2, preferably about 5:1 to about 2:1.

31. The pharmaceutical composition of claim 27, wherein the immunoconjugate targets cell bound CD138 and comprises a targeting agent for CD138.

20

32. The method of claim 31, wherein said unconjugated CD138 targeting agent is an unconjugated CD138 targeting antibody or a physiological ligand of CD138.

25

33. The pharmaceutical composition of claim 31, wherein said unconjugated targeting agent and said immunoconjugate are present at a ratio of about 10:1 to about 1:2, preferably about 5:1 to about 2:1.

34. A kit comprising:

in one container, (a) an immunoconjugate targeting cell bound antigen expressed on target cells comprising a targeting agent for said antigen that is functionally attached to an effector molecule;

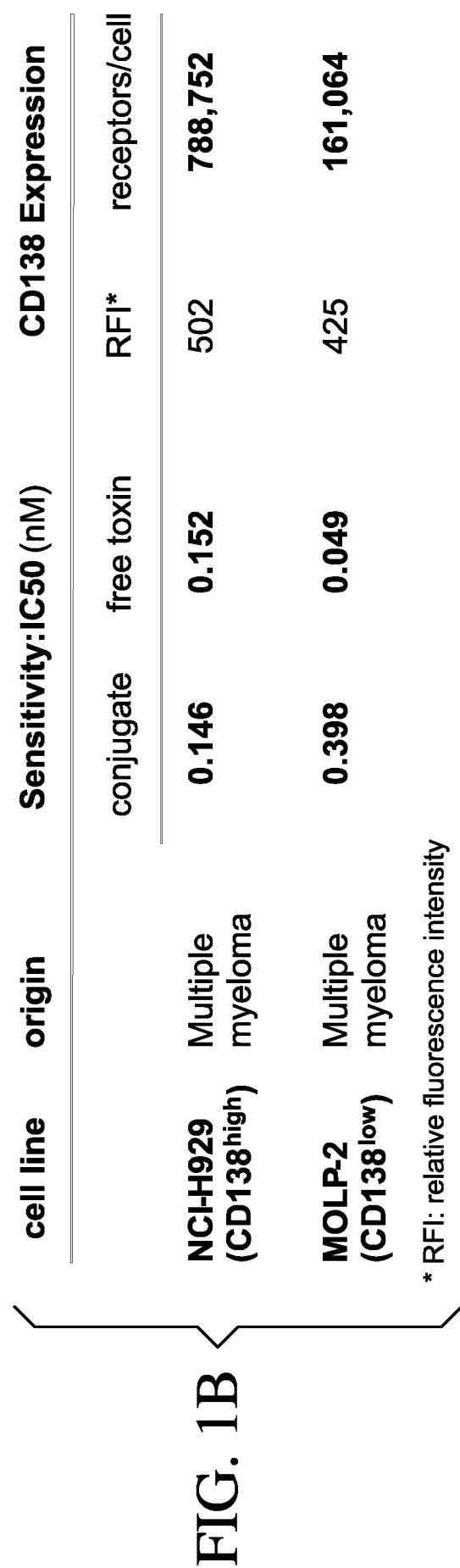
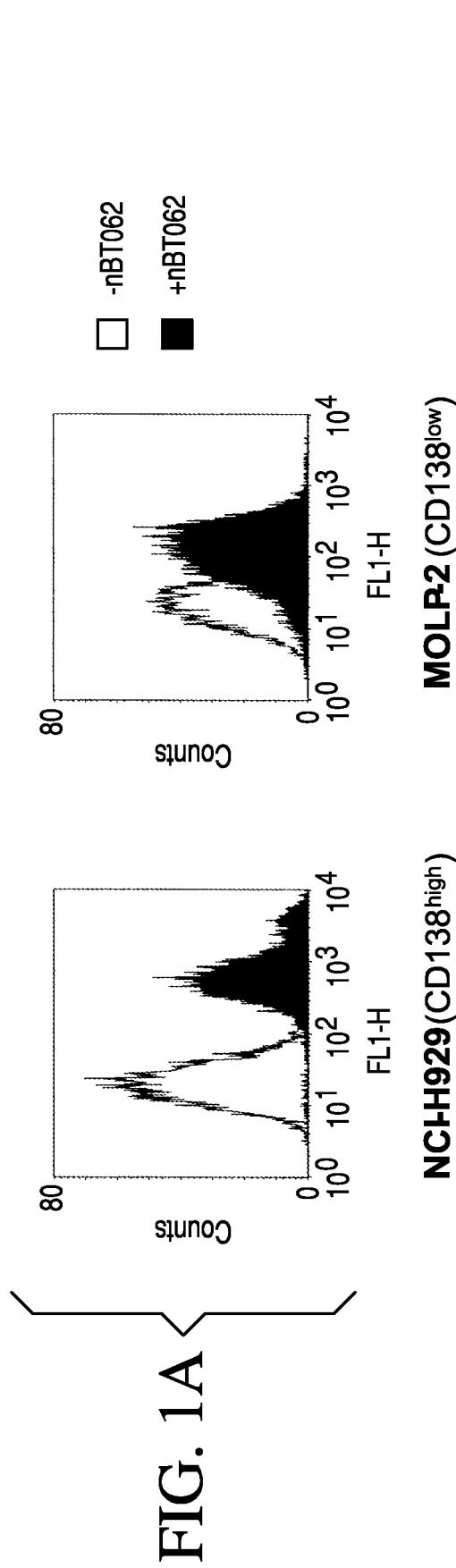
in a separate container, (b) an unconjugated targeting agent, wherein said unconjugated targeting agent is not systemically cytotoxic;

optionally in a third container, (c) a vasodilatory agent, and

optionally, in a fourth container, (d) instructions of how to use (a) and (b) and, optionally (c).

35. The kit of claim 34, wherein the immunoconjugate in container (a) targets cell bound  
5 CD138 and comprises a targeting agent for CD138.

36. The kit of claim 34, wherein the instructions in (d) delineate a treatment regime comprising ratios of said unconjugated targeting agent to said immunoconjugate of about 10:1 to about 1:2, preferably about 5:1 to about 2:1.



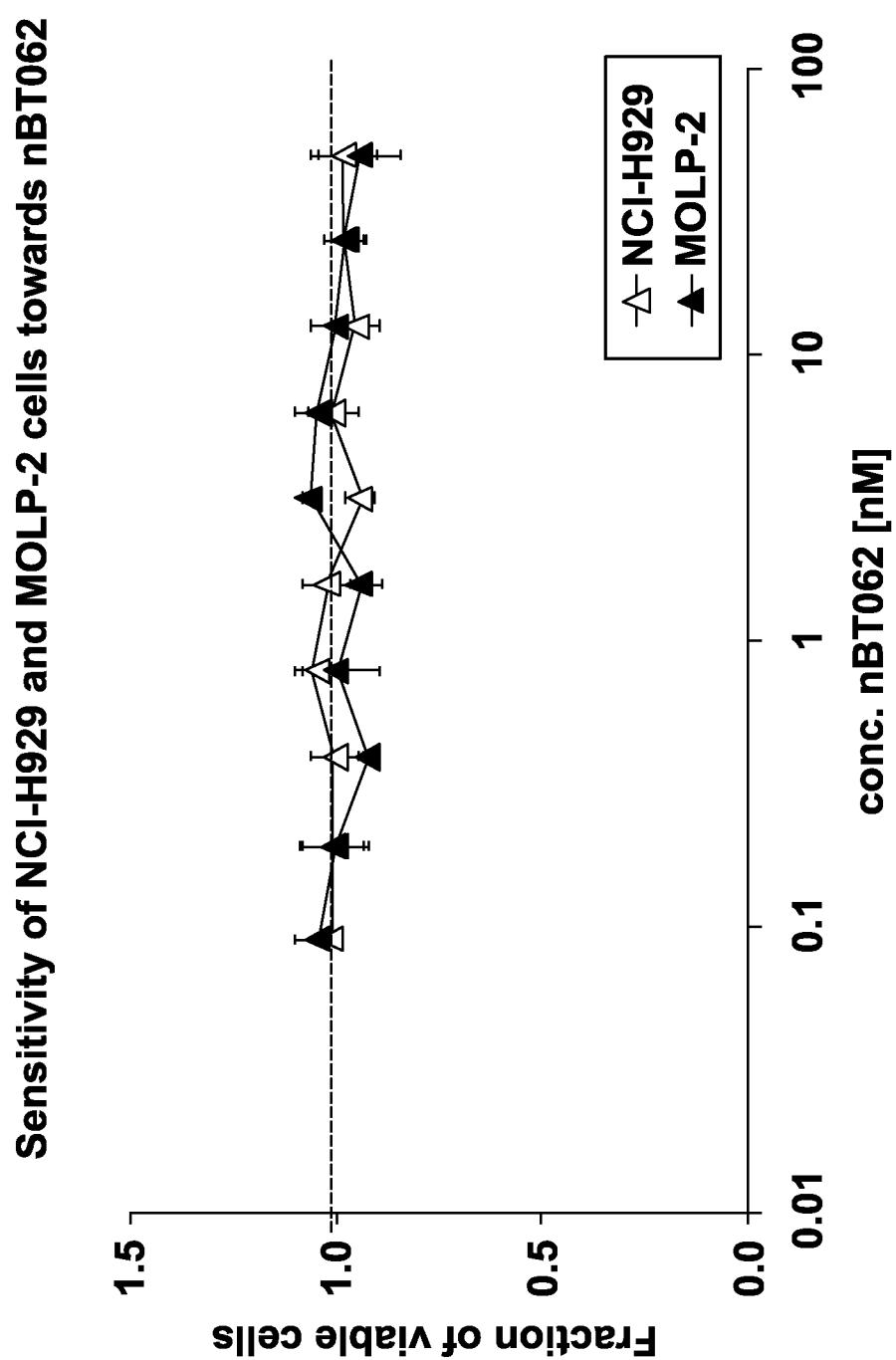
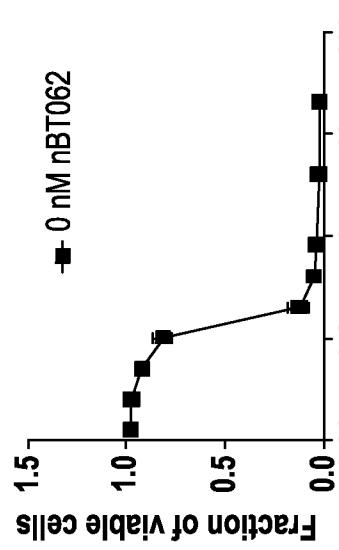
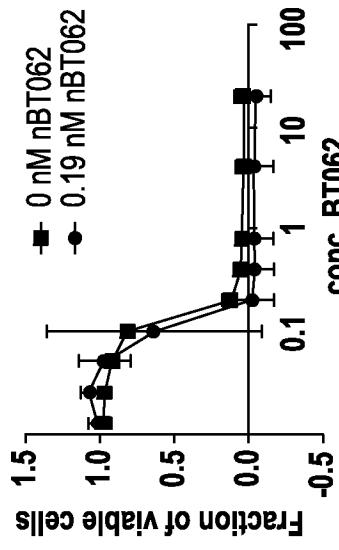


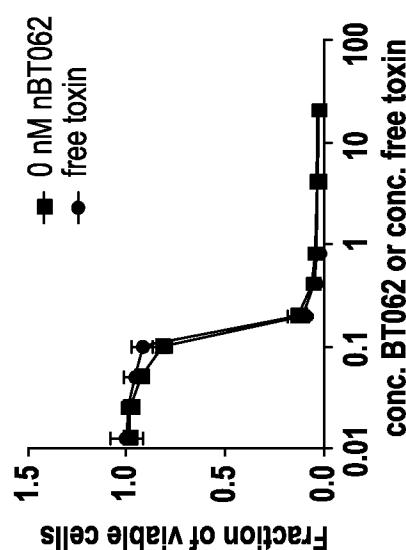
FIG. 1C

**NCI-H929, CD138<sup>high</sup>****FIG. 2A****NCI-H929; 0 nM nBT062**

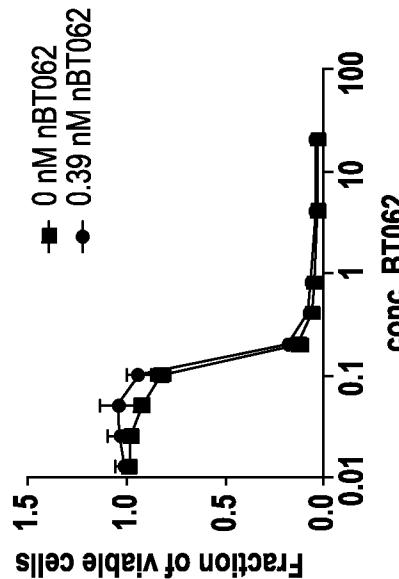
**BT062**  
 $IC_{50} = 0.146 \text{ nM}$

**NCI-H929; 0.19 nM nBT062**

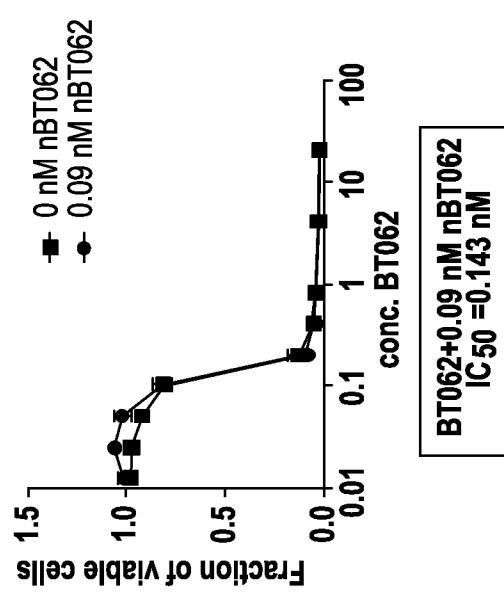
**BT062+0.19 nM nBT062**  
 $IC_{50} = 0.122 \text{ nM}$

**NCI-H929; free toxin**

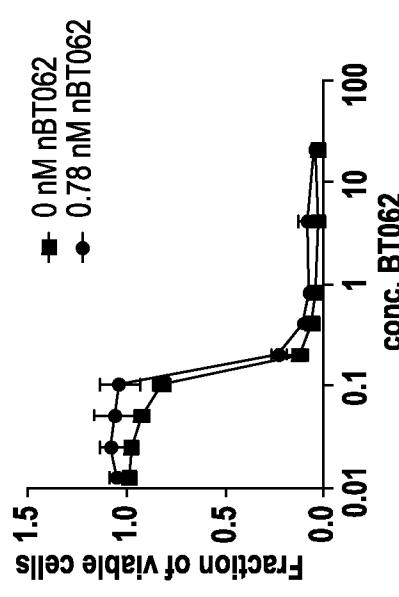
**Free toxin:**  
 $IC_{50} = 0.152 \text{ nM}$

**NCI-H929; 0.39 nM nBT062**

**BT062+0.39 nM nBT062**  
 $IC_{50} = 0.158 \text{ nM}$

**NCI-H929; 0.09 nM nBT062**

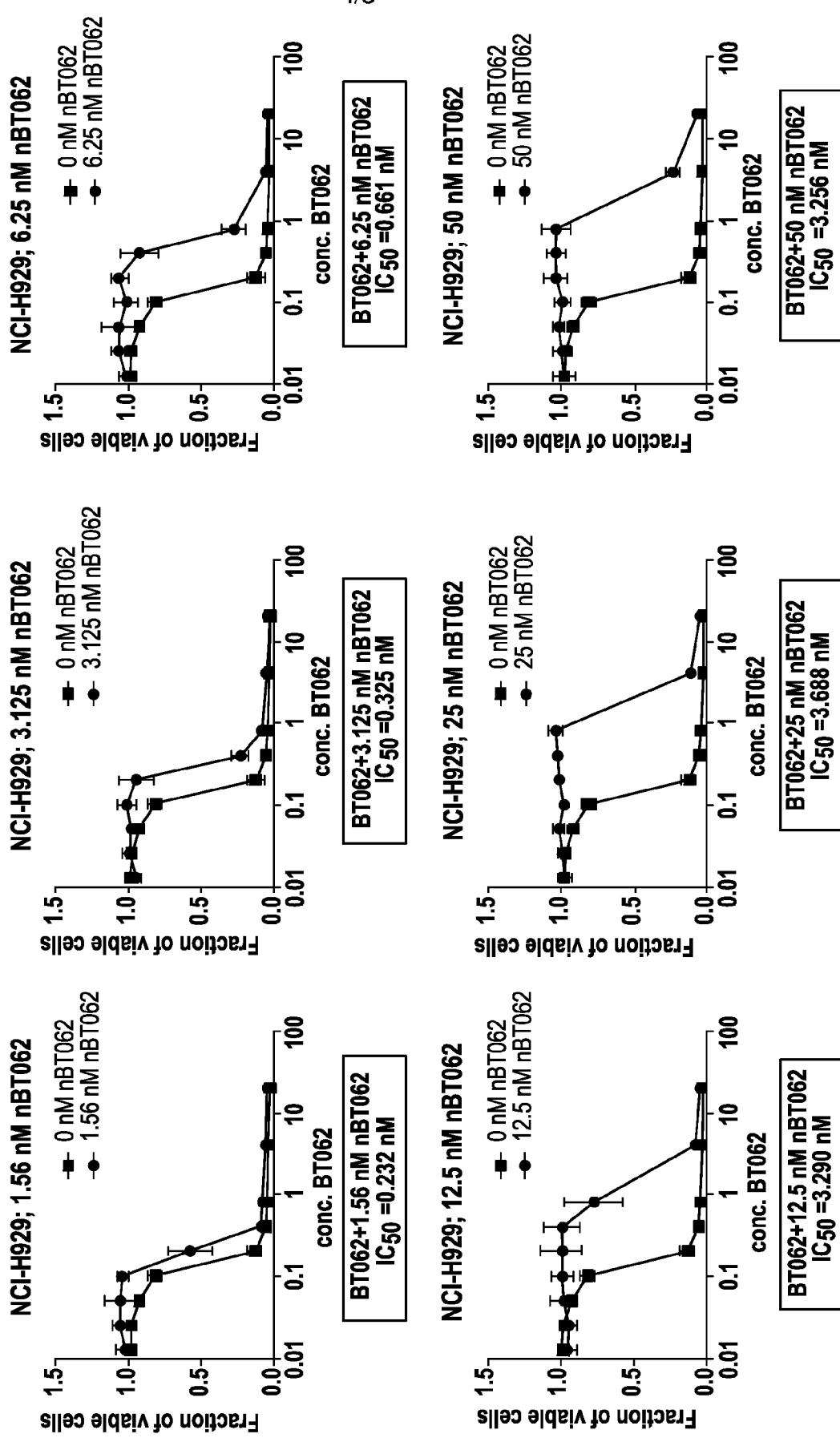
**BT062+0.09 nM nBT062**  
 $IC_{50} = 0.143 \text{ nM}$

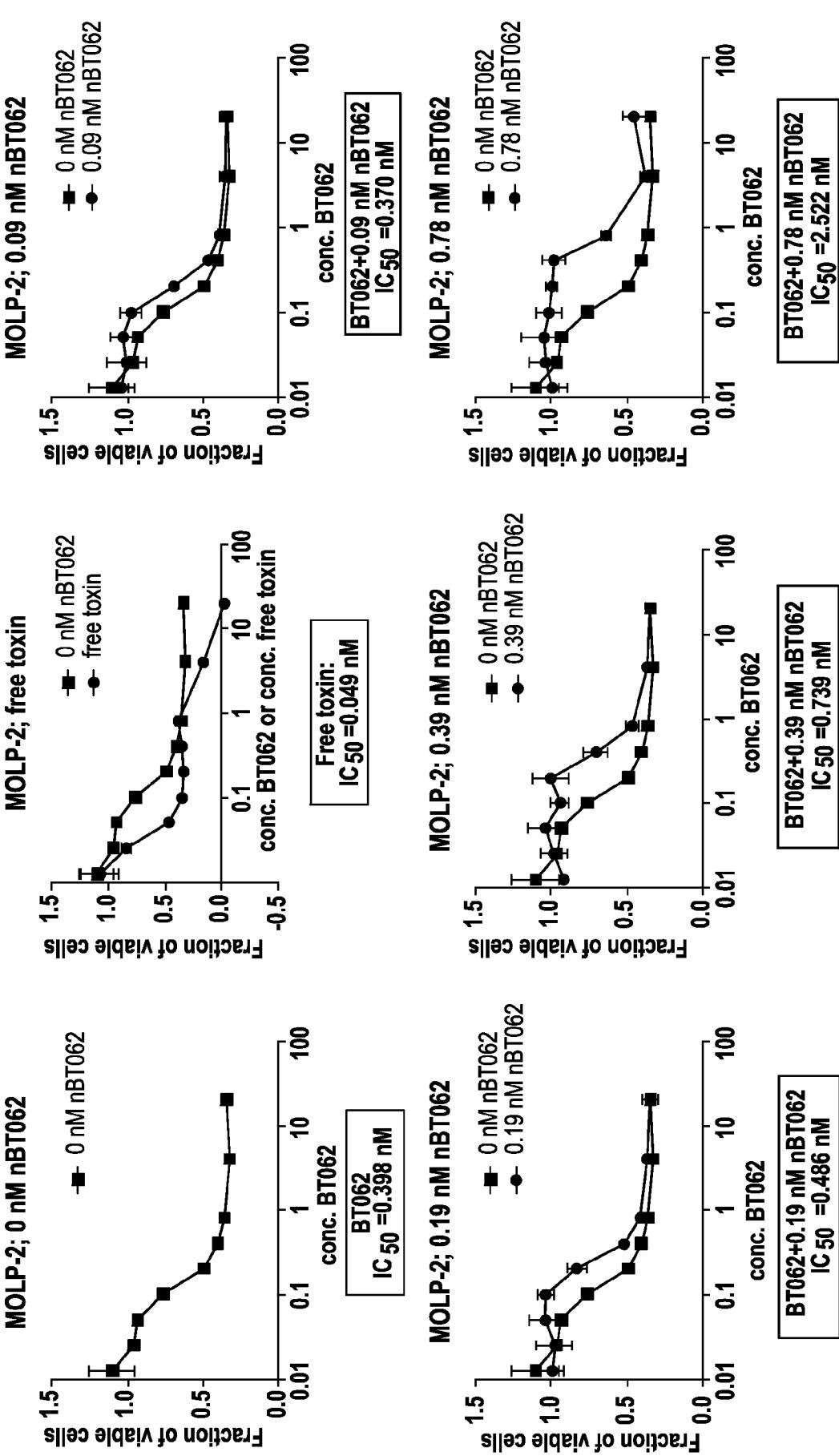
**NCI-H929; 0.78 nM nBT062**

**BT062+0.78 nM nBT062**  
 $IC_{50} = 0.166 \text{ nM}$

## NCI-H929, CD138 high

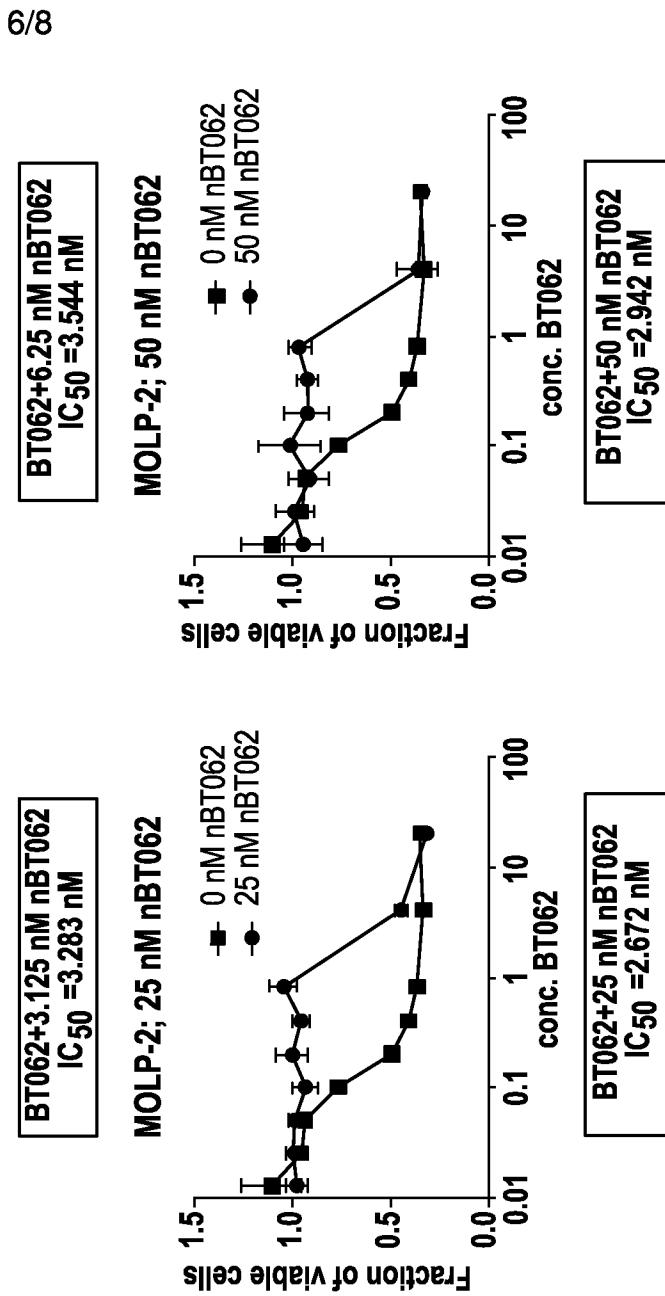
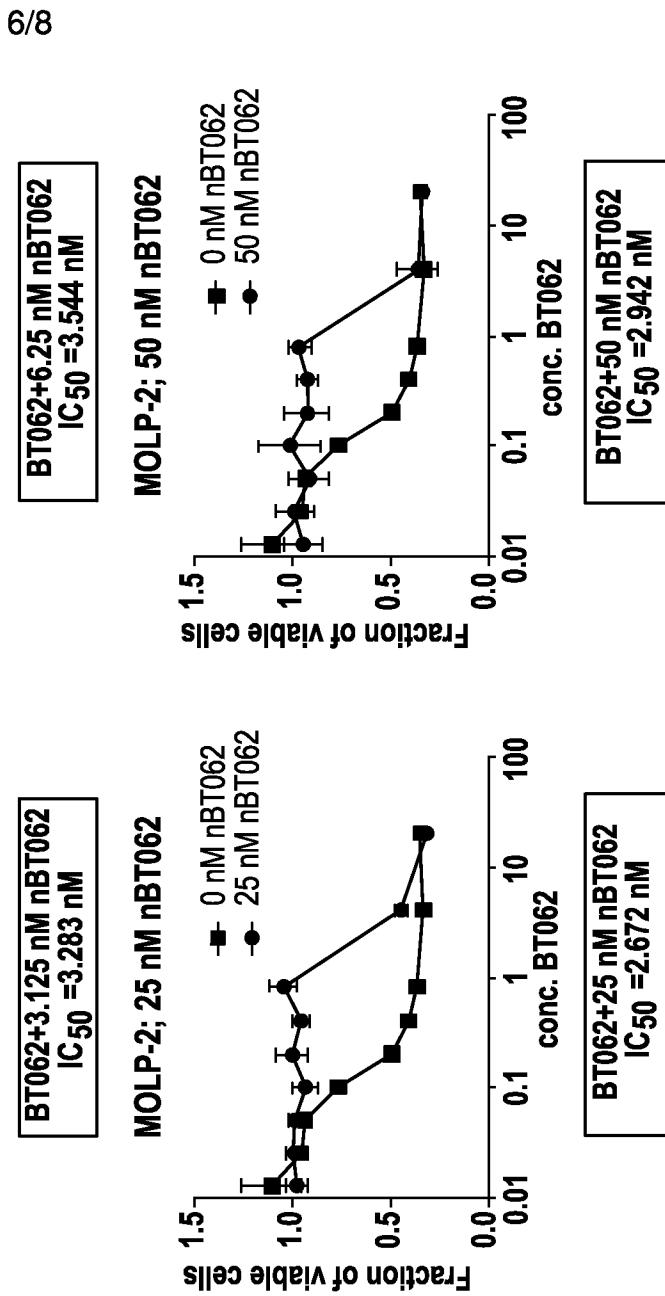
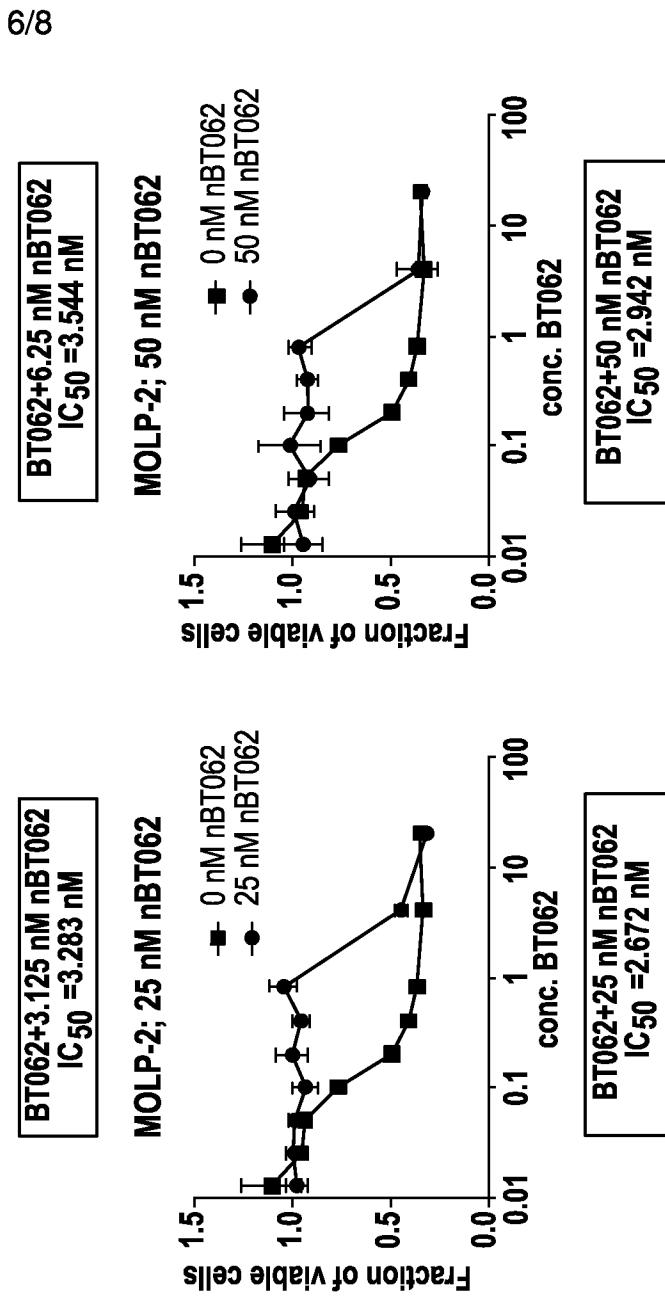
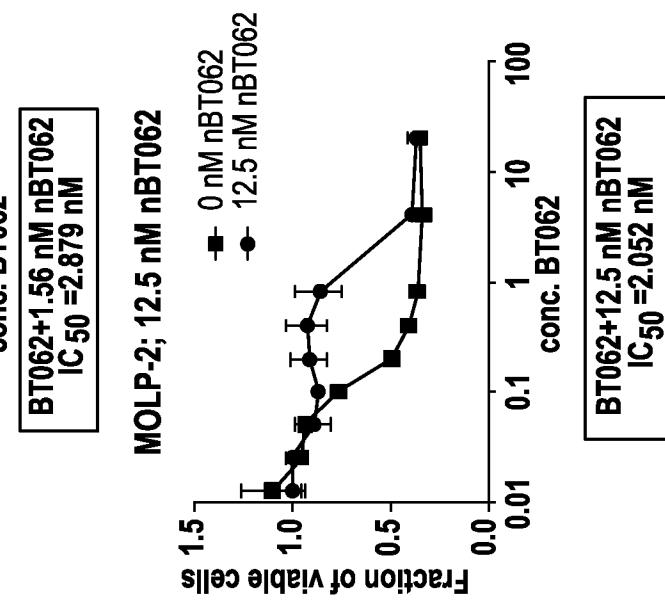
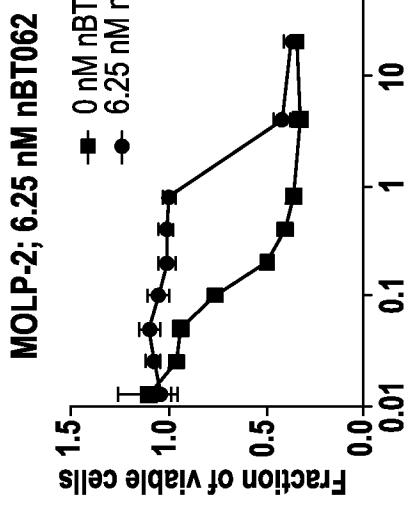
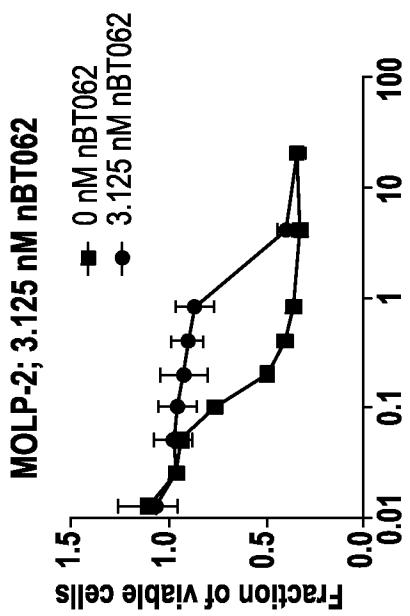
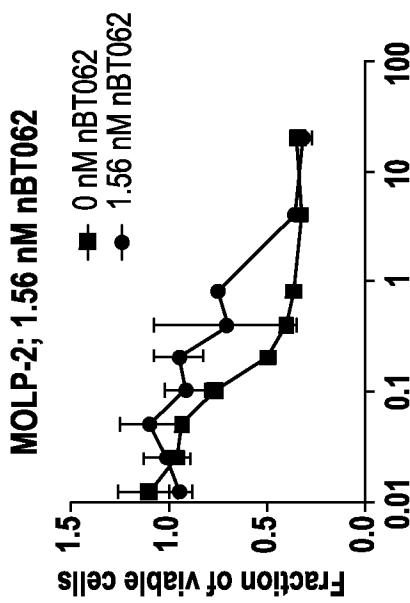
FIG. 2B



**MOLP-2, CD138<sup>low</sup>****FIG. 3A**

## MOLP-2, CD138 low

FIG. 3B



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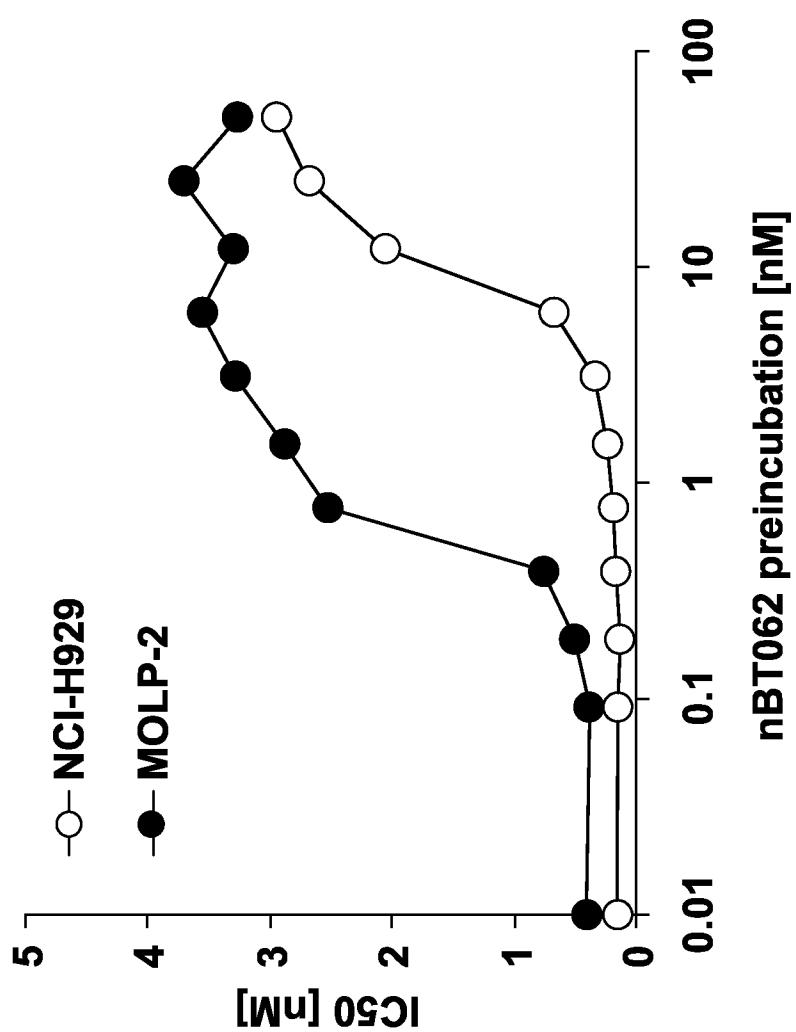
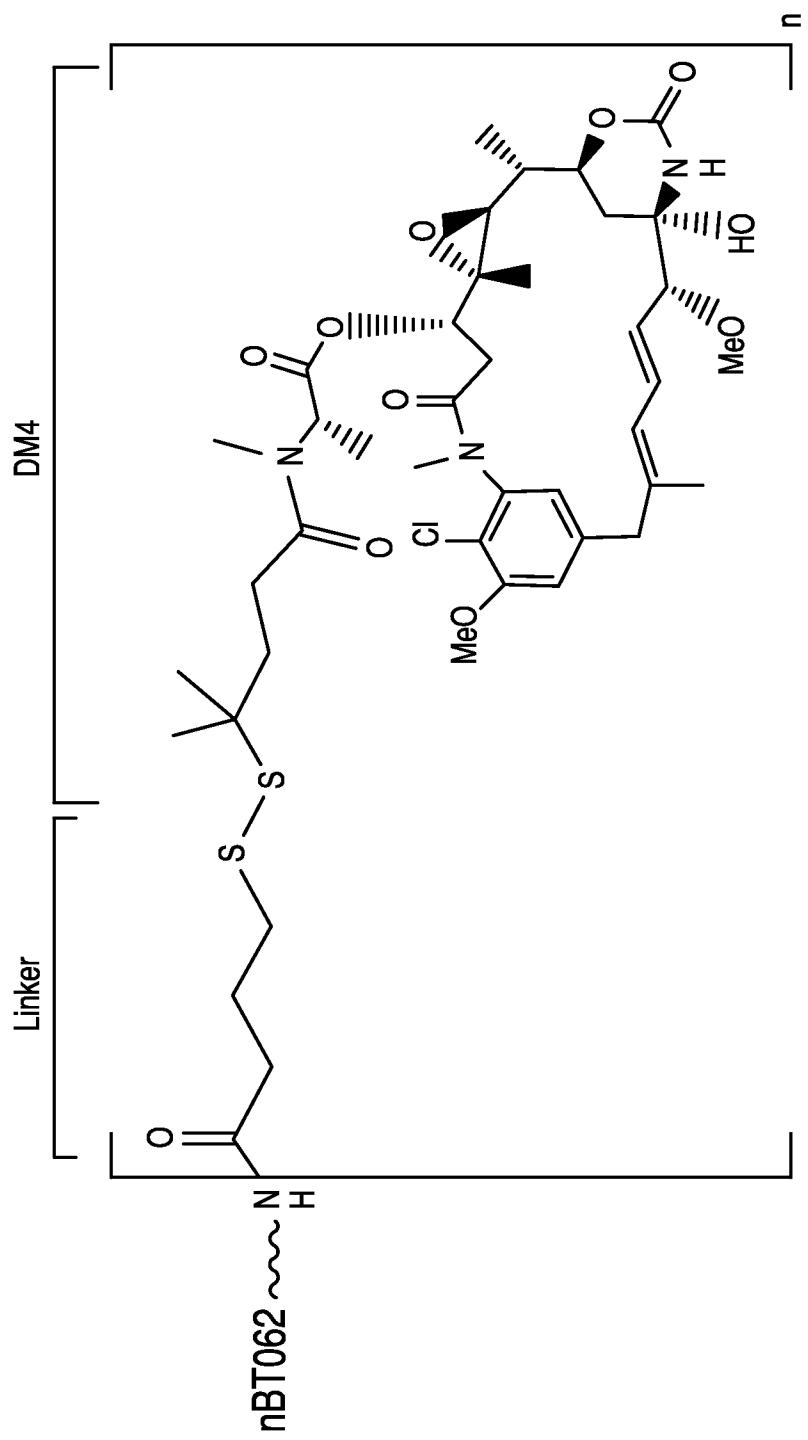


FIG. 4



\*n is approximately 3.5 drug linked per antibody molecule

FIG. 5

# INTERNATIONAL SEARCH REPORT

International application No  
PCT/EP2008/068268

A. CLASSIFICATION OF SUBJECT MATTER			
INV.	C07K16/28	C07K16/30	A61P35/00
			A61K47/48
According to International Patent Classification (IPC) or to both national classification and IPC			
B. FIELDS SEARCHED			
Minimum documentation searched (classification system followed by classification symbols)			
C07K A61K			
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched			
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)			
EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE			
C. DOCUMENTS CONSIDERED TO BE RELEVANT			
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.	
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<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C.		<input checked="" type="checkbox"/> See patent family annex.	
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1 April 2009		17/04/2009	
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer  Hermann, Patrice	

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PCT/EP2008/068268

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International application No PCT/EP2008/068268
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