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(54) Title: MIF AS THERAPEUTIC TARGET

(57) Abstract: The present invention pertains to the recognition that a specific structural alteration of MIF is involved in MIF-related disorders, providing a new approach to the treatment and prevention of MIF-related disorders, as well as other specific medical applications, e.g. for example monitoring of disease progression, drug screening and diagnostic assays and respective uses of diagnostic kits.

# MIF as Therapeutic Target

The present invention relates to applications of specific forms of the MIF protein in medicine. The invention is based upon the recognition that modification of position 81 of MIF determines the switch of MIF to its disease-associated state.

## BACKGROUND

Macrophage migration inhibitory factor (MIF) is a cytokine initially isolated based upon its ability to inhibit the *in vitro* random migration of peritoneal exudate cells from tuberculin hypersensitive guinea pigs (containing macrophages) (Bloom et al. Science 1966, 153, 80-2; David et al. PNAS 1966, 56, 72-7). Today, MIF is known as a critical upstream regulator of the innate and acquired immune response that exerts a pleiotropic spectrum of activities.

The human MIF cDNA was cloned in 1989 (Weiser et al., PNAS 1989, 86, 7522-6), and its genomic localization was mapped to chromosome 22. The product of the human MIF gene is a protein with 114 amino acids (after cleavage of the N-terminal methionine) and an apparent molecular mass of about 12.5 kDa. MIF has no significant sequence homology to any other protein. The protein crystallizes as a trimer of identical subunits. Each monomer contains two antiparallel alpha-helices that pack against a four-stranded beta-sheet. The monomer has additional two beta-strands that interact with the beta-sheets of adjacent subunits to form the interface between monomers. The three subunits are arranged to form a barrel containing a solvent-accessible channel that runs through the center of the protein along a molecular three-fold axis (Sun et al. PNAS 1996, 93, 5191-5196).

It was reported that MIF secretion from macrophages was induced at very low concentrations of glucocorticoids (Calandra et al. Nature 1995, 377, 68-71). However, MIF also counter-regulates the effects of glucocorticoids and stimulates the secretion of other cytokines such as tumor necrosis factor TNF-α and interleukin IL-1 ß (Baugh et al., Crit Care Med 2002, 30, S27-35). MIF was also shown e.g. to exhibit pro-angiogenic, pro-proliferative and anti-apoptotic properties, thereby promoting tumor cell growth (Mitchell, R.A., Cellular Signalling, 2004. 16(1): p. 13-19; Lue, H. et al., Oncogene 2007. 26(35): p. 5046-59). It is also e.g. directly associated with the growth of lymphoma, melanoma, and colon cancer (Nishihira et al. J Interferon Cytokine Res. 2000, 20:751-62).

MIF is a mediator of many pathologic conditions (i.e., MIF-related disorders) and thus associated with a variety of diseases including *inter alia* inflammatory bowel disease (IBD), rheumatoid arthritis (RA), acute respiratory distress syndrome (ARDS), asthma, glomerulonephritis, IgA nephropathy, myocardial infarction (MI), sepsis and cancer, though not limited thereto, and MIF also mediates many other MIF-related disorders. Polyclonal and monoclonal anti-MIF antibodies have been developed against recombinant human MIF (Shimizu et al., FEBS Lett. 1996; 381, 199-202; Kawaguchi et al, Leukoc. Biol. 1986, 39, 223-232, and Weiser et al., Cell. Immunol. 1985, 90, 16778).

Anti-MIF antibodies have been suggested for therapeutic use. Calandra et al., (J. Inflamm. (1995); 47, 39-51) reportedly used anti-MIF antibodies to protect animals from experimentally induced gram-negative and gram-

positive septic shock. Anti-MIF antibodies were suggested as a means of therapy to modulate cytokine production in septic shock and other inflammatory disease states.

US 6,645,493 discloses monoclonal anti-MIF antibodies derived from hybridoma cells, which neutralize the biological activity of MIF. It could be shown in an animal model that these mouse-derived anti-MIF antibodies had a beneficial effect in the treatment of endotoxin induced shock.

US 200310235584 discloses methods of preparing high affinity antibodies to MIF in animals in which the MIF gene has been homozygously knocked-out.

Glycosylation-inhibiting factor (GIF) is a protein described by Galat et al. (Eur. J. Biochem, 1994, 224, 417-21). MIF and GIF are now recognized to be identical. Watarai et al. (PNAS 2000, 97, 13251-6) described polyclonal antibodies binding to different GIF epitopes to identify the biochemical nature of the posttranslational modification of GIF in Ts cells. Watarai et al, *supra*, reported that GIF occurs in different conformational isoforms *in vitro*. According to Watarai et al., one isoform occurs by chemical modification of a single cysteine residue, namely cysteine 60, which occurs in suppressor T (Ts) cells and, according to Watarai et al., leads to a bioactive form of GIF.

It has been shown over the past decades that MIF is a molecule which is involved in a multitude of different interactions. WO2013/050453 discloses that an oxidised form of MIF (oxMIF) can be detected after onset of (MIF-related) disorders, e.g. in body fluid samples, or on cells or cell surfaces and that oxMIF is correlated with a disease state and/or disease progression. Antibodies that are specific to the disease-related, oxidised form of MIF (oxMIF-specific antibodies) are known. However, there is a need for more information on the involvement of MIF in MIF-related disorders in order to further improve the treatment and prevention of MIF-related disorders and related medical applications.

**DESCRIPTION OF THE INVENTION**

The present invention achieves this objective by revealing that the switch of MIF from a non-pathogenic to a disease-related state, i.e. the redox-switch from redMIF to oxMIF is associated with and promoted by the presence of a modification of position 81. The disease-related, oxidised form of MIF – oxMIF – is defined by the binding of oxMIF-specific antibodies, i.e. antibodies which bind preferentially to oxMIF as opposed to redMIF which is not particularly correlated with disease. The inventors have shown that the modification of MIF at position 81 (cysteine 81) by treatment with sulfhydryl –reactive agents induces binding of such oxMIF-specific antibodies. In contrast, when the wild-type cysteine 81 was mutated to serine, which is isosteric to cysteine but is not modified by sulfhydryl –reactive agents, treatment with sulfhydryl –reactive agents did not induce binding of oxMIF-specific antibodies. The inventors have thus established a clear link between modification of MIF position 81 and the oxMIF conformation of the protein which is involved in MIF-related disorders.

The sequence of human MIF is represented by SEQ ID NO: 15.

The present invention thus provides the use of MIF carrying a modification at 81 as a target for the treatment or prevention of a MIF-related disorder.

The present invention thus relates to therapeutic methods of treating or preventing a disease state or disease progression in a subject in need thereof by administering to said subject an effective amount of a compound that

(a) prevents modification of MIF at position 81, or

(b) binds preferentially to MIF carrying a modification at position 81 compared to MIF which does not contain said modification of position 81.

Said preferentially-binding compound may be an is an antibody or a molecule comprising an antigen-binding portion of an antibody and/or said preferentially-binding compound may induce a form of said modified MIF that binds the antibody RAM0, RAB0, RAM9 or RAB9 (or any other oxMIF-specific antibody, e.g. selected from the oxMIF-specific antibodies disclosed herein) to a lesser extent than does said modified MIF in the absence of the compound.

Herein, references to "modification of MIF at position 81" or "MIF modified at position 81" or "MIF carrying a modification at position 81", and the like, may be abbreviated by a reference to "modified MIF". Such modification refers throughout this application and in all embodiments to a modification of MIF whereby the MIF in its modified form or state binds to the antibody RAM9 (and/or to RAB9, RAM0, RAB0, and/or any other oxMIF-specific antibody, e.g. selected from any of the oxMIF-specific antibodies disclosed herein below). Generally, as position 81 of MIF is cysteine, such references to modified MIF may be used interchangeably with e.g., "MIF carrying a modification at cysteine 81", and the like, or "cysteine-81-modified MIF" (or, equivalently, C81-modified MIF).

Preferably, herein, a modification (in particular at MIF position 81) may be a modification compared to an unmodified cysteine residue, or compared to the free cysteine sulfhydryl group. That is, in particular, a modification at MIF position 81 may be a modification of MIF cysteine 81, and is preferably a modification on the sulphur atom of cysteine 81. That is, if herein reference is made to MIF position 81 carrying a modification, preferably MIF cysteine 81 carries a modification compared to unmodified cysteine, or more preferably the sulphur atom of cysteine 81 carries a modification compared to the free cysteine sulfhydryl group, as described herein.

The modification does not encompass the mere replacement of the cysteine sulphur atom by oxygen, i.e. the mere mutation of cysteine 81 to serine. Herein, modification of the sulfhydryl group of a cysteine residue refers to derivatization of the sulfhydryl group, i.e. the sulfhydryl group is derivatized. That is, the sulfur atom of a cysteine residue carries a modification compared to the free sulfhydryl group of cysteine, i.e. the sulfur atom of the cysteine is attached to a moiety other than H (i.e. to a modifying moiety).

The invention also encompasses a method of monitoring the effectiveness of a treatment of a MIF-related disorder, comprising a step of determining in samples isolated from a subject before and after said treatment whether MIF position 81 carries a modification, wherein the treatment is identified as effective if MIF position 81 is modified to a lesser extent after treatment than before treatment.

The invention also provides a method of assaying a test compound for preferential binding to a form of MIF which is modified at position 81, said method comprising the steps of

(a) modifying MIF at position 81 to obtain cysteine-81-modified MIF,

(b) combining a compound to be tested with said modified MIF in a test sample and in a control sample in which MIF is not modified at position 81,

(c) assessing binding of said compound to the modified MIF in the test sample and in the control sample

wherein the compound is selected if said compound binds to the modified MIF in the test sample to a greater extent than in the control sample.

For example, the compound may be selected if said compound binds to the modified MIF in the test sample preferentially or differentially compared to the control sample. For example, the compound may be selected if said compound binds to the modified MIF in the test sample with greater affinity than in the control sample.

In methods according to the invention disclosed herein, a test compound may, without limitation, preferably be an antibody or a molecule comprising an antigen-binding portion of an antibody.

The invention also provides a method of screening for a compound that prevents the modification of MIF at cysteine 81, said method comprising the steps of

(a) combining MIF with a compound to be tested in a test sample,

(b) treating MIF with a sulfhydryl-reactive reagent in the test sample containing the compound and in a control sample from which the compound is absent, under conditions in which the reagent modifies the sulfhydryl group of cysteine 81 of MIF at least in the control sample,

(c) assessing modification of cysteine 81 by the sulfhydryl-reactive reagent,

wherein the compound is selected if the sulfhydryl-reactive reagent modifies cysteine 81 to a lesser extent in the test sample than in the control sample.

The invention also provides a method of assaying a test compound for a conformational effect upon MIF which is modified at position 81, said method comprising the steps of

(a) providing a test sample containing MIF which is modified at position 81,

(b) combining a test compound with said modified MIF in the test sample,

(c) assessing the conformation of the modified MIF in the test sample in comparison to the conformation (i) of MIF in which position 81 carries a modification (control 1), and the conformation (ii) of MIF containing an unmodified cysteine residue at position 81 (control 2),

wherein the compound is selected if said conformational assessment indicates a detectable degree of similarity of the conformation of the modified MIF in the test sample with conformation (ii) compared to conformation (i), or if said conformational assessment indicates that the conformation of the modified MIF in the test sample otherwise detectably deviates from conformation (i).

The conformation of MIF may be assessed by an immunoassay. Advantageously, the immunoassay employs an antibody selected from RAM0, RAB0, RAM9 and RAB9 (and/or any other oxMIF-specific antibody, e.g. selected from oxMIF-specific antibodies disclosed herein), and the compound is selected if the modified MIF in the test sample (i.e. in the presence of the test compound) binds the antibody to a lesser extent than does said modified MIF in the absence of the compound (control 1).

Preferably, the modification of MIF position 81 in the case of "control 1" is the same as that in the test sample.

Generally, in methods according to the invention, the conformation of MIF may be assessed by any methodology that is sensitive to a conformational change in MIF that depends on the presence of a modification of MIF at position 81, in particular the presence or absence of a modification of MIF cysteine 81, in particular on the sulphur atom of MIF at position 81. For example, the conformation of MIF may be assessed by immunoassay, e.g. using one or more conformation-sensitive anti-MIF antibodies as described herein, i.e. the binding of which is dependent on the modification state of MIF position 81. E.g., the presence and/or absence of a modification of position 81 may be assessed by binding of a conformation-sensitive position-81-modification-selective antibody (and/or a conformation-selective cysteine 81 sulfhydryl-specific antibody), as described herein. For example, the conformation may be assessed by ELISA or surface plasmon resonance.

In other embodiments, the conformation may be assessed by X-ray crystallographic analysis. The conformation may, for example, also be assessed by nuclear magnetic resonance (NMR) analysis or by circular dichroism.

In accordance with the above, the invention also provides a method of assaying a test compound for a conformational effect upon MIF which is modified at position 81, said method comprising the steps of

(a) providing a test sample containing MIF which is modified at position 81,
(b) combining a test compound with said modified MIF in the test sample,
(c) assessing the binding of the modified MIF in the test sample to an antibody selected from RAM0, RAB0, RAM9 and RAB9 and/or any other oxMIF-specific, oxMIF conformation-sensitive antibody, e.g. selected from oxMIF-specific antibodies disclosed herein, in comparison to (i) MIF in which the sulphur atom of MIF cysteine 81 carries a modification compared to the free cysteine sulfhydryl group in the absence of the test compound (control 1),

wherein the compound is selected if the modified MIF in the test sample (i.e. in the presence of the test compound) binds the antibody to a lesser extent than does said modified MIF in the absence of the compound (control 1).

The invention also provides a method of assaying a test compound for binding to a modified form of MIF by X-ray crystallography, said method comprising the steps of

(a) modifying MIF at position 81 to obtain modified MIF,
(b) providing crystals of said modified MIF either by crystallising said modified MIF in the presence of said test compound or by crystallising said modified MIF and subsequently contacting said test compound with crystals of said modified MIF, and
(c) determining the three-dimensional structure of MIF using said crystals.

Optionally, the same X-ray crystallographic method may encompass additionally carrying out said crystallisation and structure determination steps with same test compound but with MIF in which position 81 is not modified.

The invention also provides a crystal containing MIF with a modification of position 81. Said crystal may optionally further contain another compound, for example a test compound, e.g. a test compound, the binding of which to said modified MIF is to be assessed.

Herein, a compound, e.g. for binding to MIF that is modified at position 81 (e.g. a compound for use in a therapeutic or diagnostic method or a test compound to be assayed), is not particularly limited, but may e.g. be a small molecule, an organic molecule, a molecule of less than 1000 Da, of less than 500 Da, or any form of antibody or antibody derivative (for example an Fab fragment, or any molecule comprising an antigen-binding portion of an antibody) which binds to MIF (in particular to a position-81-modified form thereof).

The invention also provides the use of position 81 of the MIF protein as a diagnostic marker and/or for monitoring MIF-related disorders. In particular, the invention provides the use of the modification state of position 81 of the MIF protein as a diagnostic marker and/or for monitoring MIF-related disorders.

The present invention provides a method of diagnosing a MIF-related disorder, comprising a step of determining in a sample isolated from a subject whether MIF position 81 carries a modification, wherein, if MIF position 81 is identified as carrying such a modification, the subject is diagnosed with a MIF-related disorder. That is, a finding that MIF position 81 carries such a modification is indicative of a MIF-related disorder.

The present invention provides a method of diagnosing a MIF-related disorder, comprising a step of determining in a sample isolated from a subject whether the sulphur atom of MIF cysteine 81 carries a modification compared to the free cysteine sulfhydryl group, wherein, if the sulphur atom of MIF cysteine 81 is identified as carrying such a modification, the subject is diagnosed with a MIF-related disorder. That is, a finding that the sulphur atom of cysteine 81 carries such a modification is indicative of a MIF-related disorder.

The present invention provides the use of the modification state of MIF position 81 as a diagnostic marker, wherein a finding that MIF position 81 carries a modification is indicative of a MIF-related disorder.

The present invention provides the use of the modification state of the sulfhydryl group of MIF cysteine 81 as a diagnostic marker, wherein a finding that the sulphur atom of MIF cysteine 81 carries a modification compared to the free cysteine sulfhydryl group is indicative of a MIF-related disorder.

Such methods and uses according to the invention encompass methods and uses of determining whether modified MIF is involved in, or correlated with, a given disorder or pathologic condition

The invention also provides the use of a diagnostic kit in a method or use according to the invention, wherein the diagnostic kit comprises a compound that preferentially binds to MIF in which cysteine 81 is present, or to MIF carrying a modification at position 81 (i.e., in particular, on the sulfur atom of cysteine 81). Such a kit may additionally comprise buffers, control reagents (e.g. recombinant MIF in which cysteine 81 is present or absent, or MIF with or without a modification position 81, e.g. a modification on the sulfur atom of cysteine 81, a compound that preferentially binds to any such forms of MIF), polyclonal anti-MIF antibody, and/or conjugated detection antibody.

Herein, the expression "MIF-related disorder" encompasses, for example, a (MIF-related) disease, a (MIF-related) disease state, the state of progression of a (MIF-related) disease.

In certain embodiments of methods of screening or assaying test compounds and of crystals according to the present invention, position 81 (e.g. the sulfhydryl group of MIF cysteine 81) is modified selectively. That is, the MIF is not substantially modified by the same modifying moiety at other positions, or, in particular, the MIF is not substantially modified by the same modifying moiety (i.e., does not substantially carry the same modification) at the sulphur atom of other cysteines in the MIF sequence. E.g., MIF does not substantially

carry the same modification on the sulphur atom of cysteine 57. E.g., MIF does not substantially carry the same modification on cysteine 60. In certain preferred embodiments, neither cysteine 57 nor cysteine 60 of MIF are substantially modified by the same modifying moiety as cysteine 81. In certain preferred embodiments, either or both of cysteine 57 and cysteine 60 may be substituted by (i.e. mutated to, i.e. replaced by) an amino acid other than cysteine, e.g. by serine. In other embodiments, cysteine 57 may be unmodified, i.e. may comprise a free sulfhydryl group. In other embodiments, cysteine 60 may be unmodified, i.e. may comprise a free sulfhydryl group. In other embodiments, both cysteine 57 and cysteine 60 are unmodified, i.e. comprise a free sulfhydryl group.

Assessment of whether position (cysteine ) 81 (in particular its sulphur atom or sulfhydryl group) is modified within the meaning of the present invention may be performed by mass spectroscopy, e.g. by preparing a peptide mass fingerprint (peptide mapping and mass-spectroscopic analysis). That is, the methods of the invention (e.g. diagnostic methods or methods of screening compounds) may encompass subjecting MIF to a mass-spectroscopic analysis. That is, the methods of the invention (e.g. diagnostic methods or methods of screening compounds) may encompass preparing a sample comprising MIF for mass-spectroscopic analysis. Methods of peptide mass fingerprinting to determine the modification of amino acid residues are well known to the skilled person in the relevant technical area, as part of the skilled person's common general knowledge. Methods of the invention may thus also encompass digesting MIF in a sample to be assessed with a protease (e.g. trypsin). Subsequently, peptides (MIF fragments) resulting form said digestion, in particularly a peptide containing cysteine 81 (and preferably containing no other cysteine residue) may be analysed by mass spectroscopy.

The assessment of whether position (cysteine) 81 is modified may also be performed by an immunoassay, i.e. using one or more antibodies. That is, the methods of the invention (e.g. diagnostic methods or methods of screening compounds) may encompass contacting MIF with one or more antibodies. This may lead to the formation of a complex between MIF (e.g. C81-modified MIF) and an antibody. The assessment of whether position 81 is modified may thus be performed using an antibody that selectively binds to MIF in which position 81 (e.g. the sulfhydryl group of cysteine 81 is modified), i.e. binds a form of MIF in which position 81 (e.g. the sulfhydryl group of cysteine 81) is modified with preference compared to MIF with a free sulfhydryl group at cysteine 81.

Methods of preparing specific antibodies (e.g. monoclonal antibodies) are also well known to the relevant skilled person. Such binding of an antibody may depend upon the presence of a modification (e.g. of the sulfhydryl group) of position 81 of the MIF protein for (or at least for) the binding of the antibody to the MIF protein under non-reducing and/or oxidising and/or native conditions. The binding epitope may include or may not include position 81. The antibody may be an anti-MIF antibody (e.g. a conformation-sensitive antibody, the binding of which is dependent on the modification state of position 81), e.g. a cysteine 81 modification-specific antibody which binds to MIF specifically, selectively or preferentially when the sulphur atom of MIF cysteine 81 carries a modification compared to the free cysteine sulfhydryl group, compared to its binding to MIF in the absence of such a modification (i.e. MIF containing a free cysteine sulfhydryl group at position 81). E.g. the binding epitope of the antibody may be an epitope (e.g. a linear or conformational epitope, which may

or may not include cysteine 81), the accessibility of which for antibody binding is enhanced when position 81 is modified, compared to when MIF position 81 is unmodified.

For example, the binding of the antibody to MIF may depend on the presence of a modification of cysteine at position 81 of the MIF protein, i.e. the antibody may bind MIF preferentially (e.g. under non-reducing conditions) when cysteine 81 is modified, compared to its binding to MIF when cysteine 81 is not modified (e.g. under reducing conditions), or when cysteine 81 is absent. For example, the antibody may be RAM9 or RAB9. The antibody may also be RAM0 or RAB0. Said binding may be dependent on the presence of cysteine 57 and/or cysteine 60 (as in the case of RAM9 or RAB9) or may be independent of the presence of cysteine 57 and/or cysteine 60, and/or dependent exclusively on the presence of a modification on the sulphur atom of MIF cysteine 81.

Methods and uses of the invention may also employ anti-MIF antibodies (e.g. conformation-sensitive antibodies, the binding of which is dependent on the modification state of cysteine 81), e.g. cysteine 81 sulfhydryl-selective antibodies, which bind to MIF preferentially when the sulphur atom of MIF cysteine 81 carries a free cysteine sulfhydryl group, compared to their binding to MIF in which cysteine 81 is modified. Such antibodies may be employed, e.g., as controls.

Within the meaning of the present invention, position 81 (e.g. the sulfhydryl group of MIF cysteine 81) may be identified as being modified if the mass of a MIF peptide containing cysteine 81 (but no other cysteine) is determined to be altered compared to the corresponding peptide containing a free sulfhydryl group at cysteine 81.

Preferably, assessment or determination of which amino acid the MIF gene encodes cysteine at position 81 (e.g. whether the MIF gene encodes cysteine at position 81) of the MIF protein is performed by a method involving a polymerase chain reaction (PCR).

The present invention also relates to antibodies as disclosed herein which specifically recognize the presence of a modification at MIF position 81. The invention also relates an anti-MIF antibody which specifically binds to MIF in which position 81 carries a modification (e.g. on the sulfur atom, compared to the free cysteine sulfhydryl group), wherein the antibody does not detectably bind, or binds with lower affinity, to MIF which does not contain said modification. The present invention also relates to complexes between MIF having a modification at position 81 and antibodies as mentioned herein.

Herein, an antibody may be of any type, *e.g.* preferably of the IgG1 type or the IgG4 type. Herein, an antibody referred to as "RAM" is of the IgG1 type, and an antibody referred to RAB" is of the IgG4 type.

Herein, references to binding (e.g. of an antibody or a test compound) are to binding under physiologically relevant conditions (at any temperature between, e.g. 1 and 45°C), unless otherwise indicated. In the context of X-ray crystallographic methods, references to binding encompass binding under crystallization conditions, which are well known to the person skilled in the art.

Preferential binding, or enhanced binding, etc., (e.g. of an antibody or another compound) may herein also be referred to as differential binding. For example, preferential, enhanced or differential binding means that a compound, in particular the antibodies as described herein, binds (e.g. with a $K_D$ value of less than 100 nM, preferably less than 50 nM, yet more preferably less than 10nM) to a first form of MIF; and binds to a lesser

extent (e.g. with lower affinity, e.g. characterized e.g. by a $K_D$ of more than 400 nM, or by an absence of detectable binding, i.e. the compound does not bind) to a second form of MIF. Said first form of MIF may, e.g., be a form of MIF that is associated with a MIF-related disorder (e.g. in particular C81-modified MIF), and said second form of MIF may be a form which is not thus associated (e.g. does not contain a modified sulphur atom of C81), or vice versa.

For example, differential binding may refer to binding with a $K_D$ value of less than 100 nM (preferably less than 50 nM, yet more preferably less than 10nM) to MIF carrying a modification at position 81 (e.g. on the sulfur atom of cysteine 81) and lesser binding, characterized e.g. by a $K_D$ of more than 400 nM, or an absence of detectable binding (i.e., the compound does not bind) to MIF which does not contain said modification on the sulfur atom of cysteine 81 (or vice versa).

Herein, terminology such as "diagnostic marker", or "marker in the diagnosis of a (MIF related) disease" in the context of the present invention shall encompass the possibility for an evaluation whether or not MIF is a factor involved in this (MIF related) disease. In that regard, modified MIF as a marker supplies information about the disease state, its progression and serves as a marker to determine effectiveness of a given treatment; in addition, modified MIF detection in a sample, e.g. a body fluid sample or a cell sample, can serve as an indicator for a preferred anti-MIF therapy. The detection of modified MIF thus serves to improve known diagnostic techniques in a given disease or disorder. It assists the practitioner in his or her decision how to treat a given disease or disorder and helps to improve specificity of the diagnosis. Modified MIF is thus a specific and suitable secondary marker. Its detection can serve as an adjunctive test in the management of patients afflicted with MIF related diseases. The disease in question is in a preferred embodiment a disease which is known or suspected to be MIF related (see the diseases mentioned in detail below) but can also be a disease which had so far not been suspected to be MIF related.

In a preferred embodiment, the detection of the presence of modified MIF in a sample would indicate to the practitioner that the subject, from whom (or which) the sample has been taken, might benefit from a therapy directed against MIF. Such a therapy could be selected from anti-MIF molecules, e.g. anti-modified MIF antibodies or small molecules which are directed against modified MIF.

Elevated MIF levels, i.e. levels of MIF in general are detected after the onset of various diseases, *inter alia* after the onset of cancer. However, MIF circulates also in healthy subjects, which makes a clear differentiation difficult. oxMIF, on the contrary, which the present invention links to modified MIF, is not present in healthy subjects and therefore is a much stronger diagnostic marker for MIF-related disorders. According to the present invention, patient samples such as blood, serum and urine may be assayed for increased levels of modified MIF compared to healthy subjects.

As shown herein, in particular derivatisation of the C81 sulfhydryl group by attachment of a moiety to the C81 sulfur atom promotes the switch of MIF to the disease-associated state oxMIF. oxMIF- specific antibodies (which specifically bind to oxMIF and are incapable of binding to redMIF) may be useful in the context of the present invention. Non-limiting examples of oxMIF-specific antibodies are Baxter antibodies RAM9, RAB9, RAM4, RAB4, RAM0 and RAB0.

Oxidative procedures such as cystine-mediated oxidation, GSSG (ox. Glutathione)-mediated oxidation or incubation of MIF with Proclin300 or protein crosslinkers (e.g. BMOE) may all cause binding to the above mentioned antibodies.

The above-mentioned antibodies are characterized and supported by both their sequences as well as by deposits as plasmids in *E.coli* (strain TG1), comprising either the light or the heavy chain of each of the above mentioned antibodies. The plasmids are characterized by their DSM number which is the official number as obtained upon deposit under the Budapest Treaty with the German Collection of Microorganisms and Cell Cultures (DSMZ), Mascheroder Weg 1b, Braunschweig, Germany. The plasmids were deposited in *E. coli* strains, respectively.

The plasmid with the DSM 25110 number comprises the light chain sequence of the anti-MIF antibody RAB4. The plasmid with the DSM 25112 number comprises the heavy chain (IgG4) sequence of the anti-MIF antibody RAB4. The co-expression of plasmids DSM 25110 and DSM 25112 in a suitable host cell results in the production of preferred anti-MIF antibody RAB4.

The plasmid with the DSM 25111 number comprises the light chain sequence of the anti-MIF antibody RAB9. The plasmid with the DSM 25113 number comprises the heavy chain (IgG4) sequence of the anti-MIF antibody RAB9. The co-expression of plasmids DSM 25111 and DSM 25113 in a suitable host cell results in the production of preferred anti-MIF antibody RAB9.

The plasmid with the DSM 25114 number comprises the light chain sequence of the anti-MIF antibody RAB0. The plasmid with the DSM 25115 number comprises the heavy chain (IgG4) sequence of the anti-MIF antibody RAB0. The co-expression of plasmids DSM 25114 and DSM 25115 in a suitable host cell results in the production of preferred anti-MIF antibody RAB0.

Also deposited are antibodies RAM0, RAM9 and RAM4; all have been deposited with the DSZM, Braunschweig, Germany on April 12, 2012 according to the Budapest Treaty, with the following designations:

RAM9 – heavy chain: E.coli GA.662-01.pRAM9hc – DSM 25860.

RAM4 – light chain: E.coli GA.906-04.pRAM4lc – DSM 25861.

RAM9 – light chain: E.coli GA.661-01.pRAM9lc – DSM 25859.

RAM4 – heavy chain: E.coli GA.657-02.pRAM4hc – DSM 25862.

RAM0 – light chain: E.coli GA.906-01.pRAM0lc – DSM 25863.

RAM0 – heavy chain: E.coli GA.784-01.pRAM0hc – DSM 25864.

A biological sample in the context of the present disclosure is preferably a body fluid sample of the subject on which/whom the diagnosis shall be performed. A body fluid sample is any sample of a body fluid as known to a person skilled in the art. Exemplary, but not limiting, such a sample can be blood, plasma, serum, saliva, urine, nasal fluid, ascites, ocular fluid, amniotic fluid, aqueous humour, vitreous humour, tear fluid, Cowper's fluid, semen, interstitial fluid, lymph, breast milk, mucus (incl. snot and phlegm), pleural fluid, pus, menses, vaginal lubrication, sebum, cerebrospinal fluid and synovial fluid. Further biological samples in the context of this application can be lavages (washing outs) of a (hollow) body organ (e.g. bronchoalveolar lavage, stomach lavage and bowel lavage).

A biological sample in the context of this application in an alternative embodiment, is a cell sample, most preferably a cell sample from the circulation or the diseased tissue, more preferably as a single cell suspension sample, of the subject on which the diagnosis shall be performed.

According to the invention, the diagnostic methods, assays and uses disclosed herein, in particular methods of diagnosing a MIF-related disorder as disclosed herein, encompass methods of determining whether C81-modified MIF is involved in, or correlated with, a given disorder or pathologic condition.

The present invention thus also pertains to a method for evaluating the progression of a disease; in the present context the term "state of a disease" or "disease state" is to be understood as synonymous with the term "severity of a disease" and refers to the seriousness, degree or state (i.e. stage) of a disease or condition. For example, a disease may be characterised as mild, moderate or severe. The determination or assessment of the degree of severity or the degree, i.e. state of the disease is well known to a person skilled in the art. The actual method which will be carried out for this assessment of course depends on the disease or condition in question. For example, the state of a disease may be determined by comparing the likelihood or length of survival of a subject having a disease with the likelihood or length of survival in other subjects having the same disease.

In other embodiments the state of the disease may be determined by comparing the symptoms of a disease in a subject having a disease with the symptoms in other subjects having the same disease. In yet another embodiment the state of the disease and its progression is reflected by the change of symptoms within one and the same patient over a period of time.

In a further preferred aspect, the present invention can also be directed to a method of selecting a subject as being eligible for a treatment with an anti-modified MIF compound, wherein the subject has a (MIF-related) disorder, or is at risk of developing a (MIF-related) disorder, comprising detecting the existence and/or level and/or change of level of modified MIF in said subject. A subject having an elevated level of modified MIF can be selected for a prophylactic or therapeutic treatment with an anti modified MIF compound as defined above. The term "prophylactic" or "therapeutic" treatment is art-recognized and refers to administration of a drug to a patient. If it is administered prior to clinical manifestation of the unwanted condition (e.g. disease or other unwanted state of the host, e.g. a human or an animal) then the treatment is prophylactic, i.e., it protects the host against developing the unwanted condition, whereas if administered after manifestation of the unwanted condition, the treatment is therapeutic (i.e., it is intended to diminish, ameliorate or maintain the existing unwanted condition or side effects thereof).

As used herein an anti-modified MIF compound refers to any agent that attenuates, inhibits, opposes, counteracts, or decreases the biological activity of modified MIF. An anti-modified MIF compound may be an agent that inhibits or neutralizes modified MIF activity, for example a small molecule or an antibody. Preferred antibodies are the antibodies as described herein, particularly, RAM9, RAB9, RAM4, RAB4, RAM0 and RAB0; preferably RAB9 or RAB0; or RAM9 or RAM0.

Diagnostic methods or assays disclosed herein can be used to determine the presence or level of modified MIF in e.g. body fluid samples or cellular samples of patients. The presence or absence of oxMIF is suitable to

distinguish, if the disease if MIF relevant or to decide of oxMIF treatment is reasonable. OxMIF levels indicate disease progression or treatment efficacy.

Further disclosed in the context of the present invention are kits comprising an anti- modified MIF antibody or an antigen-binding portion thereof. A kit may include in addition to the antibody, further diagnostic or therapeutic agents and uses thereof. A kit also can include instructions for use in a diagnostic or therapeutic method.

**Definitions and General Techniques**

Unless otherwise defined herein, scientific and technical terms used in connection with the present invention shall have the meanings that are commonly understood by those of ordinary skill in the art. Generally, nomenclatures used in connection with, and techniques of, cell and tissue culture, molecular biology, immunology, microbiology, genetics and protein and nucleic acid chemistry described herein are those well known and commonly used in the art. The methods and techniques of the present invention are generally performed according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification unless otherwise indicated. See, e.g., Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989) and Ausubel et al., Current Protocols in Molecular Biology, Greene Publishing Associates (1992), and Harlow and Lane Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1990), which are each incorporated herein by reference in their entirety.

"MIF" or "macrophage migration inhibitory factor" refers to the protein, which is known as a critical mediator in the immune and inflammatory response, and as a counterregulator of glucocorticoids. MIF includes mammalian MIF, specifically human MIF (Swiss-Prot primary accession number: P14174), the sequence of which is represented by SEQ ID NO: 15. The monomeric form is encoded as a 115 amino acid protein but is produced as a 114 amino acid protein due to cleavage of the initial methionine. "MIF" also includes "GIF" (glycosylation-inhibiting factor) and other forms of MIF such as fusion proteins of MIF, e.g. with an affinity tag (which is useful for purification of the protein), such as SBP (streptavidin binding peptide), optionally via a linker (e.g. a (GGGGS)2 linker), as is well known to the person skilled in the art. The numbering of the amino acids of MIF starts with the N-terminal methionine (amino acid 1) and ends with the C-terminal alanine (amino acid 115), in accordance with SEQ ID NO: 15.

"Oxidized MIF" or oxMIF is defined herein as an isoform of MIF that occurs by treatment of MIF with mild oxidizing reagents (sulfhydryl-reactive reagents), such as cystine. Such oxMIF comprises isoform(s) of MIF that share structural rearrangements with oxMIF that (e.g.) occurs *in vivo* after challenge of animals with bacteria. It is bound specifically by the oxMIF-specific antibodies disclosed herein.

"Reduced MIF" or redMIF is defined for the purposes of this invention as reduced MIF and is MIF which does not bind to RAB0, RAB9 and/or RAB4 and/or to RAM0, RAM9 and/or RAM4.

Binding kinetics of antibodies to the various forms of MIF disclosed herein may be examined by surface plasmon resonance analysis using a Biacore 3000 System. The antibodies are coated on a CM5 (= carboxymethylated dextran)chip and MIF protein is pre-incubated with 0.2% Proclin300, and the mixture is

injected. Proclin300 consists of oxidative isothiazolones that stabilizes oxMIF structure by avoiding a conversion of oxMIF to redMIF.

Antibodies or antigen-binding portions thereof preferably bind a specific form of MIF disclosed herein (e.g. modified MIF) with a $K_D$ of less than 100 nM, preferably a $K_D$ of less than 50 nM, even more preferred with a $K_D$ of less than 10 nM. Particularly preferred, the antibodies of this invention bind to a specific form of MIF disclosed herein (e.g. modified MIF) with a $K_D$ of less than 5 nM.

(Non-)binding of an antibody can be determined as generally known to a person skilled in the art, examples being any one of the following methods: Differential Binding ELISA with recombinant MIF, or surface plasmon resonance using recombinant MIF in any of the forms disclosed herein, like the well-known Biacore assay, described above.

A preferred method for the determination of binding is surface plasmon resonance of an antibody to the forms of MIF disclosed herein (e.g. modified MIF) wherein "binding" refers to a $K_D$ of less than 100 nM, preferably less than 50 nM, even more preferred less than 10 nM whereas the non-binding (e.g. in the case of modified MIF or ox;IF-specific antibodies to redMIF) is characterized by a $K_D$ of more than 400 nM. "Binding" and "specific binding" is used interchangeably herein. "Differential binding" in the context of this application encompasses that a compound, in particular the antibodies as described herein, or test compounds, bind to modified MIF (e.g. with the $K_D$ values mentioned above) while they do not bind to redMIF and/or to MIF which does not contain a modified sulphur atom of C81.

Herein, references to "antibodies" encompass antigen-binding antibody derivatives, constructs or fragments which are known to a person skilled in the relevant art, in particular antigen-binding molecules comprising antigen-binding portions of antibodies. An "antibody" refers to an intact antibody or a molecule consisting of or comprising an antigen-binding portion of an antibody that competes with the intact antibody for (specific) binding. See generally, Fundamental Immunology, Ch. 7 (Paul, W., ed., 2nd ed. Raven Press, N.Y. (1989)) (incorporated herein by reference in its entirety). The term antibody includes human antibodies, mammalian antibodies, isolated antibodies and genetically engineered forms such as chimeric, camelized or humanized antibodies, though not being limited thereto.

The term "antigen-binding portion" of an antibody refers to one or more fragments of an antibody that retain the ability to specifically bind to an antigen (e.g. modified MIF). Molecules comprising antigen-binding portions of antibodies may be produced by recombinant DNA techniques or by enzymatic or chemical cleavage of intact antibodies. Such molecules include, though are not limited to, the following: Fab, Fab', F(ab')2, Fv, and complementarity determining region (CDR) fragments, single-domain antibodies, and single-chain Fv antibodies (scFv), chimeric antibodies, diabodies, antibodies and polypeptides that contain at least a portion of an antibody that is sufficient to confer specific binding to an antigen of interest, e.g. a form of MIF disclosed herein, e.g. to modified MIF, redMIF, oxMIF, MIF containing a free sulfhydryl group in C81, MIF which does not contain a modified sulphur atom of C81, or other forms of MIF disclosed herein. From N-terminus to C-terminus, both the mature light and heavy chain variable domains comprise the regions FR1, CDR1, FR2, CDR2, FR3, CDR3 and FR4. The assignment of amino acids to each domain is in accordance with the definitions of Kabat, Sequences of Proteins of Immunological Interest (National Institutes of Health, Bethesda,

Md. (1987 and 1991)), Chothia et al. J. Mol. Biol. 196:901-917 (1987), or Chothia et al., Nature 342:878-883 (1989). An antibody or antigen-binding portion thereof can be derivatized or linked to another functional molecule (e.g., another peptide or protein). For example, an antibody or antigen- binding portion thereof can be functionally linked to one or more other molecular entities, such as another antibody (e.g., a bispecific antibody or a diabody), a detectable agent, a cytotoxic agent, a pharmaceutical agent, and/or a linking molecule.

Within the context of the present invention, an antibody, may e.g. characterised as a human antibody, a humanized antibody, a camelized antibody. Further, an antibody may be, e.g. a chimeric antibody. Further, an antibody may be, e.g. an isolated antibody.

The term "$K_D$" refers, in accordance with the common general knowledge of a person skilled in the art to the equilibrium dissociation constant of two interaction partners, e.g. a particular antibody and its respective antigen.

The term "human antibody" refers to any antibody in which the variable and constant domains are human sequences. The term encompasses antibodies with sequences derived from human genes, but which have been changed, e.g. to decrease possible immunogenicity, increase affinity, eliminate cysteines that might cause undesirable folding, etc. The term encompasses such antibodies produced recombinantly in non-human cells, which might e.g. impart glycosylation not typical of human cells.

The term "humanized antibody" refers to antibodies comprising human sequences and containing also non-human sequences.

The term "camelized antibody" refers to antibodies wherein the antibody structure or sequences has been changed to more closely resemble antibodies from camels, also designated camelid antibodies. Methods for the design and production of camelized antibodies are part of the general knowledge of a person skilled in the art.

The term "chimeric antibody" refers to an antibody that comprises regions from two or more different species.

The term "isolated antibody" or "isolated antigen-binding portion thereof" refers to an antibody or an antigen-binding portion thereof that has been identified and selected from an antibody source such as a phage display library or a B-cell repertoire.

Antibodies disclosed herein may be produced by any method for the generation of recombinant DNA by genetic engineering, e.g. via reverse transcription of RNA and/or amplification of DNA and cloning into expression vectors. In some embodiments, the vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. In some embodiments, the vector is capable of autonomous replication in a host cell into which it is introduced (e.g. bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). In other embodiments, the vector (e.g. non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" (or simply, "expression vectors").

Antibodies disclosed herein can be produced *inter alia* by means of conventional expression vectors, such as bacterial vectors (e.g., pBR322 and its derivatives), or eukaryotic vectors. Those sequences that encode the antibody can be provided with regulatory sequences that regulate the replication, expression and/or secretion from the host cell. These regulatory sequences comprise, for instance, promoters (e.g., CMV or SV40) and signal sequences. The expression vectors can also comprise selection and amplification markers, such as the dihydrofolate reductase gene (DHFR), hygromycin-B-phosphotransferase, and thymidine-kinase. The components of the vectors used, such as selection markers, replicons, enhancers, can either be commercially obtained or prepared by means of conventional methods. The vectors can be constructed for the expression in various cell cultures, e.g., in mammalian cells such as CHO, COS, HEK293, NSO, fibroblasts, insect cells, yeast or bacteria such as *E.coli*. In some instances, cells are used that allow for optimal glycosylation of the expressed protein.

Antibody light chain gene(s) and antibody heavy chain gene(s) can be inserted into separate vectors or the genes are inserted into the same expression vector. The antibody genes are inserted into the expression vector by standard methods, e.g., ligation of complementary restriction sites on the antibody gene fragment and vector, or blunt end ligation if no restriction sites are present.

The production of antibodies or antigen-binding fragments thereof may include any method known in the art for the introduction of recombinant DNA into eukaryotic cells by transfection, e.g. via electroporation or microinjection. For example, the recombinant expression of an antibody can be achieved by introducing an expression plasmid containing the antibody encoding DNA sequence under the control of one or more regulating sequences such as a strong promoter, into a suitable host cell line, by an appropriate transfection method resulting in cells having the introduced sequences stably integrated into the genome. The lipofection method is an example of a transfection method which may be used according to the present invention.

The production of antibodies may also include any method known in the art for the cultivation of said transformed cells, e.g. in a continuous or batchwise manner, and the expression of the antibody, e.g. constitutive or upon induction. It is referred in particular to WO 2009/086920 for further reference for the production of anti-(ox)MIF antibodies.

The sequences of oxMIF-specific antibodies are partly also disclosed in WO 2009/086920. Further oxMIF-specific antibodies are characeterised by the following sequences:

SEQ ID NO: 1 for the amino acid sequence of the light chain of RAB9:
DIQMTQSPSS LSASVGDRVT ITCRSSQRIM TYLNWYQQKP GKAPKLLIFV ASHSQSGVPS RFRGSGSETD FTLTISGLQP EDSATYYCQQ SFWTPLTFGG GTKVEIKRTV AAPSVFIFPP SDEQLKSGTA SVVCLLNNFY PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYSLSSTLT LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEC,

SEQ ID NO: 2 for the amino acid sequence of the light chain of RAB4:
DIQMTQSPGT LSLSPGERAT LSCRASQGVS SSSLAWYQQK PGQAPRLLIY GTSSRATGIP DRFSGSASGT DFTLTISRLQ PEDFAVYYCQ QYGRSLTFGG GTKVEIKRTV AAPSVFIFPP SDEQLKSGTA SVVCLLNNFY PREAKVQWKV

DNALQSGNSQ ESVTEQDSKD STYSLSSTLT LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEC,

SEQ ID NO: 3 for the amino acid sequence of the light chain of RAB0:

DIQMTQSPGT LSLSPGERAT LSCRASQGVS SSSLAWYQQK PGQAPRLLIY GTSSRATGIP DRFSGSASGT DFTLTISRLQ PEDFAVYYCQ QYGRSLTFGG GTKVEIKRTV AAPSVFIFPP SDEQLKSGTA SVVCLLNNFY PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYSLSSTLT LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEC,

SEQ ID NO: 4 for the amino acid sequence of the light chain of RAB2:

DIQMTQSPVT LSLSPGERAT LSCRASQSVR SSYLAWYQQK PGQTPRLLIY GASNRATGIP DRFSGSGSGT DFTLTISRLE PEDFAVYYCQ QYGNSLTFGG GTKVEIK$^{R}$TV AAPSVFIFPP SDEQLKSGTA SVVCLLNNFY PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYSLSSTLT LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEC,

SEQ ID NO: 5 for the amino acid sequence of the heavy chain of RAB9:

EVQLLESGGG LVQPGGSLRL SCAASGFTFS IYSMNWVRQA PGKGLEWVSS IGSSGGTTYY ADSVKGRFTI SRDNSKNTLY LQMNSLRAED TAVYYCAGSQ WLYGMDVWGQ GTTVTVSSAS TKGPSVFPLA PCSRSTSEST AALGCLVKDY FPEPVTVSWN SGALTSGVHT FPAVLQSSGL YSLSSVVTVP SSSLGTKTYT CNVDHKPSNT KVDKRVESKY GPPCPPCPAP EFLGGPSVFL FPPKPKDTLM ISRTPEVTCV VVDVSQEDPE VQFNWYVDGV EVHNAKTKPR EEQFNSTYRV VSVLTVLHQD WLNGKEYKCK VSNKGLPSSI EKTISKAKGQ PREPQVYTLP PSQEEMTKNQ VSLTCLVKGF YPSDIAVEWE SNGQPENNYK TTPPVLDSDG SFFLYSRLTV DKSRWQEGNV FSCSVMHEAL HNHYTQKSLS LSLGK,

SEQ ID NO: 6 for the amino acid sequence of the heavy chain of RAB4:

EVQLLESGGG LVQPGGSLRL SCAASGFTFS IYAMDWVRQA PGKGLEWVSG IVPSGGFTKY ADSVKGRFTI SRDNSKNTLY LQMNSLRAED TAVYYCARVN VIAVAGTGYY YYGMDVWGQG TTVTVSSAST KGPSVFPLAP CSRSTSESTA ALGCLVKDYF PEPVTVSWNS GALTSGVHTF PAVLQSSGLY SLSSVVTVPS SSLGTKTYTC NVDHKPSNTK VDKRVESKYG PPCPPCPAPE FLGGPSVFLF PPKPKDTLMI SRTPEVTCVV VDVSQEDPEV QFNWYVDGVE VHNAKTKPRE EQFNSTYRVV SVLTVLHQDW LNGKEYKCKV SNKGLPSSIE KTISKAKGQP REPQVYTLPP SQEEMTKNQV SLTCLVKGFY PSDIAVEWES NGQPENNYKT TPPVLDSDGS FFLYSRLTVD KSRWQEGNVF SCSVMHEALH NHYTQKSLSL SLGK,

SEQ ID NO: 7 for the amino acid sequence of the heavy chain of RAB0:

EVQLLESGGG LVQPGGSLRL SCAASGFTFS WYAMDWVRQA PGKGLEWVSG IYPSGGRTKY ADSVKGRFTI SRDNSKNTLY LQMNSLRAED TAVYYCARVN

VIAVAGTGYY YYGMDVWGQG TTVTVSSAST KGPSVFPLAP CSRSTSESTA
ALGCLVKDYF PEPVTVSWNS GALTSGVHTF PAVLQSSGLY SLSSVVTVPS
SSLGTKTYTC NVDHKPSNTK VDKRVESKYG PPCPPCPAPE FLGGPSVFLF
PPKPKDTLMI SRTPEVTCVV VDVSQEDPEV QFNWYVDGVE VHNAKTKPRE
EQFNSTYRVV SVLTVLHQDW LNGKEYKCKV SNKGLPSSIE KTISKAKGQP
REPQVYTLPP SQEEMTKNQV SLTCLVKGFY PSDIAVEWES NGQPENNYKT
TPPVLDSDGS FFLYSRLTVD KSRWQEGNVF SCSVMHEALH NHYTQKSLSL
SLGK,

SEQ ID NO: 8 for the amino acid sequence of the heavy chain of RAB2:
EVQLLESGGG LVQPGGSLRL SCAASGFTFS IYAMDWVRQA PGKGLEWVSG
IVPSGGFTKY ADSVKGRFTI SRDNSKNTLY LQMNSLRAED TAVYYCARVN
VIAVAGTGYY YYGMDVWGQG TTVTVSSAST KGPSVFPLAP CSRSTSESTA
ALGCLVKDYF PEPVTVSWNS GALTSGVHTF PAVLQSSGLY SLSSVVTVPS
SSLGTKTYTC NVDHKPSNTK VDKRVESKYG PPCPPCPAPE FLGGPSVFLF
PPKPKDTLMI SRTPEVTCVV VDVSQEDPEV QFNWYVDGVE VHNAKTKPRE
EQFNSTYRVV SVLTVLHQDW LNGKEYKCKV SNKGLPSSIE KTISKAKGQP
REPQVYTLPP SQEEMTKNQV SLTCLVKGFY PSDIAVEWES NGQPENNYKT
TPPVLDSDGS FFLYSRLTVD KSRWQEGNVF SCSVMHEALH NHYTQKSLSL
SLGK.

SEQ ID NO: 9 for the amino acid sequence of RAM0hc:
EVQLLESGGG LVQPGGSLRL SCAASGFTFS WYAMDWVRQA PGKGLEWVSG
IYPSGGRTKY ADSVKGRFTI SRDNSKNTLY LQMNSLRAED TAVYYCARVN
VIAVAGTGYY YYGMDVWGQG TTVTVSSAST KGPSVFPLAP SSKSTSGGTA
ALGCLVKDYF PEPVTVSWNS GALTSGVHTF PAVLQSSGLY SLSSVVTVPS
SSLGTQTYIC NVNHKPSNTK VDKRVEPKSC DKTHTCPPCP APELLGGPSV
FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD GVEVHNAKTK
PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK
GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN
YKTTPPVLDS DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS
LSLSPGK.

SEQ ID NO: 10 for the amino acid sequence of RAM0lc:
DIQMTQSPGT LSLSPGERAT LSCRASQGVS SSSLAWYQQK PGQAPRLLIY
GTSSRATGIP DRFSGSASGT DFTLTISRLQ PEDFAVYYCQ QYGRSLTFGG
GTKVEIKRTV AAPSVFIFPP SDEQLKSGTA SVVCLLNNFY PREAKVQWKV
DNALQSGNSQ ESVTEQDSKD STYSLSSTLT LSKADYEKHK VYACEVTHQG
LSSPVTKSFN RGEC.

SEQ ID NO: 11 for the amino acid sequence of RAM9hc:

EVQLLESGGG LVQPGGSLRL SCAASGFTFS IYSMNWVRQA PGKGLEWVSS IGSSGGTTYY ADSVKGRFTI SRDNSKNTLY LQMNSLRAED TAVYYCAGSQ WLYGMDVWGQ GTTVTVSSAS TKGPSVFPLA PSSKSTSGGT AALGCLVKDY FPEPVTVSWN SGALTSGVHT FPAVLQSSGL YSLSSVVTVP SSSLGTQTYI CNVNHKPSNT KVDKRVEPKS CDKTHTCPPC PAPELLGGPS VFLFPPKPKD TLMISRTPEV TCVVVDVSHE DPEVKFNWYV DGVEVHNAKT KPREEQYNST YRVVSVLTVL HQDWLNGKEY KCKVSNKALP APIEKTISKA KGQPREPQVY TLPPSREEMT KNQVSLTCLV KGFYPSDIAV EWESNGQPEN NYKTTPPVLD SDGSFFLYSK LTVDKSRWQQ GNVFSCSVMH EALHNHYTQK SLSLSPGK.

SEQ ID NO: 12 for the amino acid sequence of RAM9lc:

DIQMTQSPSS LSASVGDRVT ITCRSSQRIM TYLNWYQQKP GKAPKLLIFV ASHSQSGVPS RFRGSGSETD FTLTISGLQP EDSATYYCQQ SFWTPLTFGG GTKVEIKRTV AAPSVFIFPP SDEQLKSGTA SVVCLLNNFY PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYSLSSTLT LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEC.

SEQ ID NO: 13 for the amino acid sequence of RAM4hc:

EVQLLESGGG LVQPGGSLRL SCAASGFTFS IYAMDWVRQA PGKGLEWVSG IVPSGGFTKY ADSVKGRFTI SRDNSKNTLY LQMNSLRAED TAVYYCARVN VIAVAGTGYY YYGMDVWGQG TTVTVSSAST KGPSVFPLAP SSKSTSGGTA ALGCLVKDYF PEPVTVSWNS GALTSGVHTF PAVLQSSGLY SLSSVVTVPS SSLGTQTYIC NVNHKPSNTK VDKRVEPKSC DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPGK.

SEQ ID NO: 14 for the amino acid sequence of RAM4lc:

DIQMTQSPGT LSLSPGERAT LSCRASQGVS SSSLAWYQQK PGQAPRLLIY GTSSRATGIP DRFSGSASGT DFTLTISRLQ PEDFAVYYCQ QYGRSLTFGG GTKVEIKRTV AAPSVFIFPP SDEQLKSGTA SVVCLLNNFY PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYSLSSTLT LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEC.

SEQ ID NO: 15 for the sequence of human MIF

Met Pro Met Phe Ile Val Asn Thr Asn Val Pro Arg Ala Ser Val Pro
Asp Gly Phe Leu Ser Glu Leu Thr Gln Gln Leu Ala Gln Ala Thr Gly
Lys Pro Pro Gln Tyr Ile Ala Val His Val Val Pro Asp Gln Leu Met
Ala Phe Gly Gly Ser Ser Glu Pro Cys Ala Leu Cys Ser Leu His Ser
Ile Gly Lys Ile Gly Gly Ala Gln Asn Arg Ser Tyr Ser Lys Leu Leu

Cys Gly Leu Leu Ala Glu Arg Leu Arg Ile Ser Pro Asp Arg Val Tyr
Ile Asn Tyr Tyr Asp Met Asn Ala Ala Asn Val Gly Trp Asn Asn Ser
Thr Phe Ala

An antibody according to the present disclosure is preferably an isolated monoclonal antibody. The anti-MIF antibody can be an IgG, an IgM, an IgE, an IgA, or an IgD molecule. In other embodiments, the anti-MIF antibody is an IgG1, IgG2, IgG3 or IgG4 subclass. In other embodiments, the antibody is either subclass IgG1 or IgG4. In other embodiments, the antibody is subclass IgG4. In some embodiments, the IgG4 antibody has a single mutation changing the serine (serine228, according to the Kabat numbering scheme) to proline. Accordingly, the CPSC sub-sequence in the Fc region of IgG4 becomes CPPC, which is a sub-sequence in IgG1 (Angal et al. Mol Immunol. 1993, 30, 105-108).

Antibodies can be recovered from the culture medium using standard protein purification methods, e.g. via anion exchange chromatography or affinity chromatography. In one embodiment the anti-(ox)MIF antibody can be purified from cell culture supernatants by size exclusion chromatography.

The terms "center region" and "C-terminal region" of MIF refer to the region of human MIF comprising amino acids 35-68 and aa 86-115, respectively, preferably aa 50-68 and aa 86 to 102 of human MIF, respectively.

Particularly preferred anti-oxMIF antibodies bind to either region aa 50-68 or region aa 86-102 of human MIF. This is also reflected by the binding of the preferred antibodies RAB0, RAB4 RAB2 and RAB9 as well as RAM4, RAM9 and RAM0 which bind as follows:

RAB4 and RAM4: aa 86-102
RAB9 and RAM9: aa 50-68
RAB0 and RAM0: aa 86-102
RAB2: aa 86 - 102

The term "epitope" includes any protein determinant capable of specific binding to an immunoglobulin or an antibody fragment. Epitopic determinants usually consist of chemically active surface groupings of molecules such as exposed amino acids, amino sugars, or other carbohydrate side chains and usually have specific three-dimensional structural characteristics, as well as specific charge characteristics.

The term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. In some embodiments, the vector is a plasmid, i.e., a circular double stranded DNA loop into which additional DNA segments may be ligated.

The term "host cell" refers to a cell line, which is capable of producing a recombinant protein after introducing an expression vector. The term "recombinant cell line", refers to a cell line into which a recombinant expression vector has been introduced. It should be understood that "recombinant cell line" means not only the particular subject cell line but also the progeny of such a cell line. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term "recombinant cell line" as used herein.

The host cell type according to the present invention is e.g. a COS cell, a CHO cell or e.g. an HEK293 cell, or any other host cell known to a person skilled in the art, thus also for example including bacterial cells, like e.g.

*E.coli* cells. In one embodiment, the antibody is expressed in a DHFR-deficient CHO cell line, e.g., DXB11, and with the addition of G418 as a selection marker. When recombinant expression vectors encoding antibody genes are introduced into CHO host cells, the antibodies are produced by culturing the host cells for a period of time sufficient to allow for expression of the antibody in the host cells or secretion of the antibody into the culture medium in which the host cells are grown.

A "MIF-related disorder" in the present context includes but is not limited to infectious diseases, inflammation, autoimmunity, cancer, cell differentiation, atherogenesis and angiogenesis-related diseases. MIF-related disorders are e.g., type I and II-diabetes, acute lung injury, asthma, allograft-rejection, graft-versus-host-disease, wound healing disturbances and inflammatory bowel disease. Cancer is a further MIF-related disorder. In particular, MIF-related cancers include lymphoma, sarcoma, prostatic cancer and colon cancer, bladder cancer, pancreas cancer, ovarian cancer, melanoma, hepatocellular carcinoma, ovarian cancer, breast cancer and pancreatic cancer.

Further, atherosclerosis is a MIF-related disorder.

Also, malignant ascites is a MIF-related disorder.

Further MIF-related disorders include sarcoidosis, scleroderma, psoriasis, (ulcerative) colitis, as well atopic dermatitis, as well as septic shock, delayed hypersensitivity, acute respiratory distress syndrome (ARDS), multiple sclerosis, pancreatitis and ischemic cardiac injury.

Immune and inflammatory disorders, which are MIF-related, include gram negative and gram positive sepsis, e.g. *P. aeruginosa* infections or sepsis, DTH, glomerulonephritis, arthritis, adjuvant arthritis, juvenile arthritis, (autoimmune) encephalomyelitis/encephalitis, (autoimmune) myocarditis, allergic encephalitis, gastritis, colitis; (immune)glomerulonephritis; pneumonia, toxic shock syndrome, viral infections, tuberculosis, hepatitis B, dengue fever, parasitic and helminthic MIF-related infections, in particular malaria, leishmaniasis, trypanosomiasis, toxoplasmosis, amoebiasis, schistosomiasis, cysticercosis, trichenellosis and filariasis; kidney diseases, like leukocyte-mediated renal injury, non-proliferative renal disease, proliferative renal disease, renal allograft rejection and congenital nephritic syndrome of the Finnish type, nephritis, nephropathy like uric acid nephropathies and hypertensive nephropathy, ureteric obstruction and diabetic nephropathy.

Neuropathic pain is a further MIF-related disorder.

Most preferred diseases to be diagnosed according to the present invention are: glomerulonephritis, sepsis, lymphoma, lupus nephritis, psoriasis, ulcerative colitits and ophthalmological conditions, as well as Burkitt's lymphoma, leukemia, malignant ascites, prostate adenocarcinoma, pancreatic adenocarcinoma, ovarian carcinoma, colorectal carcinoma, head and neck cancer, renal cell carcinoma, hepatocellular carcinoma, breast cancer and lung cancer. Both k-*ras* wild-type as well as k-*ras* mutated cancers can be treated in accordance with the present invention.

In particular, the present invention also covers third line treatments of all above mentioned disorders.

One aspect of the present invention is directed to detection of modified MIF in a sample of a subject. Such detection allows a skilled practitioner, e.g., to determine whether or not MIF is a therapeutically important component of a disease or disorder afflicting the subject. This determination will aid the practitioner's decision whether or not an (additional) anti-(ox)MIF treatment could be beneficial for the subject in question.

Modified MIF is also useful as a marker to determine a health or disease condition of a given subject in general; an elevated level of modified MIF will allow the finding that the subject is afflicted with a MIF related disease; modified MIF can thus also be used as a (secondary) general marker for a health/disease condition of a subject, similar e.g. to the determination of C-reactive protein (CRP) which is currently and widely used as such a (secondary) marker.

According to the present invention, the presence of cysteine 81 (C81) of MIF is essential for the switch of MIF to a disease-related state, i.e. the redox-switch from redMIF to oxMIF, and it is the derivatisation of the C81 sulfhydryl group which promotes (or is responsible for) this switch to the oxMIF form. Thus, the invention provides the use of the modification state of the sulfhydryl group of MIF cysteine 81 as a diagnostic marker, for the diagnosis of MIF-related disorders.

"Diagnosis" in the context of this specification encompasses detection of a disease, evaluation of a disease state and monitoring of a disease progression, which also allows monitoring efficacy of a therapeutic treatment.

In a preferred embodiment, diagnosis of said MIF-related disorders according to the invention, may encompass the use, or the further use, of compounds binding to modified MIF for the detection of modified MIF. These compounds, which differentially bind modified MIF can be antibodies or small molecules, which differentially bind to modified MIF. The diagnostic assay which can be used in the present invention can be any diagnostic assay which is well-known to a person skilled in the art. In particular, the diagnostic assay can be carried out e.g. in an ELISA format, a sandwich (ELISA) format with use of FACS, immunofluorescence, immunohistochemistry, and any of various other suitable methods, all of which are well-known in the art.

When assessing a sample for the presence of modified C81 according to the invention, false positive results resulting from oxidised forms of MIF such as C81-modifed MIF may occur. For example, oxidation of cysteine residues in the MIF protein (and thus C81) can be induced by redox-active iron and heme in biosamples (e.g. hemolytic blood samples) or if oxidizing agents are added to the sample. Such false positive results may be avoided, e.g., by taking measures as described herein below, e.g. by de-activating redox-active iron and heme, and by avoiding the addition of e.g. oxidizing agents.

For the analysis of MIF circulating in blood, a special sample procedure is preferably employed, involving the following measures and steps. Citrated plasma is preferred. Citrated **plasma** from fresh blood (stored at +4°C not longer than 12 h) is centrifuged at 40 g for 5 min. The supernatant is transferred into a new tube and centrifuged again at 2000 g for 3 min. The cell free supernatant is transferred again into a new tube and centrifuged at 16000 g for 3 min. After the three centrifuge steps, the cell free supernatant can be stored at -80°C or directly used for the analysis of MIF. If MIF is to be analysed in **sera**, cells and insoluble fragments are preferably removed by the same three centrifugation steps prior storage by freezing or prior running the MIF ELISA.

Sediments in **urine** samples should also preferably be removed by a centrifugation step (16000 g for 5 min) prior to use in the MIF ELISAs. Generally, cells and other common particles occurring in biological fluids (e.g. tear fluid, saliva) have to be removed prior by a centrifugation step and then stored for testing of MIF.

Furthermore, as denatured MIF may also be recognized by antibodies which specifically bind to oxMIF or to modified MIF, the MIF protein to be tested is to be kept in its native conformation during sample preparation (e.g. during the isolation and preparation of body fluids). Therefore denaturating conditions/steps such as for example boiling, immobilization (on membranes, plastic (plate) or chips) and chemical treatments (e.g. with reducing agents, oxidizing agents and organic solvents), are avoided.

For the analysis of MIF on cellular surfaces, preferably a flow cytometry assay is used. It is particularly important that the samples do not undergo hemolysis during sample preparation. Therefore, all samples for the present flow cytometry analysis have been prepared without any step which would lead to a hemolysis of the cells within the sample.

**Summary of preferred embodiments of the invention**

1. A method of treating or preventing a MIF-related disorder in a subject in need thereof by administering to said subject an effective amount of a compound that
   (a) prevents modification of MIF at position 81, or
   (b) binds preferentially to MIF carrying a modification at position 81 compared to MIF which does not contain said modification of cysteine 81.
2. The method of embodiment 1, wherein said preferentially-binding compound is an antibody or a molecule comprising an antigen-binding portion of an antibody and/or said preferentially-binding compound induces a form of said modified MIF that binds the antibody RAM0, RAB0, RAM9 or RAB9, or another oxMIF-specific antibody to a lesser extent than does said modified MIF in the absence of the compound.
3. The use of MIF carrying a modification at position 81 as a target for the treatment or prevention of a MIF-related disorder.
   In a preferred embodiment, the MIF carrying a modification at position 81 (of SEQ ID NO:15) is used as a target for a drug discovery assay. A drug discovery assay is an assay well known to a person skilled in the art. It is an assay whereby the (potential) usefulness of given compounds/a given compound for the treatment or prevention of a disease or disorder (*"as a drug"*) is assessed.
4. A method of monitoring the effectiveness of a treatment of a MIF-related disorder, comprising a step of determining in samples isolated from a subject before and after said treatment whether MIF position 81 carries a modification, wherein the treatment is identified as effective if MIF position 81 is modified to a lesser extent after treatment than before treatment.
   "Effectiveness" and "efficacy" are both well-known terms and are used here interchangeably.
5. An anti-MIF antibody or molecule comprising an antigen-binding portion of an antibody which antibody or molecule binds a MIF which is modified at position 81 but does not bind MIF which is not modified at position 81.
6. A method of assaying a test compound for preferential binding to a form of MIF which is modified at position 81, said method comprising the steps of
   (a) modifying MIF at position 81 to obtain modified MIF,

(b) combining a compound to be tested with said modified MIF in a test sample and in a control sample in which MIF is not modified at position 81,

(c) assessing binding of said compound to the modified MIF in the test sample and in the control sample

wherein the compound is selected if said compound binds to the modified MIF in the test sample to a greater extent than in the control sample.

7. A method of assaying a test compound for a conformational effect upon MIF which is modified at position 81, said method comprising the steps of

(a) providing a test sample containing mo MIF which is modified at position 81,

(b) combining a compound to be tested with said modified MIF in the test sample,

(c) assessing the conformation of the modified MIF in the test sample in comparison to the conformation (i) of MIF in which position 81 carries a modification (control 1), and/or the conformation (ii) of MIF containing an unmodified of cysteine residue at position 81 (control 2),

wherein the compound is selected if said conformational assessment indicates a detectable degree of similarity of the conformation of the modified MIF in the test sample with conformation (ii) compared to conformation (i), or if said conformational assessment indicates that the conformation of the modified MIF in the test sample otherwise detectably deviates from conformation (i).

8. The method of embodiment 7, wherein in step (c) the conformation of MIF is assessed by an immunoassay, preferably by ELISA.

9. The method of embodiment 7, wherein in step (c) the conformation of MIF is assessed by X-ray crystallography.

10. A method of assaying a test compound for a conformational effect upon MIF which is modified at position 81, said method comprising the steps of

(a) providing a test sample containing MIF which is modified at position 81,

(b) combining a test compound with said modified MIF in the test sample,

(c) assessing the binding of the modified MIF in the test sample to an antibody selected from RAM0, RAB0, RAM9 and RAB9 and/or any other oxMIF-specific, oxMIF conformation-sensitive antibody, e.g. selected from oxMIF-specific antibodies disclosed herein, in comparison to (i) said modified MIF in the absence of the test compound (control 1),

wherein the compound is selected if the modified MIF in the test sample (i.e. in the presence of the test compound) binds the antibody to a lesser extent than does said modified MIF in the absence of the compound (control 1).

11. A method of screening for a compound that prevents the modification of MIF at cysteine 81, said method comprising the steps of

(a) combining MIF with a compound to be tested in a test sample,

(b) treating MIF with a sulfhydryl-reactive reagent in the test sample containing the compound and in a control sample from which the compound is absent, under conditions in which the reagent modifies the sulfhydryl group of cysteine 81 of MIF at least in the control sample,

(c) assessing modification of cysteine 81 by the sulfhydryl-reactive reagent,

wherein the compound is selected if the sulfhydryl-reactive reagent modifies cysteine 81 to a lesser extent in the test sample than in the control sample.

12. A method of assaying a test compound for binding to a modified form of MIF by X-ray crystallography, said method comprising the steps of

(a) modifying MIF at position 81 to obtain modified MIF,

(b) providing crystals of said modified MIF either by crystallising said modified MIF in the presence of said test compound or by crystallising said modified MIF in the absence of the test compound and subsequently contacting the resulting crystals with said test compound, and

(c) determining the three-dimensional structure of MIF using said crystals.

13. A crystal containing MIF with a modification at position 81.

14. The crystal of embodiment 13 further containing another compound.

15. The use of position 81 of the MIF protein as a diagnostic marker for a MIF-related disorder and/or for monitoring a MIF-related disorder.

16. A use according to embodiment 15, wherein the modification state of position 81 of the MIF protein is used a diagnostic marker for a MIF-related disorder and/or for monitoring a MIF-related disorder.

17. The use of the modification state of MIF position 81 as a diagnostic marker, wherein a finding that MIF position 81 carries a modification is indicative of a MIF-related disorder.

18. A use according to embodiment 16, wherein the modification state of the sulfhydryl group of MIF cysteine 81 is used as a diagnostic marker, wherein a finding that the sulphur atom of MIF cysteine 81 carries a modification compared to the free cysteine sulfhydryl group is indicative of a MIF-related disorder.

19. A method of diagnosing a MIF-related disorder, comprising a step of determining in a sample isolated from a subject whether MIF position 81 carries a modification, wherein, MIF position 81 is identified as being modified, the subject is diagnosed with a MIF-related disorder, or as being susceptible thereto.

20. A method of diagnosing a MIF-related disorder, comprising a step of determining in a sample isolated from a subject whether the sulphur atom of MIF cysteine 81 carries a modification compared to the free cysteine sulfhydryl group, wherein, if the sulphur atom of MIF cysteine 81 is identified as carrying such a modification, the subject is diagnosed with a MIF-related disorder, or as being susceptible thereto.

21. A use according to any of embodiments 15-18 or a method according to embodiment 19 or 20, which is a method of determining whether MIF that is modified at position 81 is involved in, or correlated with, a given disorder or pathologic condition.

22. Use of a diagnostic kit in a use according to any one of embodiments 15-18, or a method according to any one of embodiments 19-21, wherein the diagnostic kit comprises a compound that preferentially binds to MIF in which cysteine 81 is present, or to MIF carrying a modification at position 81.

23. A use according to embodiment 22, wherein the kit additionally comprises buffers, control reagents (e.g. recombinant MIF in which cysteine 81 is present or absent, or MIF with or without a modification at position 81, e.g. a modification on the sulphur atom of cysteine 81 compared to the free cysteine sulfhydryl group, a compound that preferentially binds to any such forms of MIF), polyclonal anti-MIF antibody, and/or a labelled detection antibody.

24. A method according to any one of embodiments 1, 2 or 6-12 or a use according to embodiments 22 or 23, wherein said compound is an antibody or a molecule comprising an antigen-binding portion of an antibody.

25. A method according to any one of embodiments 1, 2, 4, 19, 20 or 24 -or a use according to any one of embodiments 3, 15-18, 21-23, wherein the MIF-related disorder is a MIF-related disease, a MIF-related disease state, or the state of progression of a MIF-related disease.

26. A use or method according to embodiment 25, wherein the MIF-related disorder is an inflammatory disease or a neoplastic disease.

27. A use or method according to embodiment 25, wherein the MIF-related disorder is selected from the group consisting of colon cancer, prostate cancer, bladder cancer, pancreas cancer, ovarian cancer, melanoma, lymphoma, hepatocellular carcinoma, asthma, ARDS, rheumatoid arthritis, sepsis, IgA nephropathy, glomerulonephritis, Lupus Nephritis (LN), hepatitis, pancreatitis (+/- acute lung injury), Crohn's disease, ulcerative colitis, gastric ulcer, Alzheimer's disease, multiple sclerosis, Guillain-Barre syndrome, cardiac dysfunction, angioplasty, atherosclerosis, myocarditis, type 1 diabetes, diabetic retinopathy, age-related macular degeneration (AMD), atopic dermatitis, psoriasis, endometriosis, neuropathic pain and uveitis.

28. A use or method according to item 25, wherein the MIF-related disorder is malignant ascites, preferably in a third line treatment.

29. A MIF molecule which is modified on position 81 of SEQ ID NO: 15.

The following Examples and the Figures as described below illustrate the present invention, and methods that may be useful in performing or assessing the invention. They are in no way intended to limit the invention.

**BRIEF DESCRIPTION OF THE FIGURES:**

Figure 1 shows schematic representations of wild-type and mutant recombinant fusion constructs of MIF as employed in the present Examples, in comparison to wild-type MIF

Figure 2 shows Western blot assays of the MIF constructs of Figure 1 using the antibody RAM9.

Figure 3 shows Western blot assays of the MIF constructs of Figure 1 using the antibody RAM0.

Figure 4 shows examples of sulfhydryl-reactive reagents for derivatisation of sulfhydryl groups on a compound R or MIF, the resulting reaction products, and the expected increases in molecular weight in Da for R or MIF after derivatisation.

Figure 5 shows the general setup of an ELISA assay for assessing MIF and its mutant and/or derivatised forms.

Figure 6 shows the binding of wild-type human MIF in its reduced form (huMIFred), in cysteinylated form (Cys-MIF), and when derivatised using 5,5´-dithiobis-(2-nitrobenzoic acid/DTNB (DTNB-MIF), to the antibody RAM9 in an ELISA assay.

Figure 7 shows the assessment by ELISA of the binding of MIF in its wild-type (MIF), wild-type fusion (MIF(wt)SBP), and mutant fusion (MIF(C57S)SBP, etc) forms when unmodified, when derivatised with DNTB and assessed under non-reducing conditions, or when derivatised with DNTB and assessed under reducing conditions in the presence of DTT to the antibodies RAM9 (panel A) and RAM0 (panel B)

Figure 8 (panels A and B) show mass-spectroscopic data obtained with MIF in its wild-type (MIF), wild-type fusion (MIFwt-SBP), and mutant fusion (MIF(C57S)SBP, etc), confirming derivatisation with cysteine or DTNB (+NTB). Cysteinylation or derivatisation with DTNB give rise to peaks reflecting correspondingly increased molecular weights.

Figure 9 shows a mass spectrum obtained from peptide mass fingerprinting of cysteinylated MIF

Figure 10 shows results of an analysis of cysteinylated MIF by peptide mass fingerprinting

Figure 11 shows a mass spectrum obtained from peptide mass fingerprinting of MIF derivatised by DTNB

Figure 12 shows results of an analysis of MIF derivatised by DTNB by peptide mass fingerprinting

**REFERENCE EXAMPLES AND GENERALLY APPLICABLE METHODS**

In the following, several assays or methods that are useful in the assessment of antibodies and/or MIF are described.

**A) GCO-assay for antibody screening:**

A THP1 suspension culture is centrifuged and cells are resuspended in fresh full medium to a cell density of $10^6$ cells per ml. This culture is transferred into wells of a 96-well microplate (90 µl/well) and a potential anti-MIF antibody is added to give a final concentration of 75 µg/ml. Each antibody is tested in triplicate. After o/n incubation at 37°C dexamethasone is added to give a concentration of 2 nM and after one hour incubation at 37°C LPS is added (3 ng/ml final concentration). After further six hours incubation at 37°C the supernatant is harvested and the IL-6 concentrations are determined in a commercially available ELISA. The results of the triplicates are averaged and the percentage of IL-6 secretion is determined in comparison to the control antibodies. Antibodies that result in an IL-6 secretion of less than 75% are evaluated as positive.

**B) Assay for determination of $IC_{50}$ values**

The experimental procedure is carried out as described for the screening assay with the exception that increasing amounts of antibody are used (typically from 1 – 125 nM). The resultant dose response curve is expressed as % inhibition in comparison to a negative control antibody. This curve is used for calculation of the maximum inhibitory effect of the antibody (% Inh max) and the antibody concentration that shows 50% of the maximum inhibitory effect ($IC_{50}$).

**C) Inhibition of cell proliferation**

Serum stimulates secretion of MIF in quiescent NIH/3T3 and MIF in turn stimulates cell proliferation. Antibodies inhibiting this endogenous MIF, therefore, decrease the proliferation of quiescent NIH/3T3 cells. The reduction of proliferation is determined by the incorporation of $^{3}H$-thymidine.

1000 NIH/3T3 cells per well are incubated in a 96 well plate over the weekend at 37°C in medium containing 10% serum. Cells are then starved over night at 37°C by incubation in medium containing 0.5% serum. The 0.5% medium is removed and replaced by fresh medium containing 10% serum, 75 µg/ml antibody and 5µ Ci/ml of 3H-thymidine. After 16 hours incubation in a $CO_2$ incubator at 37°C cells are washed twice with 150 µl of cold PBS per well. Using a multi-channel pipette 150 µl of a 5% (w/v) TCA solution per well are added and incubated for 30 minutes at 4°C. Plates are washed with 150 µl PBS. Per well 75 µl of a 0.5M NaOH solution with 0.5% SDS are added, mixed and stored at room temperature. Samples are measured in a ß-counter by mixing 5 ml of Ultima Gold (Packard) and 75 µl sample solution. Each determination is done in triplicate and the values are compared with the values of the control antibody by a t-test. Antibodies that significantly reduce proliferation ($P<0.05$) are evaluated as positive.

**D) Binding studies: Epitope determination of anti-MIF antibodies**

Each peptide is diluted in coupling buffer to give a peptide concentration of typically 1 µg/ml added to microplates (NUNC Immobilizer™ Amino Plate F96 Clear) and incubated over night at 4°C (100 µl/well). As controls recombinant full length MIF and PBS are used. The plate is washed 3 times with 200 µl PBST and antibodies (2-4 µg/ml in PBS) are added (100 µl/well) and incubated for 2 hours at room temperature with gentle shaking. The plate is washed 3 times with 200 µl PBST and detection antibody (e.g. Fc specific anti-human IgG/HRP labeled, Sigma) is added (100 µl/well). After incubation for 1 hour at room temperature with gentle shaking, the plate is washed 3 times with 200 µl PBST. Each well is incubated with 100 µl TMB (3,3',5,5'-tetramethylbenzidine) solution (T-0440, Sigma) for 30 minutes in the dark. Staining reaction is stopped by adding 100 µl of 1.8 M $H_2SO_4$-solution per well. Samples are measured at 450 nm.

**E) Affinity determination of anti-MIF antibodies by Biacore**

Typically, 40 RU units of human recombinant MIF are immobilized on a sensor chip with a CM5 (= carboxymethylated dextran) matrix (Biacore). Fab fragments are injected at a concentration range of typically 6 – 100 nM diluted in HBS-EP. After each cycle the chip is regenerated with 50 mM NaOH + 1 M NaCl. Affinities are calculated according to the 1:1 Langmuir model.

**F) Measurement of C81-modified MIF in serum samples by ELISA**

Microtiter plates are coated e.g. with a human anti-C81-modified MIF monoclonal antibody. Human serum samples are diluted 1:25 in 0.5% fish gelatin/PBS, pH 7.2 and applied to the plate. After washing of the plate, detection of C81-modified MIF captured by the coating antibody is performed e.g. with an affinity purified polyclonal rabbit antibody anti-human MIF. The read-out of the ELISA is done after further incubation e.g. with HRP labelled goat anti-rabbit antibodies (BioRad, Cat.: 171-6516; any other goat anti-rabbit as known in the art could be used here as well) and TMB (3,3',5,5'-tetramethylbenzidine; T-0440, Sigma) as a chromogenic substrate (any other suitable substrate could also be used, as known to a person skilled in the art). The chromogenic reaction of TMB is stopped with $H_2SO_4$ and the ELISA plate was measured at 450 nm.

Calibration of the ELISA can be done with recombinant human MIF freshly derivatised at cysteine 81 e.g. using DTNB or MIF diluted in ProClin containing buffer which leads to an artificial oxMIF conformation. The standards are diluted in 0.5% fish gelatin/PBS including 0.2% ProClin300 and 4% human control plasma (i.e. a pool of serum samples from 50 healthy donors). The range of the calibration curve may be, e.g., 10 ng/ml to 0.156 ng/ml.

**G) Measurement of C81-modified MIF on the surface of cells in sepsis patients by flow cytometry**

Blood (e.g. from a patient with sepsis) is stained in Cell Staining Buffer (Biologend) with either Alexa700-labeled anti-CD3ε (for T cells) and PerCP-Cy5.5-labeled anti-Ly6G (for granulocytes) or APC-labeled anti-CD14 (for monocytes) and PE-Cy7-labeled anti-CD19 (for B cells) in parallel with 300 nM anti-C81-modified MIF antibody or control IgG. After washing, the human antibodies are detected using the goat R-PE-labeled anti-humanIgG antibodies. After washing, the red blood cells are lysed with the BD FACS™ Lysing solution (Becton Dickinson, Franklin Lakes, USA). Data acquisition is performed using a FACS™ Canto II (Becton Dickinson) with the DIVA™ software (software version 6; Becton Dickinson) and the data are analyzed using the FlowJo™ software (Treestar, Ashland, OR, USA).

**H) Preparation of polyclonal and affinity purified polyclonal rabbit anti-hu MIF antibodies**

Immunization procedure of polyclonal rabbit anti huMIF antibodies in New Zealand White rabbits

For the initial immunization, 25 µg of rec. human MIF obtained as described in Example 1 were diluted in 100 µl PBS was mixed with 100 µl CFA (Complete Freunds Adjuvants). 200 µl (4 x 50 µl) of the mixture was applied s.c. to different body portions of each rabbit. After 2-3 weeks after the initial immunization a first boost with 25 µg of the rec. human MIF (suspended in 100 µl PBS) was mixed with 100 µl IFA (Incomplete Freunds Adjuvants). Again, 200 µl (4 x 50 µl) of the mixture was applied s.c. to different body portions of each rabbit. A second boost was performed 2-3 weeks after the first boost, 25 µg of the rec. human MIF (suspended in 100 µl PBS) was mixed with 100 µl IFA (Incomplete Freunds Adjuvants). Again, 200 µl (4 x 50 µl) of the mixture was applied s.c. to different body portions of each rabbit. The immunization procedure was terminated 2 weeks after the second boost. Typically, plasma from multiple rabbits was pooled and used for the isolation of the anti MIF antibodies.

Protein A Purification and huMIF-affinity purification procedure of polyclonal rabbit anti huMIF antibodies

The isolation of rabbit anti huMIF antibodies from immunized plasma was typically done by two affinity chromatography steps. At first the plasma was purified by a Protein A affinity column (MabSelect Sure, GE Healthcare).To that avail, the rabbit plasma was diluted 1:3 with 20 mM $Na_2HPO_4$ buffer, pH 7.0 and applied to the affinity column. After a washing step (5 column volumes with 20 mM $Na_2HPO_4$ buffer, pH 7.0) the elution of total rabbit IgG was done with 100 mM glycine, pH 2.8. The eluate was pooled and neutralized to pH 7.0 using 1 M Tris/HCl. For hu-MIF affinity purification the total rabbit IgG was again diluted 1:3 with 20 mM $Na_2HPO_4$ buffer, pH 7.0 and applied to the 5 ml NHS-affinity column (GE Healthcare) coupled with 25 mg rhuMIF as recommended by the supplier. After a washing step (5 column volumes with 20 mM $Na_2HPO_4$ buffer, pH 7.0) the elution of the specific rabbit anti huMIF antibodies was effected with 100 mM glycine, pH 2.8. The eluate was pooled and neutralized to pH 7.0 using 1 M Tris/HCl. Finally, the hu-MIF affinity purified

specific rabbit anti human MIF antibodies (in the following "anti-human MIF affinity purified polyclonal antibody") were dialyzed against PBS and stored at -20°C.

**EXAMPLES**

The following examples demonstrate, *inter alia*, that the presence of cysteine 81 (C81) of MIF is essential for the switch of MIF to a disease-related state, i.e. the redox-switch from redMIF to oxMIF, and that derivatisation of the C81 sulfhydryl group promotes (or is responsible for) this switch.

**Example 1 Preparation of recombinant wild-type and mutant Forms of MIF**

Wild-type and cysteine-mutant recombinant fusion constructs of human MIF (huMIF) with streptavidin binding peptide (SPB) tag via a $(GGGGS)_2$-linker (cf. SEQ ID NO: 16), as shown in Figure 1, were prepared, cloned and expressed by standard methods. Briefly, synthetic DNA (Invitrogen, GeneArt) was cloned in pet25b and expressed in *E. coli* Shuffle T7 Express (NEB). Soluble protein was extracted using BugBuster® Protein Extraction Master Mix (Novagen) and purified by affinity chromatography via streptavidin column and biotin elution. Further purification was done using size exclusion chromatography. The sequence of the streptavidin binding peptide (SPB) tag is: MDEKTTGWRGGHVVEGLAGELEQLRARLEHHPQGQREP (SEQ ID NO: 17). A wild-type human MIF-fusion with SBP (SEQ ID NOs: 18) and three cysteine mutant SBP-fusion constructs with single mutations of cysteine to serine at positions 57, 60 and 81 of MIF (i.e. the single mutants C57S, C60S and C81S – SEQ ID NOs: 19-21, respectively) were thus prepared.

Recombinant wild-type untagged huMIF (SEQ ID NO: 15) was produced in *E.coli* cells including an expression system with the human MIF sequence. Fresh thaw cells were cultivated in Luria Bertani medium supplemented with Ampicillin (LB/Amp) over night at +37°C. At the next day, the bacterial cell culture was diluted with an equal volume of fresh LB/Amp medium and the expression induced by addition of IPTG (final concentration: 1.0 mM) at 30°C for 4 hours. The bacterial pellet was harvested by centrifugation and stored at $\leq$-15°C.

For further purification of the intracellular human MIF proteins the frozen bacterial pellet was resuspended in 20 mM Tris/HCl buffer, pH 7.8 and cells were disrupted mechanically by glass beads. Cell debris was removed by centrifugation and filtration using a common 0.2 µm filter. The supernatant was directly applied to an anion exchange chromatography column (HiTrap 26/16 DEAE FF, GE Healthcare, Waukesha, USA) and MIF was purified by a passive binding mode. The flow through was rebuffered in 20 mM Bis/Tris pH 6.3 and further purified by a cation exchange chromatography (Source 30S, GE). Highly pure human MIF was eluted by a salt gradient of 50 mM NaCl in 20 mM Bis/Tris buffer, pH 6.3. Finally, the purified human MIF was rebuffered against PBS concentrated by ultrafiltration and characterized of purity and functionality.

**Example 2 Western blot probing of MIF cysteine mutants with anti-oxMIF antibodies**

RAM9 is a monoclonal antibody described herein above and also in WO2013/050453, which binds preferentially to the oxidized, disease-associated form of MIF (oxMIF), compared to the reduced form of MIF (redMIF). RAM0 is a monoclonal antibody described herein above and also in WO2013/050453, which binds preferentially to the oxidized, disease-associated form of MIF (oxMIF), compared to the reduced form of MIF

(redMIF). Under physiological conditions, the binding of the antibodies to MIF occurs only following the redox-dependent (and reversible) transition of MIF to oxMIF. However, under non-physiological or denaturing conditions, MIF irreversibly changes its structure, which also leads to antibody binding. Thus, e.g. immobilizing MIF on plastic or treatment with strong detergents (SDS) enables binding of the antibodies. The antibodies RAM9 and RAM0 were thus used in Western blot assays performed according to standard procedures in order to probe the MIF constructs of Example 1 under denaturing conditions. The results are shown in Figure 2. The SDS-PAGE, upon which the Western blot was performed, was performed under reducing conditions. The results show that C57 and C60, but not C81, are of fundamental importance for binding of RAM9 to MIF in a linear molecule. Residual binding of RAM9 at the highest MIF concentrations was insignificant compared to its binding to wild-type or in the case of the C81S mutation. In contrast, RAM0 binding to MIF as a linear molecule is not substantially affected by any of the three cysteine mutations, and detects the wild-type constructs and all mutants substantially equally.

**Example 3 Effects of mutations, sulfhydryl derivatisation and the lack of derivatisation of different cysteine residues**

The antibodies RAM9 and RAM0 were used as immobilised capture antibodies in an ELISA assay to probe the effects of cysteine-to-serine mutations, sulfhydryl derivatisation and the lack of derivatisation of different cysteine residues on the formation of the oxidized, disease-associated form of MIF (oxMIF). Examples of sulfhydryl-reactive reagents for derivatisation of sulfhydryl groups in MIF are shown in Figure 4. The general setup of an ELISA assay is shown in Figure 5.

The binding of wild-type human MIF in its reduced form (MIF), in cysteinylated form (Cys-MIF) or when derivatised using DTNB (DTNB-MIF) in the ELISA assay to the antibody RAM9 is shown in Figure 6. In accordance with the disclosure in WO2013/050453, RAM9 does not bind to the reduced form of MIF. However, RAM9 shows significant binding to both cysteinylated and DTNB-derivatised MIF. As RAM9 is specific for the disease-associated form described as oxMIF e.g. in WO2013/050453, this shows that derivatisation of MIF with either cysteine (by treatment with cystine) or a 2-nitro-5-thiobenzoic acid (NTB) moiety (by treatment with DTNB – leading to "DTNB-derivatised MIF" or MIF+NTB) induces the disease-associated oxMIF form of the protein. Equivalent results were obtained with RAM0.

The assessment by ELISA of the binding of MIF in its wild-type and the mutant forms, either in a reduced / unmodified form ("MIF", "MIF(wt)SBP", "MIF(C57S)SBP", etc.); when derivatised with DTNB and assessed under non-reducing conditions ("DTNB: +"), or when derivatised with DTNB and assessed under reducing conditions ("DTNB +, DTT +") to the antibodies RAM9 and RAM0 is shown in Figure 7 and Figure 8 respectively.

These data again confirm that modification of sulfhydryl groups by the sulfhydryl-reactive compound DTNB promotes the formation of the disease-associated oxMIF to which the antibodies RAM9 and RAM0 preferentially bind. For RAM9 this effect is not observed when C57 or C60 are replaced by serine. However, it appears that this is because C60 and C57 *per se* are essential for binding of RAM9 as they are part of RAM9 linear epitope (described in Kerschbaumer et al. J Biol Chem. 2012 Mar 2;287(10):7446-55). It is not

due to a lack of oxMIF formation in MIF(C57S)SBP -DTNB and MIF(C60S)SBP -DTNB. The data show that oxMIF is still formed when C57 or C60 are replaced by serine, because derivatisation of these mutants by DTNB still strongly promotes the binding of RAM0. The latter oxMIF-specific antibody preferentially binds MIF(C57S)SBP -DTNB and MIF(C60S)SBP -DTNB to a degree that is comparable to its binding to wtMIF that has been converted into oxMIF (cf. MIF-DTNB and MIF(wt)SBP-DTNB). In contrast, the data show that C81, and the derivatisation of its sulfhydryl group, plays a much more fundamental role than C57 or C60 in the formation of the disease-related oxMIF form of the protein: When C81 is replaced by serine, treatment of the mutant MIF with DTNB does not enhance the binding of either of the two oxMIF-specific antibodies in comparison to the non-derivatised form (cf. MIF(C81S)SBP and MIF(C81S)SBP-DTNB). Mass spectroscopy data (see below, Example 4) show that C57 and C60 are not modified in this case.
The data also show that all oxMIF species which are induced by DTNB in wild-type and C57S or C60S mutants can be reduced by DTT.

**Example 4 Mass-spectroscopic analysis of derivatised MIF and cysteine mutants**

Derivatisation of MIF and its cysteine mutants with cysteine or DTNB was confirmed by mass-spectroscopic analysis.

Data obtained by MALDI-TOF for full-length proteins such as those represented in Figure 8 show that cysteinylation or derivatisation with DTNB give rise to peaks reflecting correspondingly increased molecular weights.

From a sample of cysteinylated human MIF the most intense signal was the singly protonated molecule ion at m/z 12470, followed by the 2-fold protonated ion at m/z 6235, corresponding to a mass of 12468 Da. This corresponds very well to the theoretical mass of the human MIF sequence without the N-terminal methionine and with a single cysteinylation (+ 119 Da). Non-cysteinylated human MIF was visible as a weaker signal at m/z 12352. Additional peaks at m/z 12565 and m/z 12684 were attributed to adducts with the sinapinic acid of the matrix.

From a sample of human MIF derivatized with DTNB, the strongest signals were the singly protonated molecule ions at m/z 12351 (unmodified) and m/z 12549 (with an NTB moiety, +197 Da), as well as the 2-fold protonated ions at m/z 6177 and m/z 6275, corresponding to a mass of 12352 Da (unmodified) and 12549 Da (with an NTB moiety), respectively. The signal at m/z 12760 was attributed to an adduct with sinapinic acid, m/z 24693 was a dimer, and two further signals at m/z 11868 and m/z 18083 were not explained.

From a sample of human wild-type MIF fused to SBP created with DTNB (MIF wt-SBP + NTB) the strongest signals were the singly protonated molecule ions at m/z 17269 (unmodified) and m/z 17467 (with DTNB, +197 Da), as well as the 2-fold protonated ions at m/z 8636 and m/z 8732, corresponding to a mass of 17270 Da (unmodified) and 17462 Da (with DTNB), respectively. A signal at m/z 17675 was attributed to an adduct with sinapinic acid.

From a sample of human MIF(C57S)SBP created with DTNB, the strongest signals were the singly protonated molecule ions at m/z 17251 (unmodified), m/z 17453 (with DTNB) and m/z 17651 (with 2 DTNB). The 2-fold

protonated ions at m/z 8627, m/z 8726 and m/z 8824 correspond to masses of 17252 Da, 17450 Da and 17646 Da.

From a sample of human MIF(C60S)SBP-DTNB the strongest signal was the singly protonated molecule ion at m/z 17246. This corresponds very well to the theoretical mass 17247 of the sequence. The signal at m/z 17460 was assigned to the protein modified with DTNB and additional oxidation. The corresponding 2-fold protonated ions were visible at m/z 8624 (17246 Da) and m/z 8731 (17460 Da).

A sample of human MIF(C81S)SBP created with DTNB did not yield any signals indicating derivatisation of the protein. The single main peak at m/z 17249 corresponds to the singly protonated unmodified molecule ion.

Peptide mass fingerprinting (mass-spectroscopic analysis following trypsin digestion of MIF) of sulfhydryl modified MIF allows the derivatisation to be located to a particular cysteine residue, in particular cysteine 81. The peptides expected from trypsin digestion of human MIF are shown in the following Table 1:

**Table 1**

| # | RESIDUES | EXPECTED MH+ | SEQ ID NO: | SEQUENCE |
|---|---|---|---|---|
| | | | | |
| 1 | 2 – 12 | 1287.7 | 22 | <PMFIVNTNVPR> |
| 2 | 13 – 67 | 5693.8 | 23 | <ASVPDGFLSELTQQLAQATGKPPQY IAVHVVPDQLMAFGGSSEPCALCSL HSIGK> |
| 3 | 68 – 74 | 715.4 | 24 | <IGGAQNR> |
| 4 | 75 – 78 | 484.2 | 25 | <SYSK> |
| 5 | 79 – 87 | 987.6 | 26 | <LLCGLLAER> |
| 6 | 88 – 89 | 288.2 | -- | <LR> |
| 7 | 90 – 94 | 587.3 | 27 | <ISPDR> |
| 8 | 95 – 115 | 2427.1 | 28 | <VYINYYDMNAANVGWNNSTFA> |

Following such tryptic digestion of cysteinylated MIF and mass-spectroscopic analysis of the resulting peptides by the MALDI-TOF method using a 4800 Proteomics Analyzer (AB Sciex), the peptide consisting of residues 79-87 (LLCGLLAER) was identified as being cysteinylated. Apart from the signal at $MH^+$ 987.57 that is expected for this peptide in unmodified form, a signal was also observed at $MH^+$ 1106.57, corresponding to this peptide with an additional mass of +119.00 Da, i.e. an additional mass corresponding to cysteine (see Figure 9 and Figure 10). In the peptide with residues 13-67, which contains cysteines 57 and 60, no additional mass, i.e. no cysteinylation, was observed.

Following such tryptic digestion of MIF that had been treated with DTNB and mass-spectroscopic analysis of the resulting peptides by the MALDI-TOF method using a 4800 Proteomics Analyzer (AB Sciex), the peptide consisting of residues 79-87 (LLCGLLAER) was identified as being modified by the DTNB treatment. Apart from the signal at $MH^+$ 987.57 that is expected for this peptide in unmodified form, a signal was also observed at $MH^+$ 1184.54, corresponding to this peptide with an additional mass of +196.97 Da, i.e. an additional mass corresponding to a 2-nitro-5-thiobenzoic acid moiety (see Figure 11 and Figure 12). In the peptide with resides 13-67, which contains cysteines 57 and 60, no additional mass, i.e. no 2-nitro-5-thiobenzoic acid modification, was observed.

Thus, the peptide mass fingerprinting data confirm derivatisation of cysteine 81 in both cases.

**Example 5 Production of C81-modified-MIF-specific antibodies**

Antibodies are produced in mammalian cells, preferentially in CHO cells, preferentially in CHO cells where the gene encoding for MIF (endogenous CHO-MIF) has been knocked out genetically. In the knock-out cells the contamination of the antibody with endogenous CHO-MIF can be abolished, which is desirable as sensitivity of the assays can be enhanced.

Typically, C81-modified MIF specific antibodies are produced in a batch fermentation process using a disposal bioreactor (wave system) up to 25 L volume. Stable CHO cell lines harboring the genes encoding for the heavy and light chain of the produced antibody, respectively, are seeded into an PowerCHO medium (Invitrogen Inc.) and incubated at 37°C and 5% $CO_2$.

During the cultivation, the respective human antibodies are continuously expressed into the cell culture medium. At the end of cultivation (viability <50%) the cells are separated by common centrifugation and filtration steps. The clarified cell culture supernatant (ccs) is concentrated by ultrafiltration and used for the purification of antibodies.

The human antibodies are purified from the concentrated ccs by Protein A affinity chromatography (MabSelect Sure, GE Healthcare). After equilibration of the Protein A material with 5 column-volumes (cv) of 20 mM sodium phosphate running buffer, pH 7 the concentrated supernatant of the isotype control is completely applied to the affinity column. Impurities or undesirable proteins are washed out with the running buffer. The antibodies are eluted by a pH shift using 100 mM glycine, pH 3 and dialyzed against 250 mM glycine buffer, pH 5.

Alternatively, the concentrated cell culture supernatant may be applied to the Protein A column prior equilibrated with 5 cv of 20 mM Tris/HCl buffer including 150 mM sodium chloride buffer and 0.1% Tween 80, pH 7. Impurities may be washed out by two washing steps: 1.) addition of 1 M NaCl in the equilibration buffer and 2.) 100 mM sodium phosphate including 0.1% Tween 80, pH 5. Antibodies may be eluted by 100 mM glycine buffer, pH 3 including 0.1% Tween 80 and then dialyzed against 250 mM glycine buffer, pH 5.

All references cited herein are herewith incorporated by reference in their entirety.

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## CLAIMS

1. A method of treating or preventing a MIF-related disorder in a subject in need thereof by administering to said subject an effective amount of a compound that
   (a) prevents modification of MIF at position 81, or
   (b) binds preferentially to MIF carrying a modification at position 81 compared to MIF which does not contain said modification of position 81.
2. The use of MIF carrying a modification at position 81(of SEQ ID NO:15) as a target for the treatment or prevention of a MIF-related disorder, or as a target for a drug discovery assay.
3. A method of monitoring the efficacy of a treatment of a MIF-related disorder, comprising a step of determining in samples isolated from a subject before and after said treatment whether MIF position 81 carries a modification, wherein the treatment is identified as effective if MIF position 81 is modified to a lesser extent after treatment than before treatment.
4. An anti-MIF antibody or molecule comprising an antigen-binding portion of an antibody which antibody or molecule binds a MIF which is modified at position 81 but does not bind MIF which is not modified at position 81.
5. A method of assaying a test compound for preferential binding to a form of MIF which is modified at position 81, said method comprising the steps of
   (a) modifying MIF at position 81 to obtain modified MIF,
   (b) combining a compound to be tested with said modified MIF in a test sample and in a control sample in which MIF is not modified at position 81,
   (c) assessing binding of said compound to the modified MIF in the test sample and in the control sample

   wherein the compound is selected if said compound binds to the modified MIF in the test sample to a greater extent than in the control sample.
6. A method of screening for a compound that prevents the modification of MIF at cysteine 81, said method comprising the steps of
   (a) combining MIF with a compound to be tested in a test sample,
   (b) treating MIF with a sulfhydryl-reactive reagent in the test sample containing the compound and in a control sample from which the compound is absent, under conditions in which the reagent modifies the sulfhydryl group of cysteine 81 of MIF at least in the control sample,
   (c) assessing modification of cysteine 81 by the sulfhydryl-reactive reagent,

   wherein the compound is selected if the sulfhydryl-reactive reagent modifies cysteine 81 to a lesser extent in the test sample than in the control sample.
7. A method according to any one of claims 1, 2, or 6, wherein said compound is an antibody or a molecule comprising an antigen-binding portion of an antibody.
8. The use of the modification state of MIF position 81 as a diagnostic marker, wherein a finding that MIF position 81 carries a modification is indicative of a MIF-related disorder.

9. A method of diagnosing a MIF-related disorder, comprising a step of determining in a sample isolated from a subject whether MIF position 81 carries a modification, wherein, MIF position 81 is identified as being modified, the subject is diagnosed with a MIF-related disorder, or as being susceptible thereto.
10. A method according to any one of claims 1, 3, or 9, or a use according to any one of claims 2 or 10, wherein the MIF-related disorder is a MIF-related disease, a MIF-related disease state, or the state of progression of a MIF-related disease.
11. A use or method according to claim 10, wherein the MIF-related disorder is an inflammatory disease or a neoplastic disease.
12. A use or method according to claim 10, wherein the MIF-related disorder is selected from the group consisting of colon cancer, prostate cancer, bladder cancer, pancreas cancer, ovarian cancer, melanoma, lymphoma, hepatocellular carcinoma, asthma, ARDS, rheumatoid arthritis, sepsis, IgA nephropathy, glomerulonephritis, Lupus Nephritis (LN), hepatitis, pancreatitis (+/- acute lung injury), Crohn's disease, ulcerative colitis, gastric ulcer, Alzheimer's disease, multiple sclerosis, Guillain-Barre syndrome, cardiac dysfunction, angioplasty, atherosclerosis, myocarditis, type 1 diabetes, diabetic retinopathy, age-related macular degeneration (AMD), atopic dermatitis, psoriasis, endometriosis, neuropathic pain and uveitis.
13. A use or method according to Claim 10, wherein the MIF-related disorder is malignant ascites, preferably in a third line treatment.
14. A MIF molecule which is modified on position 81 of SEQ ID NO:15.

Figure 1

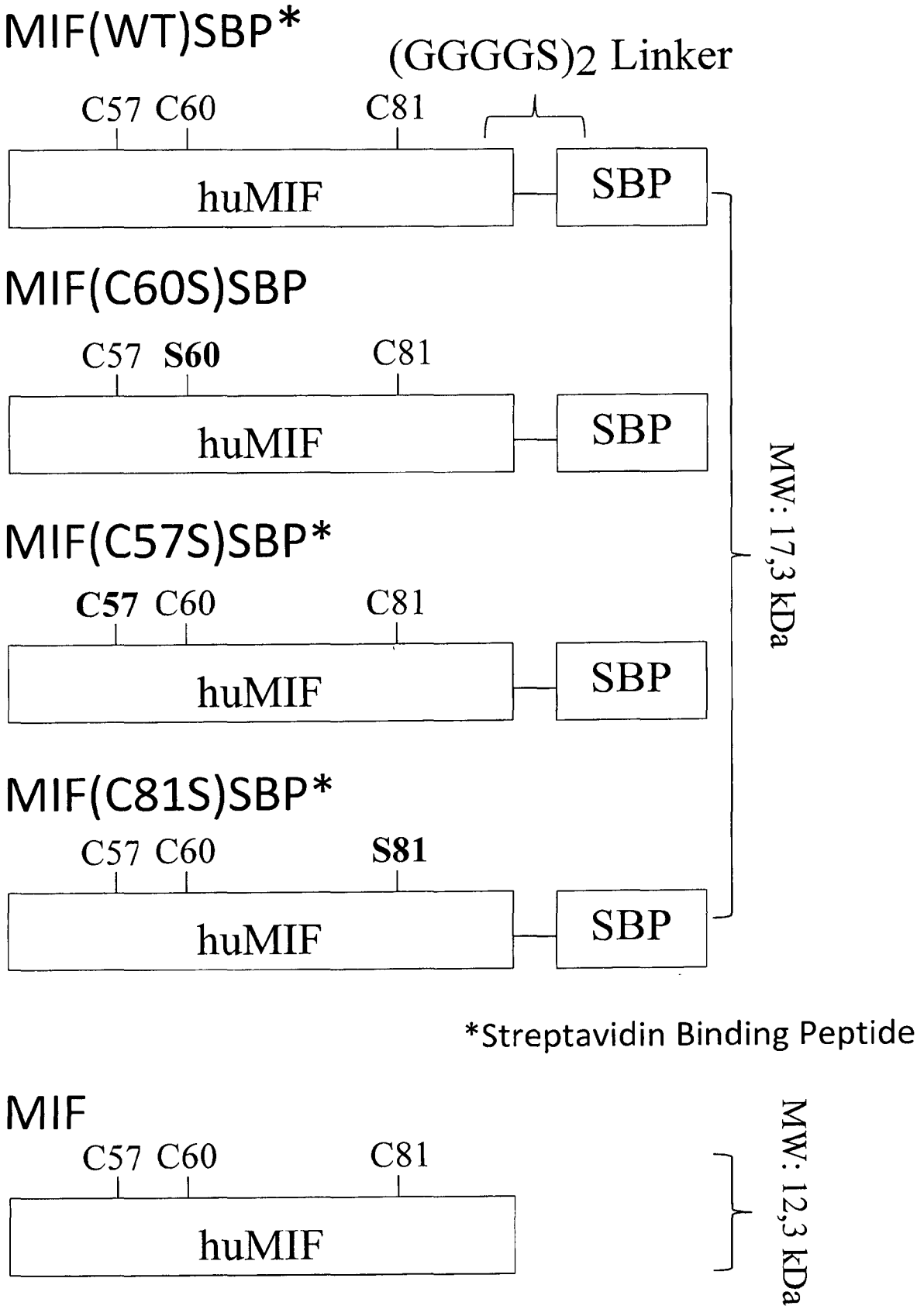
MIF(WT)SBP*
(GGGGS)2 Linker
C57 C60
C81
huMIF
SBP
MIF(C60S)SBP
C57 S60
C81
huMIF
SBP
MIF(C57S)SBP*
C57 C60
C81
huMIF
SBP
MIF(C81S)SBP*
C57 C60
S81
huMIF
SBP
MW: 17,3 kDa
*Streptavidin Binding Peptide
MIF
C57 C60
C81
huMIF
MW: 12,3 kDa

Figure 2

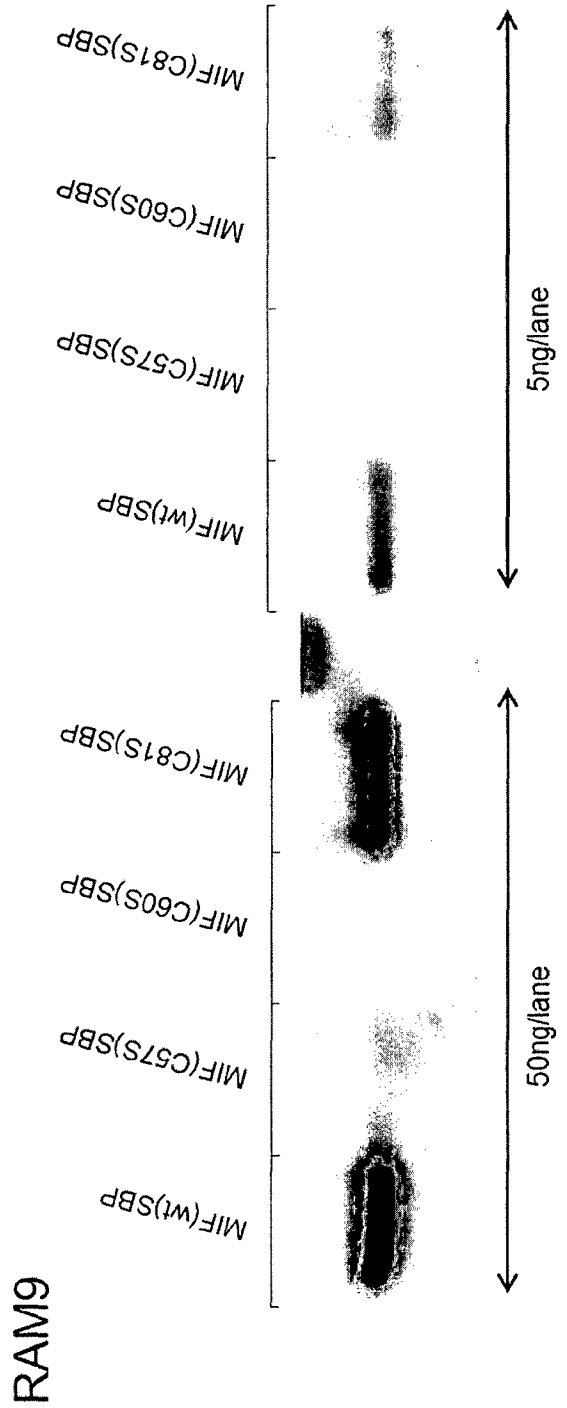
MIF(C81S)SBP
MIF(C60S)SBP
MIF(C57S)SBP
MIF(wt)SBP
5ng/lane
MIF(C81S)SBP
MIF(C60S)SBP
MIF(C57S)SBP
MIF(wt)SBP
50ng/lane
RAM9

**Figure 3**

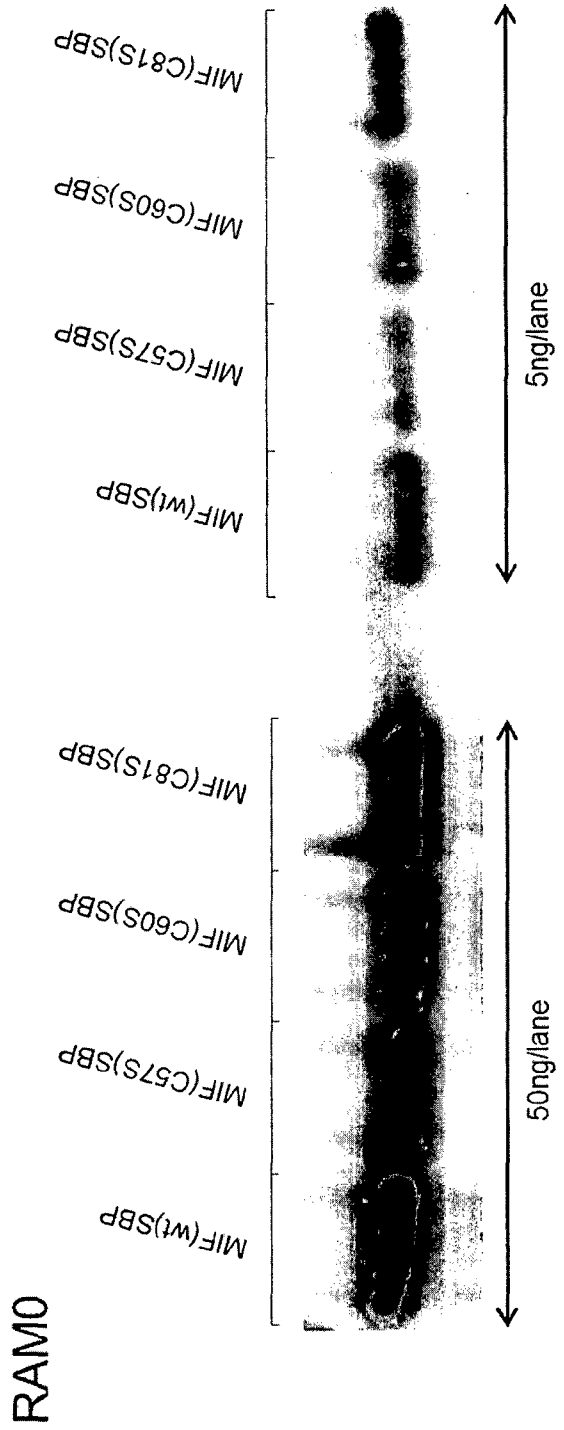
MIF(C81S)SBP
MIF(C60S)SBP
MIF(C57S)SBP
MIF(wt)SBP
5ng/lane
MIF(C81S)SBP
MIF(C60S)SBP
MIF(C57S)SBP
MIF(wt)SBP
50ng/lane
RAM0

Figure 4

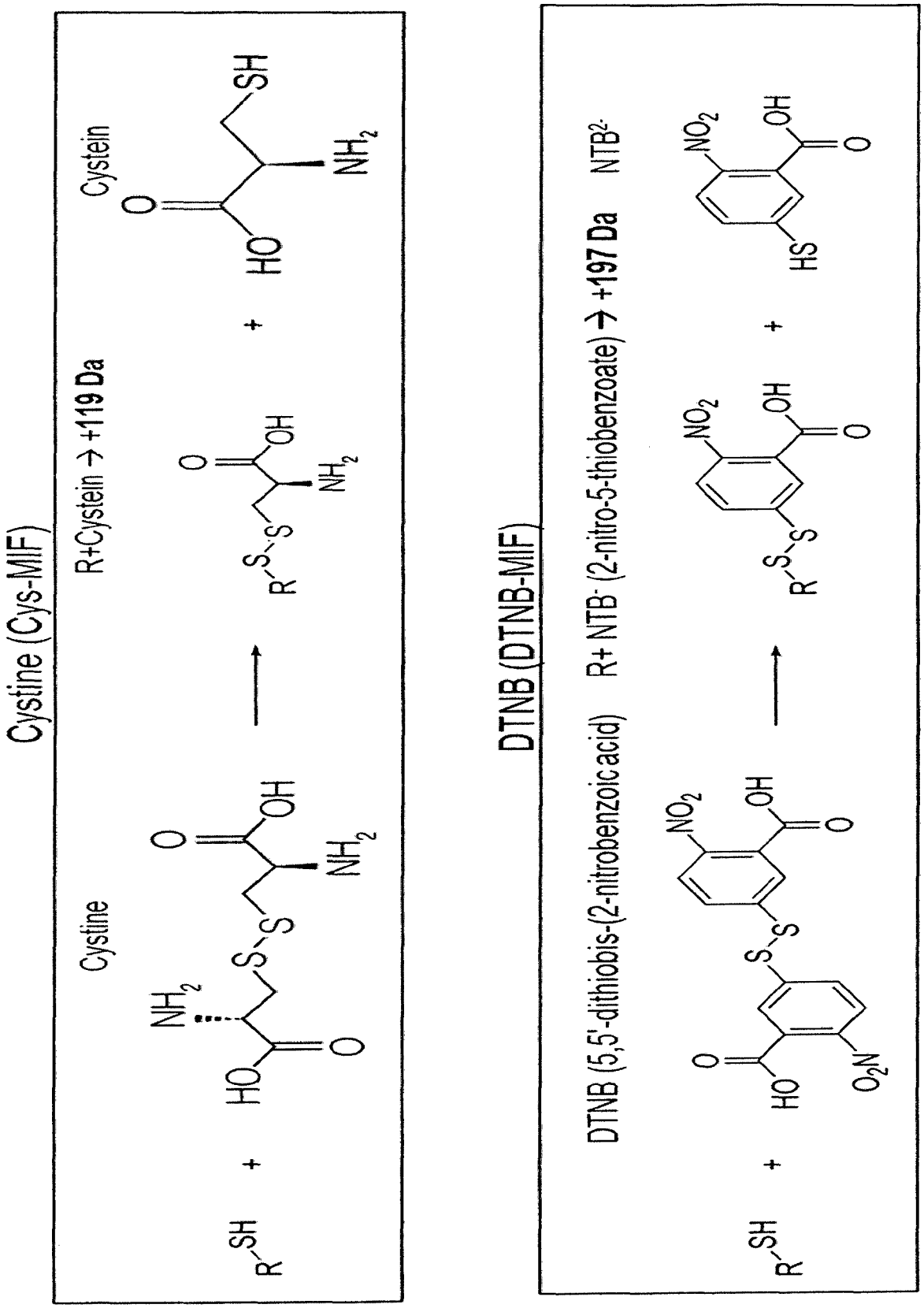
Cystine (Cys-MIF)
Cystine
Cystein
R+Cystein → +119 Da
DTNB (DTNB-MIF)
DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)
R+ NTB⁻ (2-nitro-5-thiobenzoate) → +197 Da
NTB²⁻

Figure 5

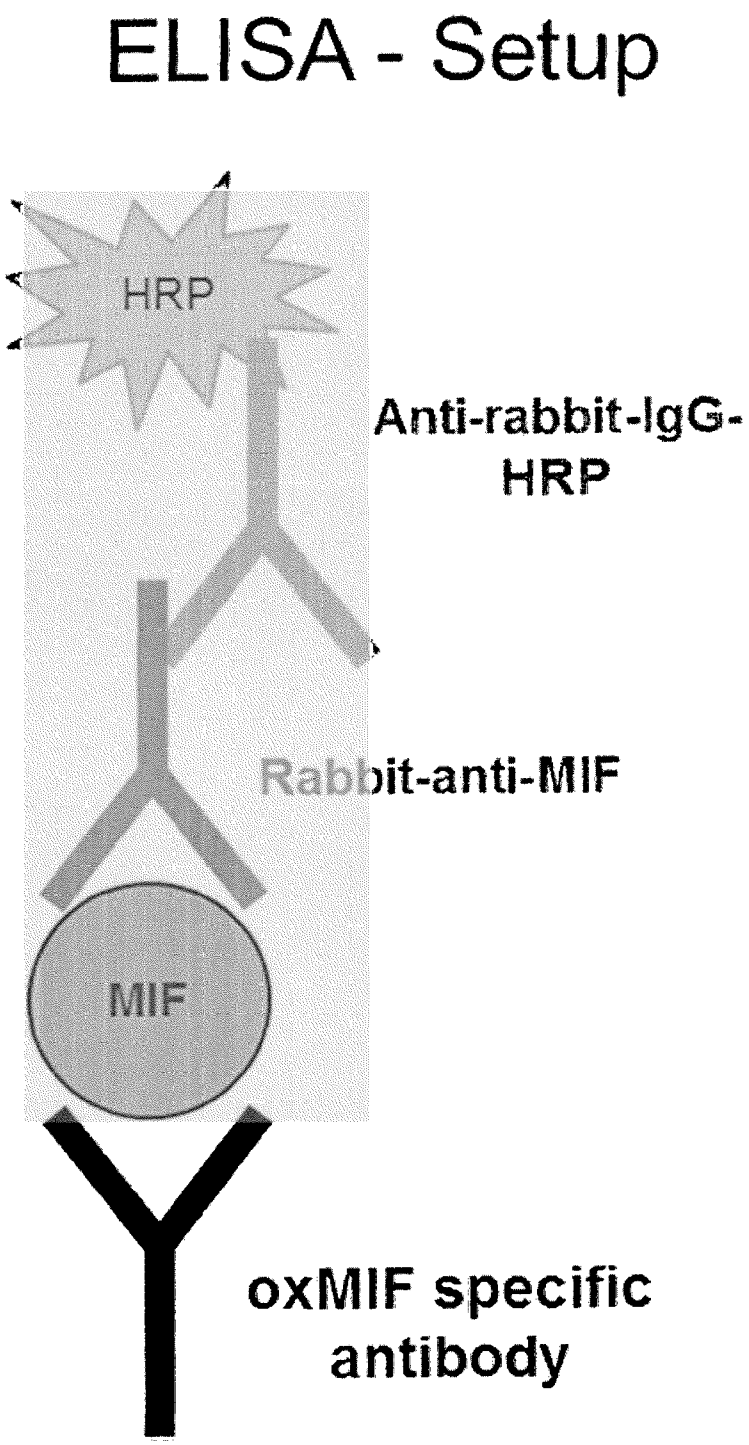
ELISA - Setup
HRP
Anti-rabbit-IgG-HRP
Rabbit-anti-MIF
MIF
oxMIF specific antibody

Figure 6

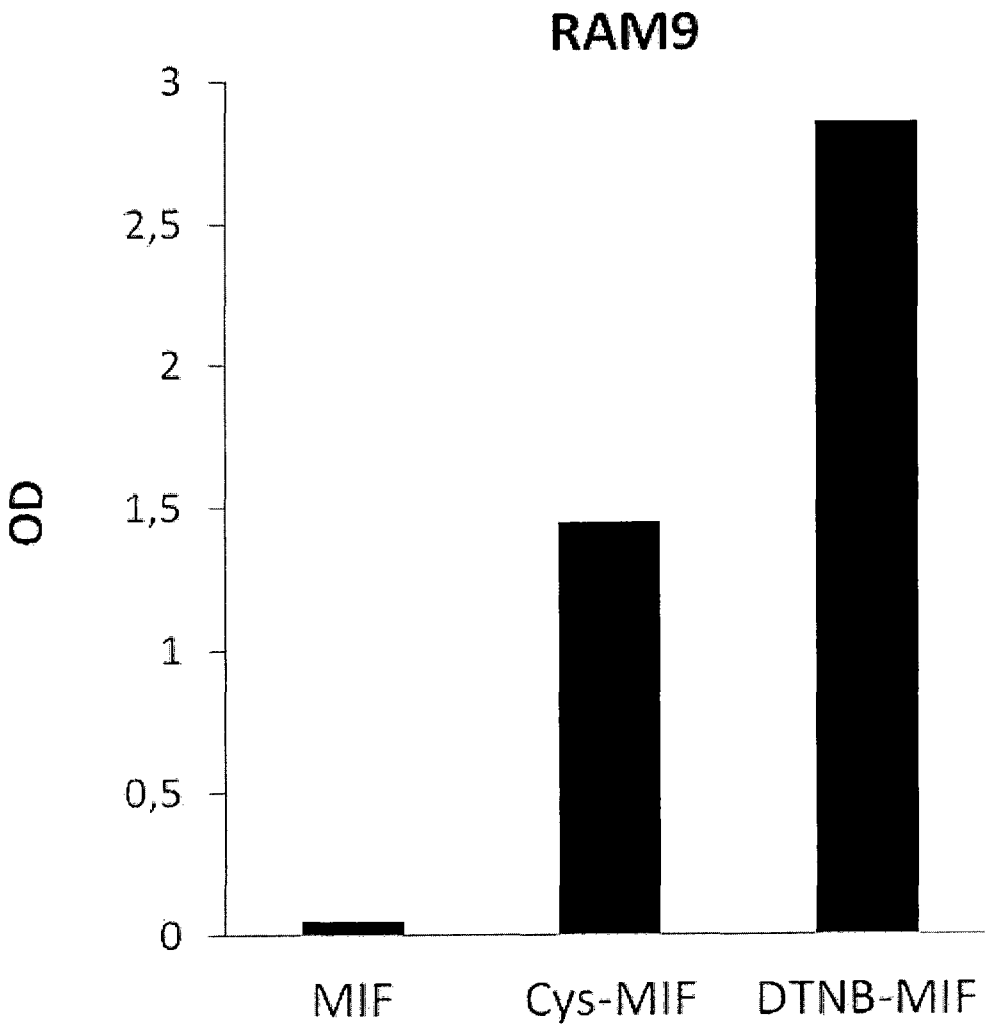

Figure 7

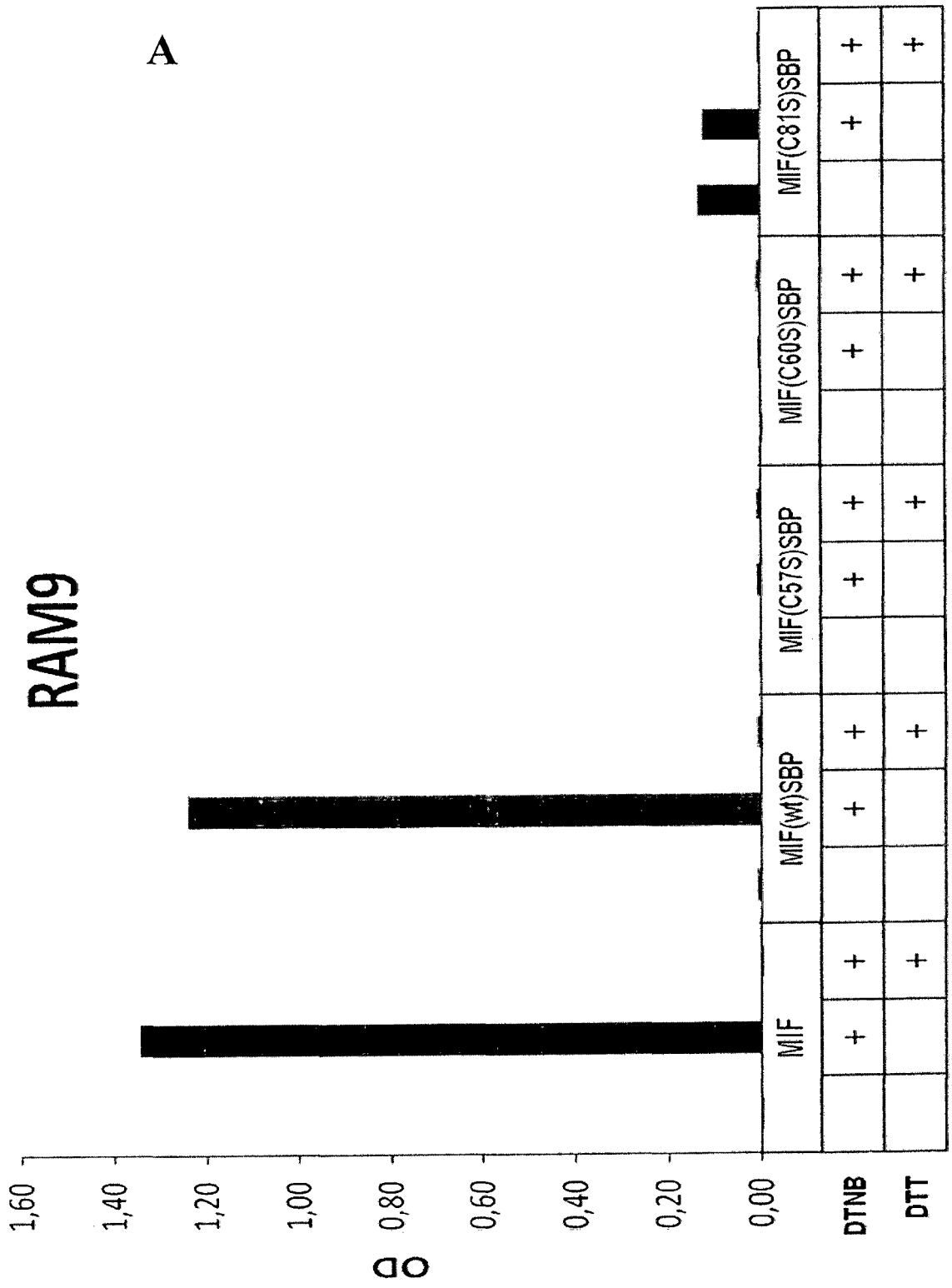
A
RAM9
OD
1,60
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
MIF
MIF(M)SBP
MIF(C57S)SBP
MIF(C60S)SBP
MIF(C81S)SBP
DTNB
DTT

Figure 7 (continued)

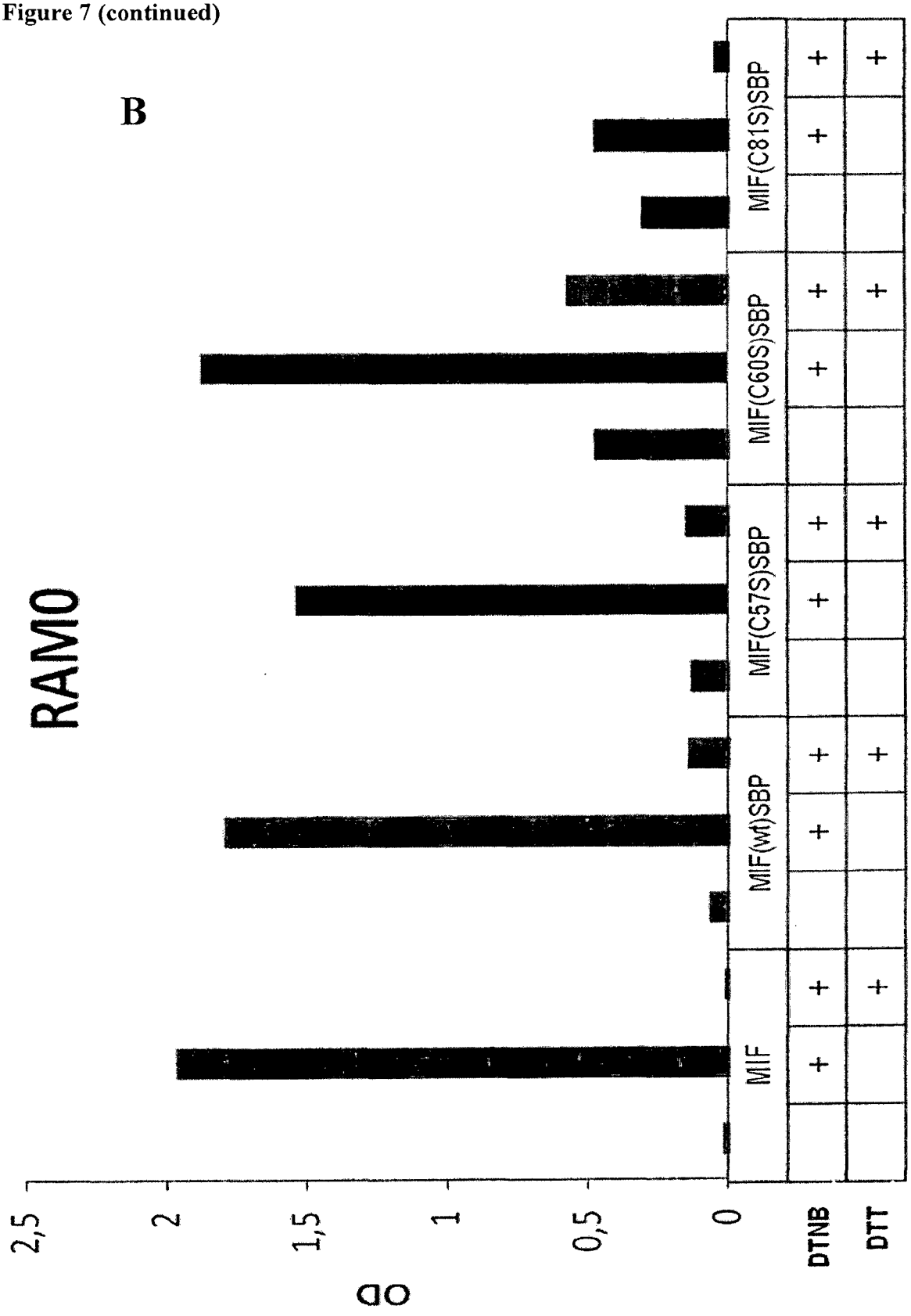

Figure 8

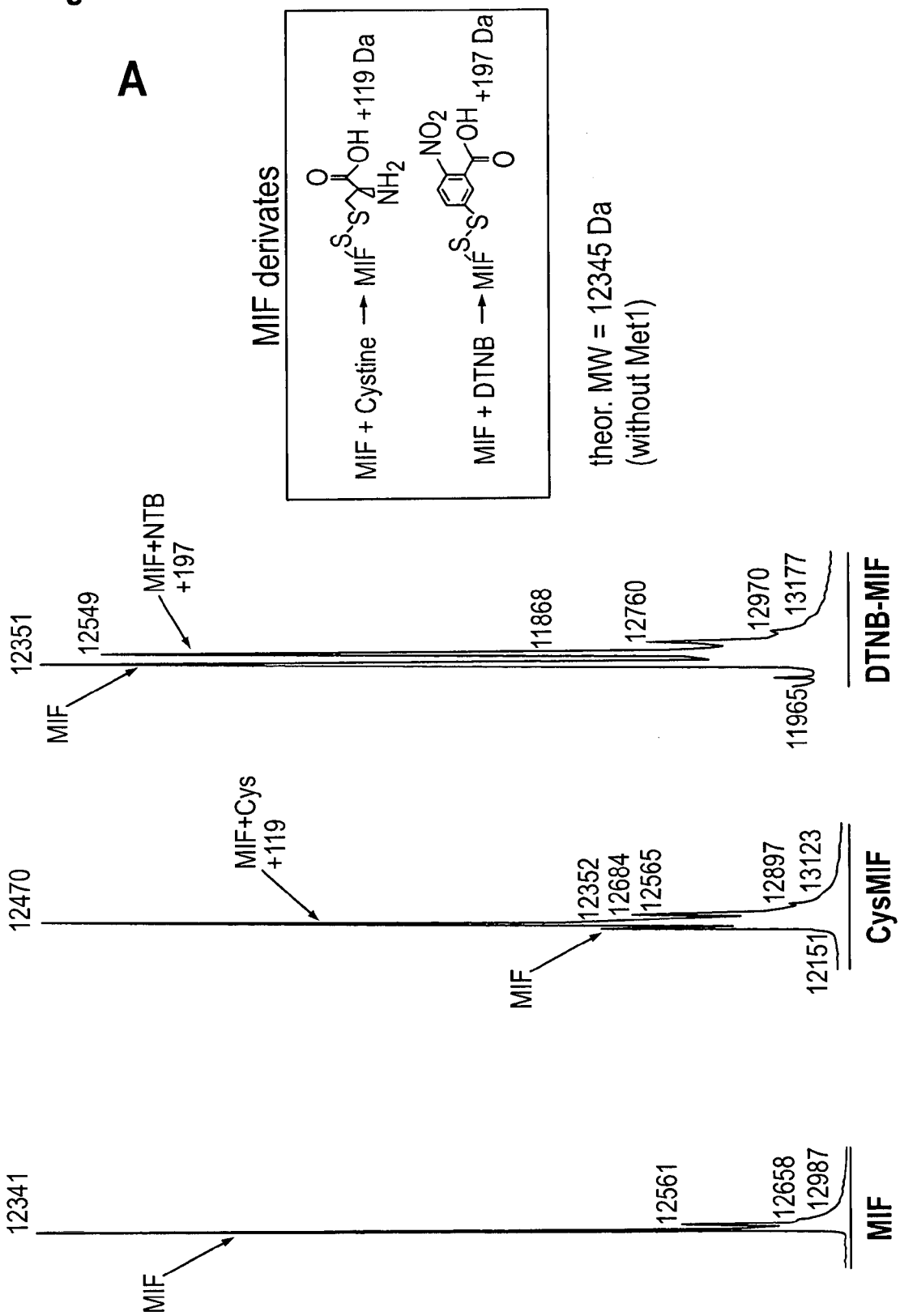
A
MIF derivates
MIF + Cystine → MIF +119 Da
MIF + DTNB → MIF +197 Da
theor. MW = 12345 Da
(without Met1)
MIF
MIF+NTB +197
MIF+Cys +119
12341
12351
12470
12549
12561
12658
12684
12760
12897
12970
12987
13123
13177
11868
11965
12151
12352
12565
DTNB-MIF
CysMIF
MIF

Figure 8
(continued)

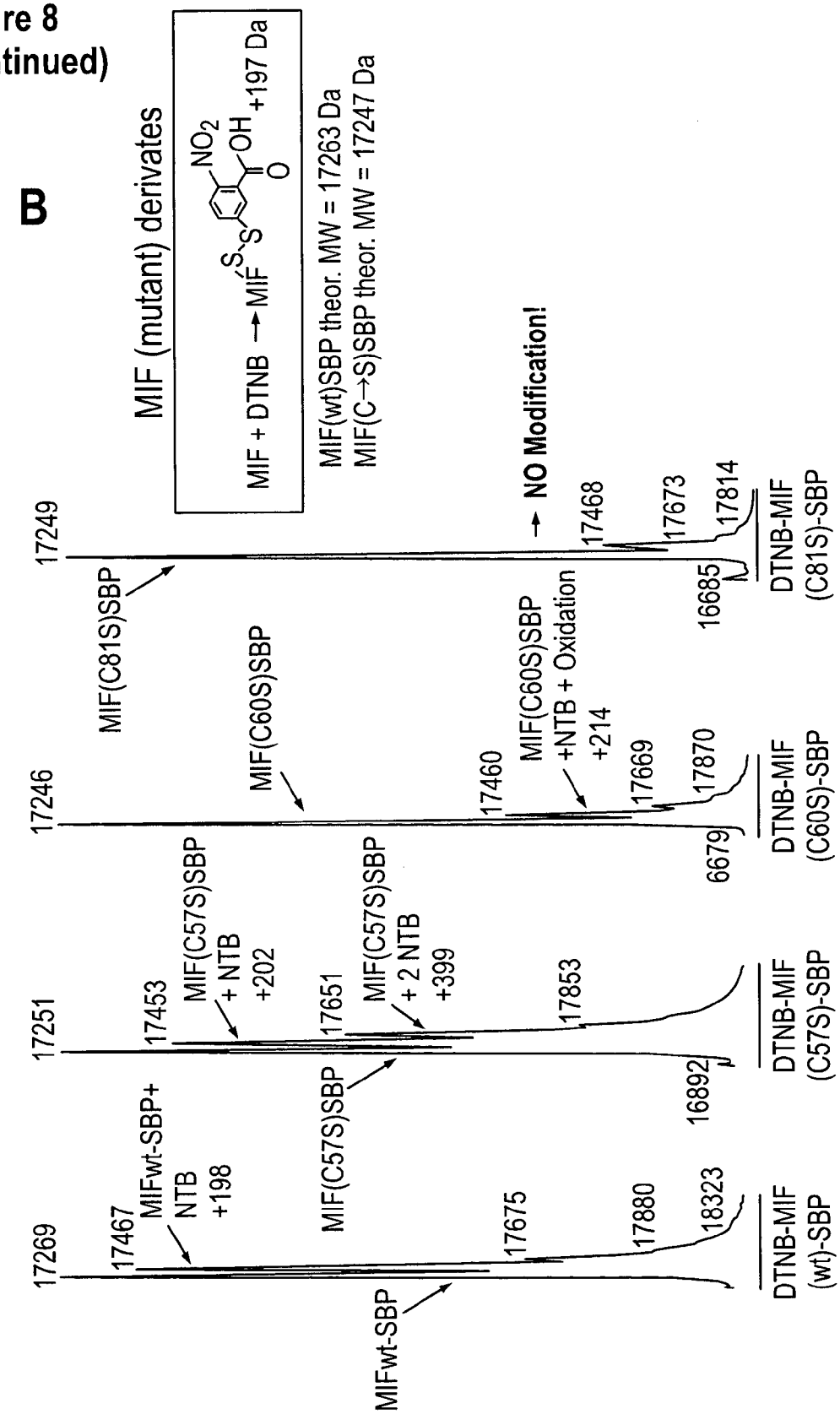

Figure 9

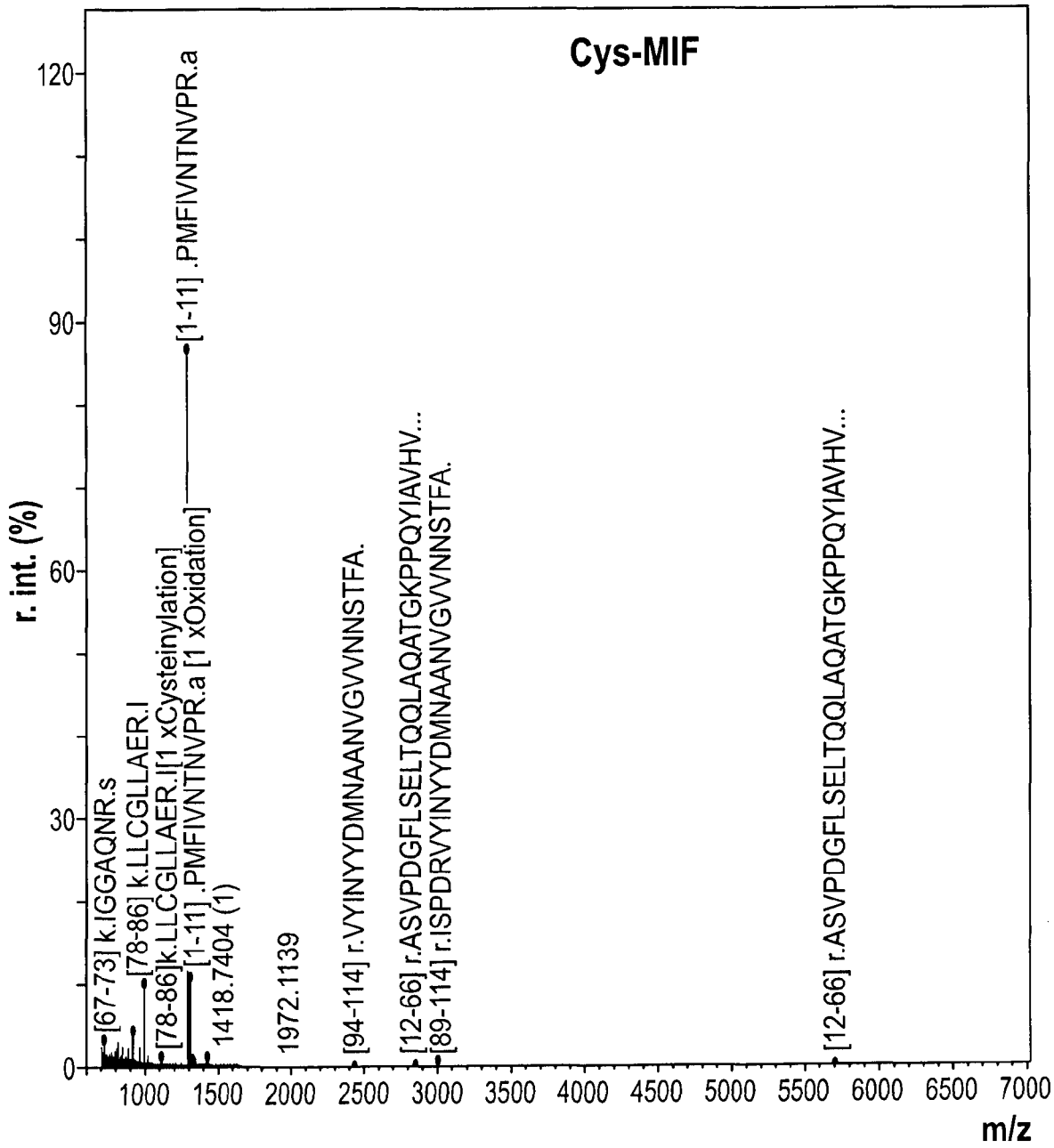
Cys-MIF
r. int. (%)
120
90
60
30
0
1000
1500
2000
2500
3000
3500
4000
4500
5000
5500
6000
6500
7000
m/z
[67-73] k.IGGAQNR.s
[78-86] k.LLCGLLAER.l
[78-86]k.LLCGLLAER.l[1 xCysteinylation]
[1-11] .PMFIVNTNVPR.a [1 xOxidation]
[1-11] .PMFIVNTNVPR.a
1418.7404 (1)
1972.1139
[94-114] r.VYINYYDMNAANVGVVNNSTFA.
[12-66] r.ASVPDGFLSELTQQLAQATGKPPQYIAVHV...
[89-114] r.ISPDRVYINYYDMNAANVGVVNNSTFA.
[12-66] r.ASVPDGFLSELTQQLAQATGKPPQYIAVHV...

Figure 10

| Accession | Length | Mo. Mass | Av. Mass | Coverage | Matched Int. |
|---|---|---|---|---|---|
| | 114 | 12784.1137 | 12792.3862 | 94.7% | 85.1% |
| PMFIVNTNVP RASVPDGFLS ELTQQLAQAT GKPPQYIAVH VVPDQLMAFG GSSEPCALCS LHSIGKIGGA QNRSYSKLLC GLLAERLRIS PDRVYINYYD MNAANVGWNN STFA | | | | | |

| Position | Modification | Type | Mo. Mass | Av. Mass | Formula |
|---|---|---|---|---|---|
| All M | Oxidation | variable | 15.9949 | 15.9994 | O |
| All C | Cysteinylation | variable | 119.0041 | 119.1435 | C3H7NO2S - H2 |
| All C | Oxidation | variable | 15.9949 | 15.9994 | O |
| All C | Disulfide | variable | -2.0157 | -2.0159 | - HH |

Figure 10 (continued)

| Meas. m/z | Calc. m/z | δ (Da) | δ (ppm) | Rel. Int. (%) | z | Annotation | Formula |
|---|---|---|---|---|---|---|---|
| 715.3700 | 715.3846 | -0.0146 | -20.4 | 3.48 | 1 | [67-73]k.IGGAQNR. s | C28H50N12O10 |
| 987.5681 | 987.5656 | 0.0026 | 2.6 | 11.46 | 1 | [78-86]k.LLCGLLAE R.l | C43H78N12O12S |
| 1106.5764 | 1106.5697 | 0.0067 | 6.1 | 1.14 | 1 | [78-86]k.LLCGLLAER .l[1xCysteinylation] | C46H83N13O14S2 |
| 1287.6936 | 1287.6878 | 0.0058 | 4.5 | 100.00 | 1 | [1-11].PMFIVNTNV PR.a | C58H94N16O15S |
| 1303.6881 | 1303.6827 | 0.0054 | 4.2 | 12.52 | 1 | [1-11].PMFIVNTNV PR.a[1xOxidation] | C58H94N16O16S |
| 2427.0635 | 2427.0710 | -0.0075 | -3.1 | 0.32 | 1 | [94-114]r.VYINYYDM NAANVGWNNSTFA. | C110H151N27O34S |

Figure 10 (continued)

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 2846.4478 | 2846.4179 | 0.0299 | 10.5 | 0.11 | 2 | [12-66]r.ASVPDGFL SELTQQLAQATGKPP QYIAVHVVPDQLMAF GGSSEPCALCSLHSIGK .i[1xDisulfide] | C252H396N66O78S3 |
| 2995.3380 | 2995.3679 | -0.0299 | -10.0 | 1.22 | 1 | [89-114]r.ISPDRVYI NYYDMNAANVGWN NSTFA.[1xOxidation] | C134H191N35O42S |
| 5691.8299 | 5691.8284 | 0.0015 | 0.3 | 0.11 | 1 | [12-66]r.ASVPDGFL SELTQQLAQATGKPP QYIAVHVVPDQLMAF GGSSEPCALCSLHSIGK .i[1xDisulfide] | C252H396N66O78S3 |

Figure 11

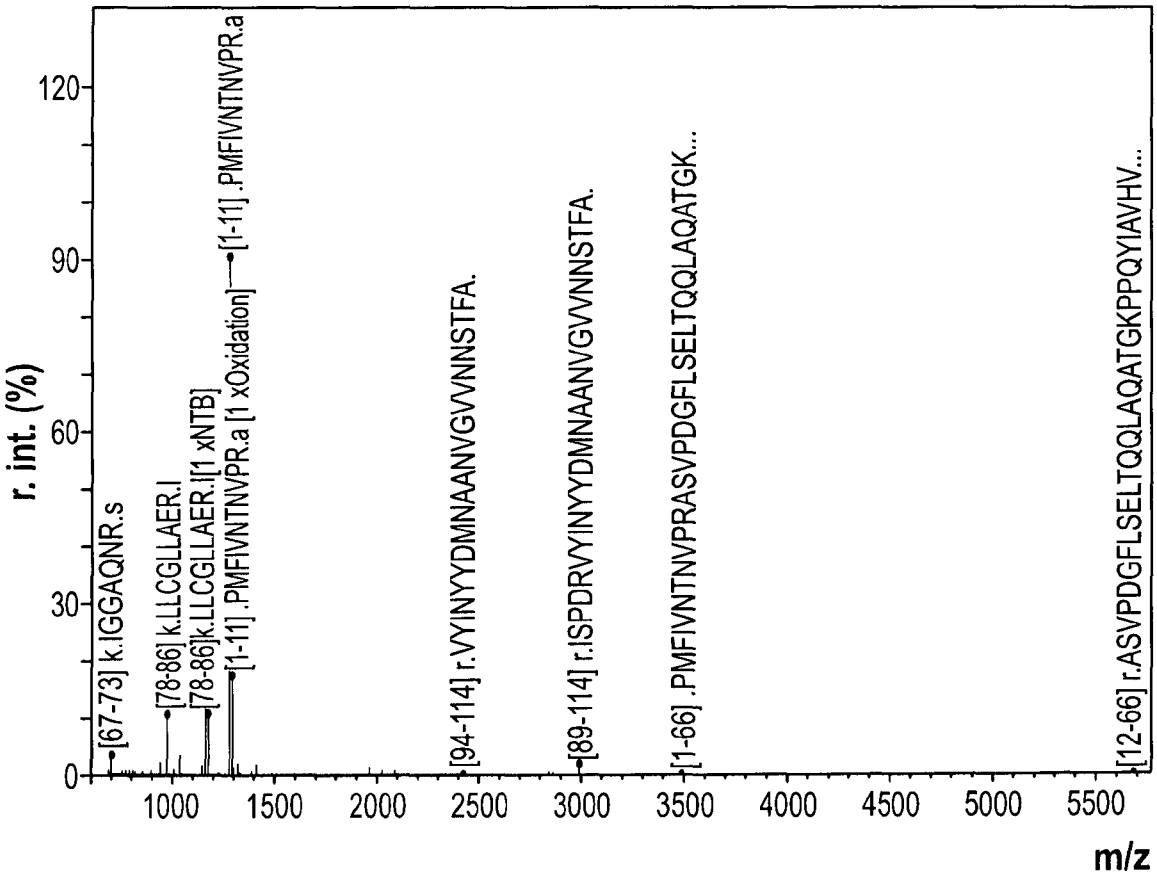
r. int. (%)
120
90
60
30
0
1000
1500
2000
2500
3000
3500
4000
4500
5000
5500
m/z
[67-73] k.IGGAQNR.s
[78-86] k.LLCGLLAER.l
[78-86]k.LLCGLLAER.l[1 xNTB]
[1-11] .PMFIVNTNVPR.a [1 xOxidation]
[1-11] .PMFIVNTNVPR.a
[94-114] r.VYINYYDMNAANVGVVNNSTFA.
[89-114] r.ISPDRVYINYYDMNAANVGVVNNSTFA.
[1-66] .PMFIVNTNVPRASVPDGFLSELTQQLAQATGK...
[12-66] r.ASVPDGFLSELTQQLAQATGKPPQYIAVHV...

Figure 12

| Accession | Length | Mo. Mass | Av. Mass | Coverage | Matched Int. |
|---|---|---|---|---|---|
| | 114 | 13018.0362 | 13026.4639 | 94.7% | 81.3% |

PMFIVNTNVP RASVPDGFLS ELTQQLAQAT GKPPQYIAVH VVPDQLMAFG GSSEPCALCS LHSIGKIGGA QNRSYSKLLC GLLAERLRIS PDRVYINYYD MNAANVGWNN STFA

| Position | Modification | Type | Mo. Mass | Av. Mass | Formula |
|---|---|---|---|---|---|
| All M | Oxidation | variable | 15.9949 | 15.9994 | O |
| All C | Disulfide | variable | -2.0157 | -2.0159 | - HH |
| All C | Oxidation | variable | 15.9949 | 15.9994 | O |
| All C | NTB | variable | 196.9783 | 197.1694 | C7H4NO4S - H |

Figure 12 (continued)

| Meas. m/z | Calc. m/z | δ (Da) | δ (ppm) | Rel. Int. (% | z | Annotation | Formula |
|---|---|---|---|---|---|---|---|
| 715.3403 | 715.3846 | -0.0442 | -61.8 | 4.27 | 1 | [67-73]k.IGGA QNR.s | C28H50N12O10 |
| 987.5548 | 987.5656 | -0.0107 | -10.9 | 11.64 | 1 | [78-86] k.LLCG LLAER.I | C43H78N12O12S |
| 1184.5437 | 1184.5438 | -0.0001 | -0.1 | 11.68 | 1 | [78-86] k.LLCG LLAER.I[1xNTB] | C50H81N13O16S2 |
| 1287.6936 | 1287.6878 | 0.0058 | 4.5 | 100.00 | 1 | [1-11] .PMFIVNT NVPR.a | C58H94N16O15S |
| 1303.6892 | 1303.6827 | 0.0065 | 4.9 | 19.21 | 1 | [1-11] .PMFIVNT NVPR.a[1xOxidatio n] | C58H94N16O16S |
| 2427.0853 | 2427.0710 | 0.0143 | 5.9 | 0.38 | 1 | [94-114]r.VYINY YDMNAANVGW NNSTFA. | C110H151N27O34S |
| 2995.3569 | 2995.3679 | -0.0110 | -3.7 | 1.98 | 1 | [89-114] r.ISPD RVYINYYDMNAA NVGWNNSTFA. | C134H191N35O42S |

Figure 12 (continued)

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 3011.3431 | 3011.3628 | -0.0197 | -6.6 | 0.11 | 1 | [89-114]r.ISPD RVYINYYDMN AANVGWNNS TFA.i[1xDisulfide] | C134H191N35O43S |
| 3489.7732 | 3489.7581 | 0.0151 | 4.3 | 0.07 | 2 | [1-66] .PMFIVNTNVPRA SVPDGFLSELTQ QLAQATGKPPQYI AVHVVPDQLMAF GGSSEPCALCSL HSIGK.I [1xOxidation] | C310H490N82O93S4 |
| 5691.4711 | 5691.8284 | -0.3573 | -52.8 | 0.11 | 1 | [12-66] r.ASVP DGFLSELTQQ LAQATGKPPQ YIAVHVVPDQL MAFGGSSEPC ALCSLHSIGK.I [1xDisulfide] | C252H396N66O78S3 |